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The Biosure Study: Influence of Composition of Diet and Food Consumption on Longevity, Degenerative Diseases and Neoplasia in Wistar Rats Studied for up to 30 Months Post Weaning

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Abbreviations: A = *ad lib.*; ALP = alkaline phosphatase; ALT = alanine aminotransferase; AST = aspartate aminotransferase; BUN = blood urea nitrogen; BW = body weight; GLDH = glucose-6-phosphate dehydrogenase; Hb = haemoglobin; Hct = haematocrit; I = interrupted (6 hr per day); LB = Low Nutrient Breeder diet; LBA = LB diet *ad lib.*; LH = luteinizing hormone; LM = Low Nutrient Maintenance (high fibre) diet; LMA = LM diet *ad lib.*; MCH = mean cell haemoglobin; MCHC = MCH concentration; MCV = mean cell volume; ME = metabolizable energy; MPV = mean platelet volume; N.S. = not significant; PCV = packed cell volume; PDW = platelet distribution width; PLT = platelet count; PR = Porton Rat diet; PRA = PR diet *ad lib.*; R = restricted to 80% *ad lib.*; RBC = red blood cell count; RDW = red cell distribution width; RH = relative humidity; SB = Standard Breeder diet; SBA = SB diet *ad lib.*; SBI = SB diet interrupted (6 hr per day); SBR = SB diet restricted to 80% *ad lib.*; SM = Standard Maintenance diet; SMA = SM diet *ad lib.*; SMI = SM diet interrupted (6–hr per day); SMR = SM diet restricted to 80% *ad lib.*; SPF = Specified Pathogen Free; VFA = volatile fatty acids; VIF = vasoactive intestinal peptides; WBC = white blood cell count.

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SYNOPSIS

The 1200-rat Biosure Study had six interrelated aims:

(1) To see whether dietary restriction (80% *ad lib.*) reduces the age-standardized incidence of fatal or potentially fatal neoplasia before the age of 30 months.

(2) To see whether the beneficial effects of diet restriction can be achieved by (a) limiting the daily period of access to food to 6 hr, or by (b) limiting the energy value of the diet.

(3) To see whether reduced calorie intake between weaning and age 4 months influences survival and/or incidence of non-neoplastic and neoplastic diseases.

(4) To compare effects of food consumption, energy intake and protein intake on survival and disease.

(5) To study the relationships between body weight at different ages with eventual survival and disease incidence.

(6) To provide a database for studying relationships between various in-life measurements and eventual survival and disease incidence in individual animals.

Twelve groups of SKF Wistar rats consisting of 50 animals of each sex were fed according to different dietary regimens from when they were weaned at the age of 3 wk until they died, or had to be killed because they were sick, or until the experiment was terminated at 30 months. For five of the 12 dietary regimens, satellite groups consisting of 30 animals per sex were maintained in parallel and used to supply information on the effect of diet on circulating hormone levels during the course of the study. During the 13 wk post weaning a Standard Breeder diet (SB) was provided either *ad lib.* (four groups), 80% *ad lib.* (three groups), or with access to food limited to 6 hr per day (one group). During this same period two other groups were fed a Low Nutrient Breeder diet (LB) *ad lib.* A further group was fed a Low Nutrient Maintenance (high fibre) diet (LM) *ad lib.* Finally, one group was fed the high protein Porton Rat diet (PR) *ad lib.* From the end of this initial 13-wk period until death or the termination of the experiment, three of the 12 groups were fed *ad lib.* (A) a Standard Maintenance diet (SM); two groups were fed the same diet with consumption restricted to 80% *ad lib.* (R); two groups were fed SM but with access to the food basket limited to 6 hr per day (I); four groups were fed LM *ad lib.*, and the group which was given the PR diet *ad lib.* during the first 90 days of the study remained on this same dietary regimen for the rest of the study. No known carcinogen was deliberately added to, or included in, any of the diets. During the study and when it was terminated, data were collected from main and satellite group animals on body weight, food consumption, water consumption, clinical chemistry, haematology and urinalysis. At the termination of the study, samples of plasma were taken for measurement of circulating hormone levels. All rats, whether decedents or killed terminally, were subjected to a full autopsy, and haematoxylin and eosin stained sections were prepared routinely from liver, kidneys, heart, spleen, lungs, pancreas, adrenals, thyroids, parathyroids, testes, epididymides, ovaries, uterine horns, skin, mammary glands, pituitary and other organs thought to be abnormal at autopsy. For terminally-killed animals, the following organ weights were recorded: liver, kidneys, heart, adrenals, testes, prostate, seminal vesicles, ovaries, pituitary and brain. The findings were analysed statistically using methods recommended by Peto *et al.* (in IARC, 1980) and also by other methods where appropriate.

The different dietary regimens were associated with highly significant differences in longevity, in the incidence of degenerative diseases (particularly myocardial degeneration, chronic progressive nephropathy, polyarteritis, prostatitis and radiculoneuropathy), and in the age-standardized incidence of benign and malignant neoplasms of virtually all sites.

The main determinant of longevity and incidence of degenerative and neoplastic disease incidence was the dietary regimen during the second phase of the study. During this phase, restriction of the intake of the SM diet to 80% *ad lib.* (SMR) had by far the most strikingly beneficial effects on most endpoints. By comparison, limiting access to SM diet to 6 hr per day, which had relatively little effect on food consumption, had little effect also on longevity or on the various disease incidence endpoints. Feeding animals *ad lib.* on the high fibre LM diet was associated with reduction in nephropathy, pituitary and mammary tumours. However, it was also associated with significantly increased incidences of adenocarcinomas of the uterine body and of haemangiomas and haemangiosarcomas of the mesenteric lymph node. Feeding the high protein PR diet *ad lib.* (PRA) was associated with somewhat higher incidences of early death, degenerative diseases and neoplasms of most sites than feeding the SM diet *ad lib.* (SMA).

By comparison with the major influence of dietary regimen during the second stage of the study, the dietary regimen during the first 90 days of the study had relatively little influence on any of the endpoints, other than the incidences of corticomedullary and pelvic nephrocalcinosis.

A particularly striking finding was that, irrespective of dietary regimen there were highly significant positive correlations between body weight six or more months after the start of the study and subsequent risk of premature death and subsequent risk of developing a fatal or potentially fatal malignant neoplasm.

Among the findings that are entirely new are the adverse effects of the high fibre (LM) diet on the incidence of the uterine and mesenteric lymph node tumours, and the significant effect of restricting intake of SM diet to 80% *ad lib.* in reducing the incidence of lung adenomas and adenocarcinomas to zero.

The findings are considered in the light of earlier studies of the effects of dietary composition and calorie intake, and also in relation to the genetically determined characteristics of the Wistar strain of rats used in the study. Finally, the implications of the results of the study in relation to testing chemicals for chronic toxicity and carcinogenicity are discussed.

All six interrelated aims of the study were achieved. However, the relative paucity of in-life measurements, particularly for circulating hormones, resulted in less being obtained from the study than had been hoped with respect to identifying in-life predictors of survival and disease.

Many other analyses could be undertaken on the mass of data from this large study. The investigators would welcome requests for access to the data, which are stored on computer.

INTRODUCTION

Numerous earlier studies have shown strong correlations between, on the one hand, calorific restriction and, on the other hand, longevity, incidence of degenerative lesions and premature ageing, incidence of hormonal changes and incidence of benign and malignant neoplasms in laboratory rodents (Baumann, 1948; Berg and Simms, 1960; Conybeare, 1980; Deerberg *et al.*, 1990; McCay *et al.*, 1935; Masoro, 1984; Masoro *et al.*, 1989; Nolen, 1972; Rehm *et al.*, 1985b; Roe and Tucker, 1974; Ross and Bras, 1965; Rowlatt *et al.*, 1973; Salmon *et al.*, 1990; Tucker, 1979; Weindruch, 1985; Yu *et al.*, 1982). Recently these associations have been discussed at length in the important book by Weindruch and Walford (1988).

The 1200-rat Biosure Study had six interrelated aims:

(1) To see whether dietary restriction (80% *ad lib.*) reduces the age-standardized incidence of fatal or potentially fatal neoplasia before the age of 30 months.

(2) To see whether the beneficial effects of diet restriction can be achieved by (a) limiting the daily period of access to food to 6 hr, or by (b) limiting the energy value of the diet.

(3) To see whether reduced calorie intake between weaning and age 4 months influences survival and/or incidence of non-neoplastic and neoplastic diseases.

(4) To compare effects of food consumption, energy intake and protein intake on survival and disease.

(5) To study the relationships between body weight at different ages with eventual survival and disease incidence.

(6) To provide a database for studying relationships between various in-life measurements and eventual survival and disease incidence in individual animals.

A preliminary report on the design and outcome of the study was presented at a Conference on the "Biological Effects of Dietary Restriction" (Roe, 1991) and the relationship between body weight in early adult life and the risks of premature death and cancer seen in the study has been highlighted in another short paper Roe *et al.* (1991). The present paper constitutes the definitive report on the study.

MATERIALS AND METHODS

Animals

All the animals used in these experiments were bred and supplied by the Smith Kline and French Research Ltd (SKF) Breeding Unit at the Frythe (Welwyn, Herts, UK). They were bred and subsequently housed under strict Specified Pathogen Free (SPF) barrier conditions. In the breeding colony, all the rats and mice were fed PR diet (see below) and given microfiltered tap water *ad lib.* The breeding animals were maintained on solid floors with softwood shavings (SMC Ltd, Standon, Herts, UK) for bedding and nesting.

The colony of barrier-maintained outbred SKF Wistar rats was originally caesarean derived from a breeding nucleus of Wistar rats obtained from ICI (Alderley Park, Cheshire, UK) in 1968. Between 1968 and the start of the study the strain was re-derived by caesarean section on five occasions. Breeding females were removed after mating so that post-partum mating did not occur. Systematic precautions were taken to avoid close in-breeding.

Animal husbandry

The animals for the study were supplied in four batches at 4-wk intervals.

Batches 1, 2 and 3, each consisting of 200 male and 200 female SKF Wistar rats, were used in the main study. Batch 4, consisting of 150 male and 150 female rats, was used to provide satellite groups of 30 per sex per group for five of the dietary regimens. Up to six animals (the number depending on survival until the due date) of each sex from each satellite group were killed at weeks 1, 24, 52, 79 and 104 to provide intermediate data, including measurements of circulating hormone levels.

The animals were transferred as weanlings from the Breeding Unit to the Research Laboratory. Batches 1, 2 and 3 were housed in different but identical animal rooms. Batch 4 was housed in the same room as batch 1. All the rooms were maintained at

Table 1A. Formulae of the five diets

Ingredient	% Content of ingredient in each diet				
	SB	LB	SM	LM	PR
Wheatfeed	3.9	47.7	19.5	69.0	20.0
Wheat	34.5	7.5	37.0	0.95	19.43
Maize	38.8	21.5	19.0	5.3	18.08
Oats	0	0	0	0	10.0
Oatfeed	0	14.0	11.2	19.25	0
Barley	0	0	0	0	5.37
Soya Ext 44	0	0	0	0	10.00
Dried skim milk	0	0	0	0	7.50
Fish meal	8.3	4.1	1.5	1.4	5.0
Pruteen[+]	5.1	1.4	5.2	0	0
Brewer's yeast	2.0	1.1	0	0	2.5
Vitamin mix	0.8	0.6	0.65	0.52	0.50
Mineral mix	1.0	0.4	0.4	0.33	0.50
Salt	0.4	0.4	0.4	0.38	0.80
Corn oil	3.3	0	3.1	0	0
Calcium carbonate	1.15	1.5	1.5	1.8	0.70
Potassium monohydrate phosphate	0.91	0	0.95	0.014	0
Methionine	0.05	0.05	0	0.02	0
Lysine	0	0	0.1	0	0
Choline chloride	0	0	0	0	0.02

SB = Standard Breeder diet LB = Low Nutrient Breeder diet
SM = Standard Maintenance diet LM = Low Nutrient Maintenance diet
PR = Porton Rat diet

[+] a single cell origin protein.

Table 1B. Comparison of manufacturer's specifications of diets in respect of main nutrients

Nutrient	% Content of nutrient in each diet				
	SB	LB	SM	LM	PR
Moisture	12.5	12.5	12.5	12.5	12.5
Crude protein	20.2	16.7	14.3	13.6	19.8
Crude oil	3.1	3.4	3.1	3.0	3.0
Ash	4.9	6.0	5.2	6.7	6.2
Crude fibre	2.5	7.8	6.3	10.6	5.4
Dietary fibre	10.3	26.4	19.4	31.7	14.6

See Table 1A for key to abbreviations used for diets.

21 ± 2°C and 50 ± 10% relative humidity (RH). Unfortunately, during certain very cold spells, there was a failure of the humidity controlling system such that overnight levels fell as low as 5% RH (see below). The rooms were illuminated by fluorescent tubes between 06.00 and 18.00 hr. Microfiltered tap water was given *ad lib.*

The rats were randomly assigned to 12 treatment groups and housed five per cage in stainless-steel, grid-floor cages (TAJ Ltd, Whitstable, Kent, UK) with no bedding. Towards the end of the study several of the animals developed sore feet and exhibited a lack of muscle tone in their hind legs. These appearances were due to their developing radiculoneuropathy, a degenerative disease of the cauda equina and lumbar spinal cord (see below). Animals exhibiting these signs were transferred to cages in which solid floor liners had been placed over the grid floor. Also, they were provided with soft greenwood granules (SMC Ltd, Standon, Herts., UK) as bedding. The cage trays were cleaned three times per week and animals were rehoused in clean, washed cages at monthly intervals. Where bedding was provided this was changed twice weekly.

Formulae of the five diets used in the study

Table 1A shows the formulae of the five diets and Table 1B lists the manufacturers' specifications for the main nutrients. The fat (expressed as 'crude oil') content of all five diets was similar (3.0–3.4%) and so was the moisture content (12.5%). The major differences between the diets were in their protein and fibre contents.

Four of the diets used were specially formulated for the experiment and are not available commercially; these were: Standard Breeder diet (SB); Standard Maintenance diet (SM); Low Nutrient Breeder diet (LB); Low Nutrient Maintenance diet (LM).

The fifth diet used was the commercially available Porton Rat diet (PR). PR had been used for many years by SKF as the standard toxicology diet, and served as a 'within experiment' control allowing, where necessary, comparison of data from this study with those from previous studies.

The five diets were formulated and supplied by Biosure (Lavender Mill, Manea, Cambs., UK) using, except PR, similar raw materials as the source of the main nutrient constituents. The two 'Standard' diets

Table 1C. Composition of diets: standard breeder (SB) diet (weeks 0–12)

Nutrients	Manufacturer's specification	Analysis
Moisture (%)	12.5	13.9
Crude protein (%)	20.2	18.3
Crude oil (%)	3.1	3.3
Ash (%)	4.9	5.2
Crude fibre (%)	2.5	1.6
Phosphorus (%)	0.72	0.74
Chloride (%)	0.41	0.41
Calcium (%)	0.72	0.70
Magnesium (%)	0.11	0.17
Sodium (%)	0.24	0.25
Potassium (%)	0.88	0.77
Zinc (mg/kg)	52	32
Manganese (mg/kg)	55	48
Copper (mg/kg)	10.5	11
Iron (mg/kg)	72	175
Vitamins		
A (iu/kg)	8300	10,100
E (mg/kg)	137	90.5
Binding agent lignosulfonate (%)	0	0

Table 1D. Composition of diets: low nutrient breeder (LB) diet (weeks 0–12)

Nutrients	Manufacturer's specification	Analysis
Moisture (%)	12.5	14.6
Crude protein (%)	16.7	15.9
Crude oil (%)	3.4	2.9
Ash (%)	6.0	5.7
Crude fibre (%)	7.8	6.9
Phosphorus (%)	0.70	0.62
Chloride (%)	0.33	0.35
Calcium (%)	0.72	0.64
Magnesium (%)	0.20	0.24
Sodium (%)	0.20	0.20
Potassium (%)	0.81	0.82
Zinc (mg/kg)	76	47
Manganese (mg/kg)	78	73
Copper (mg/kg)	13.0	11.8
Iron (mg/kg)	63.0	245
Vitamins		
A (iu/kg)	6500	6500
E (mg/kg)	117	76.1
Binding agent lignosulfonate (%)	0	0

Table 1E. Composition of diets: standard maintenance (SM) diet (weeks 13—end of test)

Nutrients	Manufacturer's specification	Analysis 1	2	3	4
Moisture (%)	12.5	13.3	13.5	14.0	12.6
Crude protein (%)	14.3	14.3	15.2	14.1	14.9
Crude oil (%)	3.1	3.1	2.9	2.9	2.8
Ash (%)	5.2	5.1	5.5	5.4	5.2
Crude fibre (%)	6.3	5.7	5.5	6.7	5.8
Phosphorus (%)	0.72	0.76	0.71	0.66	0.63
Chloride (%)	0.32	0.34	0.36	0.35	0.34
Calcium (%)	0.72	0.80	1.00	0.86	0.82
Magnesium (%)	0.13	0.24	0.16	0.14	0.13
Sodium (%)	0.18	0.19	0.21	0.18	0.17
Potassium (%)	0.93	0.85	0.85	0.73	0.72
Zinc (mg/kg)	56	36	54	39	40
Manganese (mg/kg)	64	59	63	50	50
Copper (mg/kg)	10.5	11.5	11.7	10.0	10.0
Iron (mg/kg)	72	175	170	160	125
Vitamins					
A (iu/kg)	6800	8100	7400	6300	7500
E (mg/kg)	118	96.8	85.9	75.3	65.0
Binding agent lignosulfonate (%)	0*	0.00	0.984	0.984	1.47

Analyses conducted at 8/5/85, 2/10/85, 14/3/86 and 10/4/86.
*Lignosulfonate was added to the diet to prevent spillage and crumbling after the start of the study.

Table 1F. Composition of diets: low nutrient maintenance (LM) diet (week 0—end of test)

Nutrients	Manufacturer's specification	Analysis 1	2	3	4	5	6
Moisture (%)	12.5	13.4	12.6	16.0	13.0	13.8	13.2
Crude protein (%)	13.6	13.7	13.9	13.7	13.5	13.4	13.5
Crude oil (%)	3.0	2.7	3.1	2.6	2.9	3.0	2.7
Ash (%)	6.7	6.1	6.2	6.0	5.6	6.0	6.1
Crude fibre (%)	10.6	10.4	13.3	10.2	8.9	8.7	10.3
Phosphorus (%)	0.77	0.72	0.69	0.66	0.65	0.56	0.70
Chloride (%)	0.30	0.34	0.33	0.32	0.30	0.32	0.26
Calcium (%)	0.85	0.70	1.10	0.98	0.78	1.10	0.81
Magnesium (%)	0.23	0.35	0.27	0.21	0.22	0.22	0.30
Sodium (%)	0.18	0.21	0.19	0.21	0.20	0.17	0.17
Potassium (%)	0.90	0.93	1.00	0.92	0.74	0.73	0.85
Zinc (mg/kg)	83	55	59	59	61	56	70
Manganese (mg/kg)	87	90	87	86	77	94	90
Copper (mg/kg)	13.6	13.5	16.0	12.7	10.7	12.0	13.0
Iron (mg/kg)	58	205	210	255	230	230	295
Vitamins							
A (iu/kg)	5400	7000	8400	5400	4500	7985	6720
E (mg/kg)	103	77.2	87.2	87.6	89.0	74.9	49.3
Binding agent lignosulfonate (%)	0*	0.00	0.10	0.988	1.48	1.48	1.76

Analysis conducted at 24/4/85, 29/7/85, 11/3/85, 25/7/86, 23/2/87 and 14/5/87.
*Lignosulfonate was added to the diet to prevent spillage and crumbling after the start of the study.

Table 1G. Composition of diets: Porton rat (PR) diet (week 0—end of test)

Nutrients	Manufacturer's specification	Analysis 1	2	3	4	5	6	7
Moisture (%)	12.5	13.1	13.3	12.2	13.8	13.3	12.3	13.3
Crude protein (%)	19.8	19.8	20.2	21.1	19.8	20.1	20.4	19.7
Crude oil (%)	3.0	3.0	2.9	2.8	2.7	2.7	2.8	2.9
Ash (%)	6.2	5.3	5.3	5.5	5.1	5.5	5.3	5.6
Crude fibre (%)	5.4	5.4	4.4	4.6	5.7	4.3	5.4	5.8
Phosphorus (%)	0.81	0.70	0.70	0.70	0.66	0.73	0.68	0.61
Chloride (%)	0.65	0.66	0.68	0.66	0.66	0.63	0.61	0.63
Calcium (%)	0.88	0.65	0.79	0.78	0.60	0.63	0.50	0.77
Magnesium (%)	0.19	0.17	0.17	0.16	0.17	0.16	0.17	0.15
Sodium (%)	0.40	0.40	0.39	0.38	0.35	0.36	0.33	0.35
Potassium (%)	0.85	0.87	0.80	0.86	0.82	0.79	0.80	0.80
Zinc (mg/kg)	75	40	55	45	44	47	47	70
Manganese (mg/kg)	74	65	65	62	58	61	62	68
Copper (mg/kg)	14	13.5	14.2	12.7	12.5	15.3	11.5	12.6
Iron (mg/kg)	142	150	220	225	190	230	235	160
Vitamins								
A (iu/kg)	8000	7500	8600	6600	11,900	6000	6040	11,500
E (mg/kg)	30	79.5	76.5	72.6	82.0	77.5	76.5	70.3
Binding agent lignosulfonate (%)	0	0	0	0	0	0	0	0

Analyses conducted at 29/4/85, 14/8/85, 16/10/85, 17/1/86, 14/4/86, 22/7/86 and 26/11/86.

(SB, SM) were formulated to provide nutrient levels that were typical of current breeding and maintenance diets at that time. The 'Low Nutrient' diets were intended to be similar to the standard diets except that the density of available energy was decreased by the addition of fibre.

In formulating the four experimental diets, protein was varied by the manipulation of the inclusion of the main protein sources, white fish meal and Pruteen; fibre was added in the form of wheatfeed and oatfeed, typically at the expense of the cereals, maize and wheat. The rate of inclusion of the mineral and vitamin premixes was adjusted to compensate, at least partially, for the variation in food intake that was likely to occur as the animals sought to regulate their intake of metabolizable energy (ME) on diets differing markedly in ME density.

Before the main experiment a short nutritional study was carried out at the University of Leeds to determine the ME densities of the five diets, and to ensure that the protein quality of the diets was satisfactory. A brief description of this study and the results obtained are given in Appendix 1.

All the diets were sterilized by gamma-irradiation at an average dose of 25 kGy (2.5 Mrad) by Isotron (Swindon, Daventry, UK).

Because of the relatively small amounts required for the initial part of study, the SB and LB diets were each supplied as a single batch. PR was drawn from the SKF central food store, and a total of seven batches were used over the duration of the study. SM and LM diets were manufactured solely for this study and were supplied in two and three batches, respectively. It had been previously demonstrated by chemical assay and biological tests at SKF (Spencer, 1985) that irradiated diet could be used satisfactorily up to at least one year of age.

Each batch of diet was analysed for the wide range of nutrients and contaminants typically monitored in a Good Laboratory Practice (GLP) study. The analytes were:

Moisture	Total viable count
Crude protein	Mesophilic spores
Crude oil	Salmonella
Ash	Presumptive coliforms
Crude Fibre	Faecal coliforms
Calcium	
Phosphorus	Arsenic
Magnesium	Cadmium
Sodium	Lead
Potassium	Mercury
Chloride	Selenium
Zinc	Nitrate
Manganese	Nitrite
Copper	Total DDT
Iron	Dieldrin
Vitamin A	Lindane
Vitamin E	Heptachlor
	Malathion
	Total PCBs
	Total aflatoxins

Tables 1C–1G compare the manufacturer's specification for nutrients with the results of the analysis of the different batches of the five diets. There was reasonable agreement for the main nutrients but this was not always so for the mineral constituents. One of the key aspects of the diet with respect to this study was fibre content and its influence on the ME density of the diet. There was good agreement between the estimate of dietary fibre by calculation and

Table 2. Experimental design

		Number of rats			
		Male		Female	
Group	Diet	Main	Satellite	Main	Satellite
1	SBA/SMA	50 (20,15,15)	30	50 (15,15,20)	30
2	SBA/LMA	50 (20,15,15)	30	50 (15,15,20)	30
3	SBA/SMR	50 (15,15,20)	0	50 (20,15,15)	0
4	SBA/SMI	50 (15,15,20)	0	50 (20,15,15)	0
5	SBR/SMR	50 (20,15,15)	30	50 (15,20,15)	30
6	SBR/LMA	50 (20,15,15)	30	50 (15,20,15)	30
7	SBR/SMA	50 (15,20,15)	30	50 (20,15,15)	30
8	SBI/SMI	50 (15,20,15)	0	50 (20,15,15)	0
9	LBA/SMA	50 (15,15,20)	0	50 (15,20,15)	0
10	LBA/LMA	50 (15,15,20)	0	50 (15,20,15)	0
11	LMA/LMA	50 (15,20,15)	0	50 (15,15,20)	0
12	PRA/PRA	50 (15,20,15)	0	50 (15,15,20)	0
	Total	600 (200,200,200)	150	600 (200,200,200)	150

All numbers in parentheses show the number of rats in batches 1, 2 and 3. All the satellite animals were derived from batch 4. The first diet shown was given up to 13 weeks, and the second subsequently: SB = Standard Breeder diet; LB = Low Nutrient Breeder diet; SM = Standard Maintenance diet; LM = Low Nutrient Maintenance diet; PR = Porton Rat diet; A = *ad lib.*; R = restricted to 80% *ad lib.*; I = access to food restricted to 6 hr/day.

the determination of indigestible dry matter by biological assay; the relationship could be expressed as:

indigestible matter (%)

$$= 2.8 + [1.23 \times \text{dietary fibre (\%)}]$$

The coefficient of determination (r^2) was 0.93.

A similar close relationship was observed between the calculated dietary fibre content of the diet and ME density; the relationship could be expressed as:

$$\text{ME density (kJ/g)} = 15.7 - [0.20 \times \text{dietary fibre (\%)}]$$

The coefficient of determination (r^2) was 0.94. Significantly the intercept at zero dietary fibre of 15.7 kJ/g lies close to the theoretical ME density of a diet made up primarily of carbohydrate (16 kJ/g) and protein (17 kJ/g).

It is reasonable to assume therefore that the different diets did, as planned, deliver different levels of indigestible matter, and as a consequence, provide a range of ME densities.

There was no evidence that the contaminants reached levels that would have been unacceptable for a standard long-term toxicology study. It is not normal practice to measure the activity of phyto-oestrogens in the diet in either the UK or USA for toxicology studies. However, none of the materials used in the four experimental diets contained materials, such as soya bean meal or lucerne, that have from time to time been reported as containing these materials (Liener, 1969). Unpublished data from The Netherlands have confirmed that the probability of finding such substances in typical laboratory animal diets is low. Further, phyto-oestrogens have a very low physiological activity compared with synthetic or true mammalian oestrogens, and are unlikely to have any significant effect unless the diet is grossly contaminated (Trenkle and Burroughs, 1978).

Feeding regimens

The degree of restriction employed in these experiments was designed to produce reduced calorie intake but not malnutrition.

To facilitate the feeding of the animals, particularly those given access to food during only 6 hr per day, a food hopper was specially designed by G. Conybeare and manufactured by TAJ Ltd (Whitstable, Kent, UK). By simply turning it around without removing it from the cage, animals could be given access to food or deprived of access to it. The availability of this specially designed hopper rendered it easy to regulate food availability without incurring the risks of transferring diseases from one cage to another, which could occur if hoppers were removed from cages and then accidentally returned to different cages.

The animals on the restricted feeding regimens started feeding between 09.00 and 09.15 hr daily. The animals on a time-restricted regimen (interrupted) had their hoppers turned to the non-available position 6 hours after the food was offered.

Experimental plan

The plan was to feed rats according to one dietary regimen during the first 13 wk of the study, and thereafter according to the same or another regimen. However, for the four groups (groups 5–8) which were designated to be restricted to 80% *ad lib.* during the first phase of the study, it was first necessary to measure *ad lib.* food consumption. This was done by offering all groups food (SB, LB, LM or PR) *ad lib.* during the first week of the study and measuring the amount of food consumed. During the following 12 weeks the groups designated to be fed *ad lib.* (A) continued as during the first week, whereas the 80% *ad lib.* (R) groups were restricted to 80% of the food consumed during the previous week and the group designated to have access to food for only 6 hr per

day (e.g. from 09.00 to 15.00 hr) began to be fed this interrupted (I) regimen from the start of the second week of the study. During the remainder of the experiment '80% *ad lib.*' was based on food consumption by animals on the same diet in *ad lib.*-fed groups during the most recent week in which this was measured (see below).

During the second phase of the study, which extended from the end of week 13 for a further 27 months or until before death, animals were fed one of three different diets (SM, LM or PR) either *ad lib.* (A), restricted to 80% *ad lib.* (R) or, *ad lib.* but only during 6 hr per day (I).

On the day when the four batches of animals arrived in the experimental unit (day 1 of the study = day 22 of life), they were individually weighed and allocated to the 12 different groups using a computer-generated random order printout.

The construction of the 12 different groups is indicated in Table 2.

Observations on animals in main groups

Body weights were recorded weekly. Terminal body weights were recorded on the morning of the day of autopsy.

Clinically evident physical or behavioural abnormalities were recorded weekly. Sick animals were isolated to prevent cannibalism.

Food consumption, corrected for spillage (see below), was measured each day of every week up to week 8, then once every 2 weeks by up to 20 animals per sex/group up to week 20, and thereafter in up to 20 rats/sex/group once monthly.

Water consumption was measured by weight. The weight of water consumed during the previous 24 hr was recorded daily, at the same time each day (late morning). The fitting of ball valves kept wastage to a minimum so that it was not necessary to make any allowance for wastage. Measurements were made during week 4, and then every 12 weeks from week 12 to 120 and for the last time during week 128.

Blood samples were taken from up to 20 animals per sex/group for routine clinical chemistry and haematology from the lateral tail vein (Conybeare *et al.*, 1988) during weeks 27, 53, 79 and 105 and at autopsy except in animals that were found dead. Blood samples were taken between 11.00 and 12.00 hr, that is shortly after SMR and SMI animals had eaten a main meal but rather longer after SMA, LMA and PRA animals had ceased nibbling food. The following parameters were measured:

Haematology
- White blood cell count (WBC)
- Differential white cell count
- Red blood cell count (RBC)
- Haemoglobin (Hb)
- Haematocrit (Hct)
- Mean cell volume (MCV)
- Mean cell haemoglobin (MCH)
- Mean cell haemoglobin concentration (MCHC)
- Red cell distribution width (RDW)
- Platelet count (PLT)
- Packed cell volume (PCV)
- Mean platelet volume (MPV)
- Platelet distribution width (PDW)

Clinical chemistry
- Total protein
- Albumin
- Blood urea nitrogen (BUN)
- Creatinine
- Glucose

In addition, the following enzymes were measured in blood samples derived from all the survivors killed at the end of the study: alanine aminotransferase (ALT); alkaline phosphatase (ALP); glucose-6-phosphate dehydrogenase (GLDH); aspartate aminotransferase (AST).

Urine samples were collected from up to 20 animals per sex/group during weeks 26, 52, 78, 104 and 120. The animals were placed in glass metabolism cages for 4 hr without food and water from 16.00 to 20.00 hr.

The following parameters were measured:

Volume	
pH	
Glucose	Carried out using
Ketones	B M-Test 7
Protein	Boehringer Corp.
Blood	(London) Ltd,
Bilirubin	Lewes, East Sussex, UK
Urobilinogen	

All animals found dead or killed were subjected to a full autopsy examination but no organs were weighed.

Beginning during week 129 all of the surviving animals were killed in a randomly determined order by exposure to carbon dioxide, followed by exsanguination. Blood samples were taken from the vena cava and 5-ml samples sent to Sandoz (Switzerland) for subsequent hormone assay. The hormones assayed were:

Growth hormone	(males and females)
Luteinizing hormone (LH)	(males and females)
Progesterone	(males and females)
Prolactin	(males and females)
Testosterone	(males)
17β-Oestradiol	(females)

The following organs were weighed:

- Kidneys
- Adrenals
- Testes/ovaries
- Pituitary (after 24-hr fixation)
- Heart
- Liver
- Seminal vesicles
- Prostate

These tissues along with:

Spleen
Mesenteric lymph node
Uterus
Mammary gland
Lung
Pancreas

and any abnormal-looking organ or tissue showing pathological changes were fixed in 10% formalin, cut, stained with haemotoxylin and eosin and then examined microscopically (by FJCR).

Brains were weighed and stored at −20°C for possible subsequent amine content assays.

At autopsy, samples of liver were taken from three male rats from groups 5, 11 and 12 (i.e. SBR/SMR, LMA/LMA and PRA/PRA). Hepatocyte *P*-450 levels were measured in these samples.

Observations on satellite animals [groups 1 (SBA/SMA), 2 (SBA/LMA), 5 (SBR/SMR), 6 (SBR/LMA) and 7 (SBR/SMA)]

Six male and six females from each of the five groups were killed after week 1, and thereafter similar numbers were killed at 6-monthly intervals up to 24 months.

Body weights were recorded weekly, and terminal body weights on the morning of the day of autopsy. All clinical signs and adverse observations were recorded weekly. Sick animals were isolated to prevent cannibalism.

Food and water consumption were measured as for main group animals.

During a 2-wk period before their sacrifice, vaginal smears were taken from female rats, first to determine whether animals were cycling regularly, irregularly or not at all, and secondly to see how oestrus cycling related to terminally measured circulating hormone levels. For animals cycling normally, this 2-wk period would cover at least two complete 4–5-day cycles and the data obtained would enable the stage of oestrus at sacrifice to be determined.

Satellite animals were killed at the designated times by cervical dislocation (rather than by exposure to carbon dioxide as in the main study). A large blood sample (+5 ml) was taken from the vena cava to determine the same six sex and steroid hormones determined in the main study.

At autopsy, all of the animals were given a gross macroscopic examination and the following tissues were then fixed in 10% formalin: kidneys, adrenals, testis/ovaries, pituitary, heart, liver, uterus/prostate, seminal vesicles.

The kidneys were histologically processed and subsequently examined histopathologically (by Mr K. Isaacs).

Animals of satellite groups found dead or killed because they were sick were not examined and no tissues were taken from them.

Steps taken to overcome the problem of spillage of food

In animals fed SBR or SMR, or SBI, spillage was negligible. It was also low (i.e. only about 2.5%) in animals fed SMI. In *ad lib.*-fed animals, however, spillage was a serious problem in some groups, particularly those fed the low energy diets. Spillage with PRA (10%) and SBA (12%) was of the same order as that normally seen in *ad lib.*-fed animals fed commercially marketed diets, but with the other diets used in the present study spillage was much higher: SMA, 32%; LBA, 37%; LMA, 66%. To try to overcome this problem, a binding agent (a preparation of lignosulfonates) was added to the diets in increasing concentrations up to a maximum of 1.8% in the LM diet (Tables 1C–1G). This binding agent is used commonly in the animal feed industry. However, its addition did not resolve the problem which seemed to be one more of palatability rather than of the physical structure of the diet. This was deduced from the fact that pellets of diet did not crumble spontaneously, hence the spillage came mostly from pellets that had been gnawed.

The inclusion of floor liners (see above) made the estimation of spillage of food most difficult because the spilled food became mixed with bedding material. This probably accounts for an apparent increase in food consumption (and in its standard error) towards the end of the study when floor liners were in use.

The microscopic examination of tissues

In a study as large as the present one, the microscopic examination of tissues inevitably extends over several months, and in consequence there is a real danger of diagnostic drift such that there is inconsistency in the recording and severity grading of minor non-neoplastic lesions. In an attempt to overcome this, the pathologist (FJCR) responsible for reading the sections in the main study, endeavoured, as far as was possible (i) to use only a limited number of clearly defined parameters, (ii) to read animals from all 12 groups in parallel and (iii) to re-read and check all apparent between-group differences in the incidence/severity of lesions. A six-point scale was used, wherever appropriate, for grading lesions in respect of severity. The points on this scale were *grade 0* = no lesion, *grade 1* = minimal severity, *grade 2* = slight, *grade 3* = moderate, *grade 4* = severe and *grade 5* = very severe. The distinction between grades was subjective and arbitrary. Nevertheless, the rigorous system of checking and re-checking ensured the reliability of between-group differences in severity where these were reported.

Every effort was made to account for macroscopically observed lesions in terms of the microscopic findings, and where neoplasms were observed histologically their size was recorded and those that were evident macroscopically were distinguished from those that were not. In practice, this distinction was very rarely necessary because of the extremely high quality of the macroscopic examination.

Table 3. Groups available for comparing effects of different diets

Diet from wk 13	Diet to wk 13					
	SBA	SBR	SBI	LBA	LMA	PRA
SMA	Group 1	Group 7	—	Group 9	—	—
SMI	Group 4	—	Group 8	—	—	—
SMR	Group 3	Group 5	—	—	—	—
LMA	Group 2	Group 6	—	Group 10	Group 11	—
PRA	—	—	—	—	—	Group 12

See Table 2 for key to abbreviations for diets.

The criteria used for the diagnosis of neoplastic and non-neoplastic lesions was in accordance with 1990 state-of-the-art practice and with standard works including Boorman *et al.* (1990), Burek *et al.* (1976), Cotchin & Roe (1967), Gopinath *et al.* (1987), Jones *et al.* (1983 and 1985) and Turusov (1976).

Factors contributory to death

For each animal in the study an effort was made to list factors which (a) caused it to die and (b) rendered it sick and therefore a candidate for euthanasia. In many animals more than one factor fell into these categories. For instance, one and the same animal might be found to have a large malignant neoplasm, severe chronic progressive nephropathy and severe polyarteritis of the pancreatic and/or mesenteric arteries. In such cases, all lesions were listed as contributory to death.

One of the objectives of the study was to compare the effects of different diets on the 'cancer mortality'. Strictly speaking, this was not possible in view of (a) the practice of sacrificing sick animals and (b) the fact that the experiment was terminated after 133 weeks, at which time some of the animals in all groups were still alive. Futher, among animals killed terminally, there were a number in which sizeable malignant neoplasms were found despite the animals appearing to be in reasonably good health. In such cases, the pathologist (FJCR) expressed his opinion with regard to the likelihood that the lesion in question would or would not have led to the death of the animal within 4 weeks of when it was killed.

A major problem in the identification of factors contributory to death relates to neoplasms of the pituitary gland. On the one hand, benign tumours of moderate size may be the only autopsy finding that could explain why the animal was sick or why it died. On the other hand, large malignant pituitary tumours which are actively invading the base of the brain are sometimes found in apparently healthy animals killed at the termination of studies. For the purposes of listing factors contributory to death in the present study, the clinical observations before death and the full autopsy findings were taken into account in judging whether a small malignant tumour or a large benign tumour of the pituitary in fact contributed to death.

Statistical methods

1. *Types of comparison*

Three kinds of comparison have been used in the analysis of the findings in the main study. The first was a simple overall comparison of the findings in each of the 12 groups to identify the main features of the responses to different *dietary regimens*. In comparisons of this kind the findings in specified 'test' groups were usually compared with those in group 1 (SBA/SMA) which could be regarded as the 'control' group on the grounds that it was fed according to a 'standard' dietary regimen. In tables based on analyses of this kind, the group used as the base for comparison can be identified by the fact that no probability (P) values are given for that group.

The second kind of comparison was of the effects of different *diets up to week 13* of the study. In this kind of comparison, provided that the endpoint was measured before the change of diet, direct comparison of the six possible early dietary regimens (SBA, SBR, SBI, LBA, LMA, PRA) could be carried out validly. However, for later endpoints direct comparisons were not possible because of confounding by what proved to be the much more important effects of diet after week 13. To obtain an unbiased test of the effects of diets up to week 13 in this case, it was essential to make comparisons based only on animals on the same diet from week 13. However, the results of these comparisons could then be combined to increase the power of the test. As shown in Table 3 a 'stratified' analysis, adjusted for diet from week 13, could only involve 11 of the 12 groups, it not being possible to use group 12 since no group that received PRA up to week 13 received a different diet thereafter. Table 3 also shows that, when comparing with the effects of SBA, the amount of information varies from diet to diet. Thus, the effects of SBR to week 13 can be compared based on three pairs of groups identical in diet from week 13, while those of SBI, LBA and LMA can only be compared based on 1, 2 and 1 pair(s) of groups, respectively. Note that in all cases the comparison is across the table, with aggregation of differences down the table.

The third kind of comparison made concerned the effects of *diet from week 13*. Since, for many endpoints, there was no significant effect of diet to week 13 or, if there was an effect, it was very small

compared with that of diet from week 13, many of the analyses presented in this report compare the effect of the five dietary regimens from week 13 (SMA, SMI, SMR, LMA, PRA) ignoring the effect of diet to week 13. However, some of the analyses presented are stratified for the effect of diet to week 13. Again, when comparing with the effects of the standard diet, SMA, the amount of information varies from diet to diet. Thus, the effects of SMR, SMI and LMA can be compared for, respectively, 2, 1 and 3 dietary regimens to week 13. It can be seen from Table 3 that groups 8 and 11 (as well as again group 12) cannot enter into these analyses since there are no groups with which to compare them.

2. *Sex*

Analyses are presented for males and for females separately and in some cases also for sexes combined. The analyses based on combination of the sexes have been 'stratified' for sex, that is to say they combine corresponding treatment differences for the two individual sexes, keeping to the principle that in any comparison animals are always alike apart from the diet of interest.

3. *Age-adjusted analysis of present/absent findings*

The methodology used for many of the histopathological endpoints is as described by Peto *et al.* in an IARC (1980) Monograph. In this method, the experimental period is divided into time periods and, within each time interval, the numbers of animals *observed* to have the lesion in question in each group is contrasted with the number *expected* to have it, assuming there to be no difference in incidence rate between the groups being compared. These individual observed and expected numbers are then summed over the time periods to form a total observed and expected for each group. Differences between total observed and total expected numbers represent departures from the null hypothesis that the variation in dietary regimen has no effect, and procedures are described in the above reference to enable testing, by approximate chi-squared statistics, of whether there is significant overall between-group variation in incidence or whether the incidence in a specific group differs significantly from that in the control group. Relative risk can be estimated approximately by the ratio of total observed to total expected, or more precisely by the Mantel–Haenszel statistic.

In this method, the way in which the incidence rate is calculated depends on the context in which the observation was made.

For analysis of a type of lesion that is *visible* during life, the rate is calculated by:

$$\frac{\text{number of animals developing the lesion in the time period}}{\text{total number of lesion-free animals at the start of the period}}$$

For a lesion only detectable at autopsy, the incidence rate calculation depends on whether the condition can be assumed to be *fatal*, that is to have caused the death of the animal, or to be *incidental*, that is to have been seen in an animal dying of another cause. For fatal conditions, the rate is calculated by:

$$\frac{\text{number of animals dying during the time period because of the lesion}}{\text{number of animals alive at the start of the period}}$$

For incidental conditions, the rate is calculated by:

$$\frac{\text{number of animals dying during the period with the lesion}}{\text{total number of animals dying in the period}}$$

Where a lesion can be fatal in some animals but only incidental in others, the statistical method involves first calculating the observed and expected values for the fatal occurrences based on all the animals at risk, then calculating the observed and expected values for the incidental occurrences based on all the animals that did not have a fatal occurrence, and finally combining the two sets of observed and expected values.

For analysis of visible or fatal conditions, single weeks of death were used as the time periods. For incidental conditions, which require broader time periods in order to have sufficient numbers of animals in the denominator for reliable calculations, nine time periods were used: weeks 1–52, 53–80, 81–94, 95–104, 105–112, 113–118, 119–124, >124, and the terminal sacrifice.

The techniques described above were also used to analyse survival itself, taking death before terminal sacrifice as a fatal condition. Simpler, non-age-adjusted versions of the above chi-squared tests were also used to analyse the incidence of conditions observed only at one time point.

4. *Continuous data: use of rank tests*

Although means are often provided for descriptive purposes, rank tests have normally been used to test significance. Statistics based on rank methods were generally preferred to analysis of variance since much of the continuous data tended not to be normally distributed, and rank tests are not unduly affected by extreme data values. Further, rank methods are virtually as powerful as analysis of variance methods, even when the data actually are normally distributed. For comparison of the 12 different dietary regimens, or for testing the effect of diet after 13 weeks unadjusted for the effect of diet before (or vice versa), Kruskal–Wallis one-way analysis of variance was used. For testing the effect of diet before 13 weeks adjusted for diet subsequently (or vice versa), the stratified version of this test, as described by Fry and Lee (1988) was used.

Table 4A. Food consumption of various diets in g/day (with % SBA)

Diet	Group	Wk 4 Males		Wk 4 Females		Wk 12 Males		Wk 12 Females	
SBA	1	26.50		17.34		25.11		18.01	
	2	24.98		16.51		24.22		16.66	
	3	25.65		17.31		24.94		17.81	
	4	26.34		16.88		25.12		17.55	
	mean	25.87	(100)	17.01	(100)	24.85	(100)	17.51	(100)
SBR	5	17.88		13.50		19.64		13.90	
	6	17.88		13.50		19.64		13.90	
	7	17.60		13.44		19.80		13.82	
	mean	17.79	(69)	13.48	(79)	19.69	(79)	13.87	(79)
SBI	8	19.67	(76)	13.92	(82)	21.21	(85)	14.38	(82)
LBA	9	27.11		19.72		26.92		18.93	
	10	27.40		19.88		26.97		19.02	
	mean	27.26	(105)	19.80	(116)	26.94	(108)	18.98	(108)
LMA	11	28.82	(111)	21.49	(126)	31.25	(126)	21.44	(122)
PRA	12	28.82	(111)	18.58	(109)	29.49	(119)	19.28	(110)

Table 4B. Food consumption of various diets in g/day (with % SMA)

Diet	Group	Wk 24 Males		Wk 24 Females		Wk 60 Males		Wk 60 Females	
SMA	1	23.04		16.26		23.01		18.93	
	7	22.04		16.53		22.32		18.28	
	9	23.26		17.76		24.15		18.52	
	mean	22.78	(100)	16.85	(100)	23.16	(100)	18.58	(100)
SMR	3	19.46		13.92		18.14		14.74	
	5	19.52		13.80		18.14		14.58	
	mean	19.49	(86)	13.86	(82)	18.14	(78)	14.66	(79)
SMI	4	28.16		18.65		25.95		19.14	
	8	26.03		17.19		25.05		17.82	
	mean	27.10	(119)	17.92	(106)	25.50	(110)	18.48	(99)
LMA	2	26.82		18.47		26.73		20.02	
	6	26.58		19.25		28.04		21.29	
	10	27.86		19.95		28.20		21.06	
	11	28.97		19.95		28.30		23.14	
	mean	27.56	(121)	19.41	(115)	27.82	(120)	21.38	(115)
PRA	12	27.63	(121)	19.03	(113)	26.85	(116)	20.81	(112)

Table 4C. Food consumption: significance of comparisons with SBA during weeks 1, 4, 8 and 12

	Males					Females				
	Diet									
Week	SBR	SBI	LBA	LMA	PRA	SBR	SBI	LBA	LMA	PRA
1	− − −	−	+ + +	+ + +	+ + +	− − −	− − −	+ + +	+ + +	+ +
4	− − −	− − −	+ +	+ +	+ + +	− − −	− − −	+ + +	+ + +	+ + +
8	− − −	− − −	+ +	N.S.	+ + +	− − −	− − −	+ + +	+ + +	+ + +
12	− − −	− − −	+ + +	+ + +	+ + +	− − −	− − −	+ + +	+ + +	+ + +

−, − − and − − − = significantly less than SBA $P < 0.05$, $P < 0.01$, $P < 0.001$.
+, + + and + + + = significantly more than SBA $P < 0.05$, $P < 0.01$, $P < 0.001$.
N.S. = not significant.

Table 4D. Food consumption: significance of comparisons with SMA during the second stage of the study

	Males				Females			
	Diet							
Week	SMR	SMI	LMA	PRA	SMR	SMI	LMA	PRA
14	− − −	N.S.	+ + +	+ + +	− − −	− − −	+ + +	+ +
24	− − −	+ + +	+ + +	+ + +	− − −	(+)	+ + +	+ +
36	− − −	+ + +	+ + +	+ + +	− − −	N.S.	+ + +	+ +
48	− − −	(+)	+ + +	+ + +	− − −	N.S.	+ + +	N.S.
60	− − −	+ + +	+ + +	+ + +	− − −	N.S.	+ + +	+ +
72	N.S.	+ + +	+ + +	+ + +	N.S.	+ + +	+ + +	+ +
84	− − −	+ + +	+ + +	+	− − −	+	(+)	+
96	− − −	+ + +	+ + +	(+)	− − −	+	(+)	(+)
108	− − −	(+)	+ +	+ +	− − −	+	+ + +	N.S.
120	− − −	−	+ + +	+	− − −	+	+ + +	(+)
128	(−)	−	+ + +	N.S.	N.S.	N.S.	(+)	N.S.

Key as for Table 4C except that comparison is with SMA instead of SBA. Also (−) and (+) indicate significance at the $P < 0.1$ level.
N.S. = not significant.

Table 5A. Median food and water consumption during weeks 4 and 12 relative to SBA (%)*

Sex	Week		Diet					
			SBA	SBR	SBI	LBA	LMA	PRA
Males	4	Food	100	71	76	104	110	112
		Water	100	87	91	121	124	128
	12	Food	100	80	83	107	123	118
		Water	100	81	90	112	119	114
Females	4	Food	100	79	82	113	126	108
		Water	100	82	86	99	108	117
	12	Food	100	81	82	108	124	111
		Water	100	76	72	98	110	106

*Correlation coefficients (r) between water and food consumption, calculated on the basis of the 12 group means, were 0.93 in males ($P < 0.001$) and 0.91 in females ($P < 0.001$) during week 4 and 0.99 in males ($P < 0.001$) and 0.94 in females ($P < 0.001$) during week 12.

Table 5B. Median food and water consumption during week 60 relative to SMA (%)*

Sex		Diet				
		SMA	SMR	SMI	LMA	PRA
Males	Food	100	79	111	119	116
	Water	100	82	94	128	133
Females	Food	100	79	103	113	109
	Water	100	77	90	113	120

*Correlation coefficients (r) between water and food consumption, calculated on the basis of the 12 group means, were 0.87 in males ($P < 0.001$) and 0.93 in females ($P < 0.001$) during week 60.

5. *Treatment of missing values*

For most endpoints, animals not providing data were omitted from the analysis. There were two main exceptions to this. The first concerned Peto 'fatal' analysis where animals with missing sections for pathological examination were counted in the at-risk population up to the week before they died. The second concerned analysis of microscopic findings for tissues for which sections were taken only if an abnormality was seen macroscopically at autopsy, when all animals were considered to be at risk.

6. *Unit of observation*

Normally the unit of observation was the animal. For food, water, energy and protein consumption, the cage was the unit of observation, with per rat data calculated for each cage.

RESULTS

Food consumption

Tables 4A and 4B show mean food consumption in the 12 groups of rats at weeks 4, 12, 24 and 60 of the study.

During the first 13 weeks of the study, restricted feeding of SB diet (SBR) achieved food intakes of between 69 and 79% SBA, the effect in males being greater at 4 than at 12 weeks. During the same period restriction by time of SB diet (SBI) led to reduction of food intake to between 76 and 85% SBA. Again the reduction was more marked in males at 4 than at 12 weeks. The consumption of LBA, LMA and PRA ranged in both sexes between 5 and 26% higher than that of SBA. The increases in food intake compared with the SBA groups were not surprising, since these three diets had a lower ME density and the rats on these three diets would have to eat more food to obtain the same amount of utilizable energy. Rats fed the PR diet compensated fully for the lower energy density but those fed LB and LM did not. It is possible that rats found the LB and LA diets unpalatable and that reduced food consumption for this reason partly counter-balanced the desire of the rats to compensate for the high fibre, low utilizable energy content of these diets.

During the second part of the study, SMR reduced food consumption to between 78 and 86% SMA during weeks 24 and 60. However, male rats on the

Table 6. Metabolizable energy (ME)* density of the diets

Sex	ME density for each diet				
	SB	SM	LM	LB	PR
Males	13.75	11.83	8.98	10.72	12.01
Females	13.80	12.22	9.11	10.64	12.11
Combined	13.78	12.03	9.05	10.68	12.06
Combined (as % SB)	100.0	87.3	65.7	77.5	87.5
Combined (as % SM)	114.6	100.0	75.2	88.8	100.3

$$*\text{ME density} = \frac{\text{gross energy intake} - \text{gross energy excreted in urine and faeces}}{\text{weight of food eaten}}$$

Table 7A. Mean estimated metabolizable energy consumption (kJ/rat/day) at weeks 4 and 12

Sex	Week		Diet SBA	SBR	SBI	LBA	LMA	PRA
Males	4	Mean	355.7	244.6	270.6	292.0	258.7	346.0
		%SBA	100	69	76	82	73	97
		Sig		− − −	− − −	− − −	− − −	N.S.
	12	Mean	341.7	270.8	291.7	288.8	280.6	354.1
		%SBA	100	79	85	85	82	104
		Sig		− − −	− − −	− − −	− − −	(+)
Females	4	Mean	234.7	186.0	192.1	210.7	195.7	225.0
		%SBA	100	79	82	89	83	96
		Sig		− − −	− − −	− − −	− − −	N.S.
	12	Mean	241.6	191.4	198.4	202.0	195.3	233.5
		%SBA	100	79	82	84	81	97
		Sig		− − −	− − −	− − −	− − −	(−)

Key for significance (Sig) as Table 4.

Table 7B. Mean estimated metabolizable energy consumption (kJ/rat/day) at weeks 24 and 60

Sex	Week		Diet SMA	SMR	SMI	LMA	PRA
Males	24	Mean	269.4	230.5	320.4	247.5	331.8
		%SMA	100	86	119	92	123
		Sig		− − −	+ + +	− − −	+ + +
	60	Mean	273.9	214.,6	301.6	249.8	322.4
		%SMA	100	78	110	91	118
		Sig		− − −	+ + +	− − −	+ + +
Females	24	Mean	205.9	169.4	219.0	176.7	230.4
		%SMA	100	82	106	86	112
		Sig		− −	+	− − −	+ +
	60	Mean	227.0	179.2	225.9	194.6	252.1
		%SMA	100	79	99	86	111
		Sig		− − −	N.S.	− − −	+ + +

Key for significance (Sig) as Table 4.

SMI regimen managed to consume 10–20% more food than SMA rats and SMI females ate the same amount as SMA rats or slightly more. LMA rats continued to eat 15–21% more than SMA rats and PRA rats also ate 12–21% more than SMA rats.

Table 4C indicates the statistical significance of differences in food consumption during weeks 1, 4, 8 and 12. Males in the LBA and LMA-fed groups during the first part of the study continued to consume significantly more food than SBA males during the first week of the second part of the study, even if they were switched to higher nutrient density diets. However, females in these groups decreased their food intake if they were switched to higher nutrient density diets.

Table 4D indicates the statistical significance of differences in food consumption during the second part of the study. Except during weeks 72 and 128, SMR rats of both sexes ate highly significantly ($P < 0.001$) less than SMA rats. During week 72, for reasons unknown, SMA rats consumed less food than at the other times when food consumption was measured. By contrast SMI rats, particularly males, managed to consume more of the SM diet during the 6 hr per day when they had access to the food hopper than SMA rats which had access to the hopper throughout the 24 hr. LMA rats consistently ate significantly ($P < 0.001$) more than SMA rats. Similarly PRA rats, particularly males, ate significantly more than SMA rats.

Table 8. Mean estimated metabolizable energy consumption per 100 g body weight (kJ/rat/day) at weeks 28 and 60

Sex	Week		Diet SMA	SMR	SMI	LMA	PRA
Males	28	Mean	69.4	57.6	79.3^{+++}	69.2	74.3^{+}
		%SMA	100	83	114	100	107
	60	Mean	52.5	54.0	63.6^{+++}	54.0	59.4^{++}
		%SMA	100	103	121	103	113
Females	28	Mean	93.9	70.8	98.8^{+}	84.0	98.9^{+}
		%SMA	100	75	105	89	105
	60	Mean	75.1	71.5	87.2^{+++}	75.6	81.7^{+++}
		%SMA	100	95	116	101	109

Key for significance levels as Table 4.

Water consumption

As expected, during both parts of the study there was a close correlation between food consumption and water consumption. Thus the lower food consumption in SBR and SBI groups compared with SBA during the first 13 weeks of the study was correlated with lower water consumption whereas the higher food consumption in LBA, LMA and PRA groups was associated with higher water consumption (see Table 5A). The same picture was apparent during the second part of the study (see Table 5B).

Animals tend to drink most during the same periods of the day as they eat most. This means that the rats on the SBR, SBI, SMR and SMI regimens, even though they had free access to water at all times, tended to drink only little after they had exhausted their daily ration of food (SBR and SMR) or after their access to the food hopper was curtailed each day (SBI and SMI). The overall reduction in water consumption in these groups, combined with the marked reduction in water consumption during the extensive periods of each day when no food was available, is thought to have contributed to the higher incidence of ringtail in these groups (see below).

Energy intake

Before the start of the main experiment, the ME density of the five diets, expressed as kilojoules per gram of diet (kJ/g) was measured by one of us (GT, as described in Appendix 1). Thirty male and 30 female 3-wk-old SKF Wistar rats were used in this separate study. The rats were housed in pairs in a controlled-environment room. Each diet was fed to each of three pairs of male and female rats. The rats were given 14 days to acclimatize to the diets; thereafter for 7 days food intake was measured, and urine and faeces collected. The gross energies of the diets and of the dried urine and faeces were determined by adiabatic bomb calorimetry and from these determinations the ME density of the diets was determined (McLean and Tobin, 1987). The results, as shown in Table 6, indicate that the ME of SM, LM, LB and PR are only 87.3, 65.7, 77.5 and 87.5%, respectively, of that of SB, and that whereas the ME of PR and SM are similar, the ME of LM is only about 75% of that of SM.

Using these data for the ME of the various diets, the mean daily energy consumption per rat was calculated for each diet. In Table 7A, energy consumption in animals on the six different dietary regimens used during the first part of the study (weeks 4 and 12) is shown, both as means expressed in kJ/rat/day and as percentages of that of SBA. In Table 7B comparable data are given for the second part of the study (weeks 24 and 60) with, in this case, the percentages being those of SMA.

During the first 13 weeks of the study, when rats were growing most rapidly, limiting access to SM diet to 6 hr per day (SBI) effectively reduced ME consumption to about 80% SBA. However, during the second part of the study this form of dietary restriction was not effective in reducing energy intake (c.f. SMI *v.* SMA in Table 7B). By contrast, SMR effectively reduced energy intake compared with SMA. The other point that is noteworthy is that, despite the higher consumption of food by rats on the LMA regimen, their mean daily energy intakes were always 10–20% lower than those of SMA rats. Here it is relevant to point out that whereas there is evidence that animals regulate ME intake by adjusting food intake to compensate for variation in energy density (see Hervey and Tobin, 1983), for high fibre diets physical limitations may prevent total compensation (Forbes, 1983).

In a study such as this where treatments result in widely divergent rates of weight gain, it can be difficult to interpret differences in food and energy intake, which may reflect simply differences in body weight and thus energy requirements. In an attempt to eliminate this possibility, it is conventional to adjust food or energy intake to a unit of body weight, for example per 100 g body weight or to body weight raised to the power 0.75 (or similar value). The latter figure is an estimate of metabolic body size or lean mass; in this experiment it is unlikely, because of treatment effects, that lean body mass is simply related to body weight$^{0.75}$. In the absence of any more valid basis for comparison, we have adjusted energy intakes to a simple unit of body weight; data for weeks 28 and 60 for rats on the five regimens are given in Table 8.

The following conclusions may be drawn from these data:

First, the rats fed restricted amounts of diet appear, in general, to need less energy per 100 g body weight than animals fed the same diet *ad lib.* This suggests that diet restriction by amount may lead to an increased efficiency of energy utilization for maintenance and growth, possibly resulting from a reduced rate of protein turnover in the body, a process that is known to be expensive of energy. This is consistent with findings in animals of agricultural importance (Blaxter, 1989).

Secondly, the time-restricted-fed animals appeared to require more energy per unit of body weight than the *ad lib.*-fed animals. While it may be correct that these animals do require more energy per unit of body weight (i.e. that they have a raised level of metabolism), it is also possible that the data are misleading. When the ME density of the diets was determined, all diets were fed *ad lib.* It is possible that a large bolus of food may not be digested as efficiently as the same amount of food eaten more gradually, as, for example, in *ad lib.* feeding (Conybeare, 1988). If this is correct, the ME density of diets fed in a time-restricted regimen will be overestimated.

Thirdly, while the rats fed the high fibre diet (LM) only partially compensated for the lower ME density

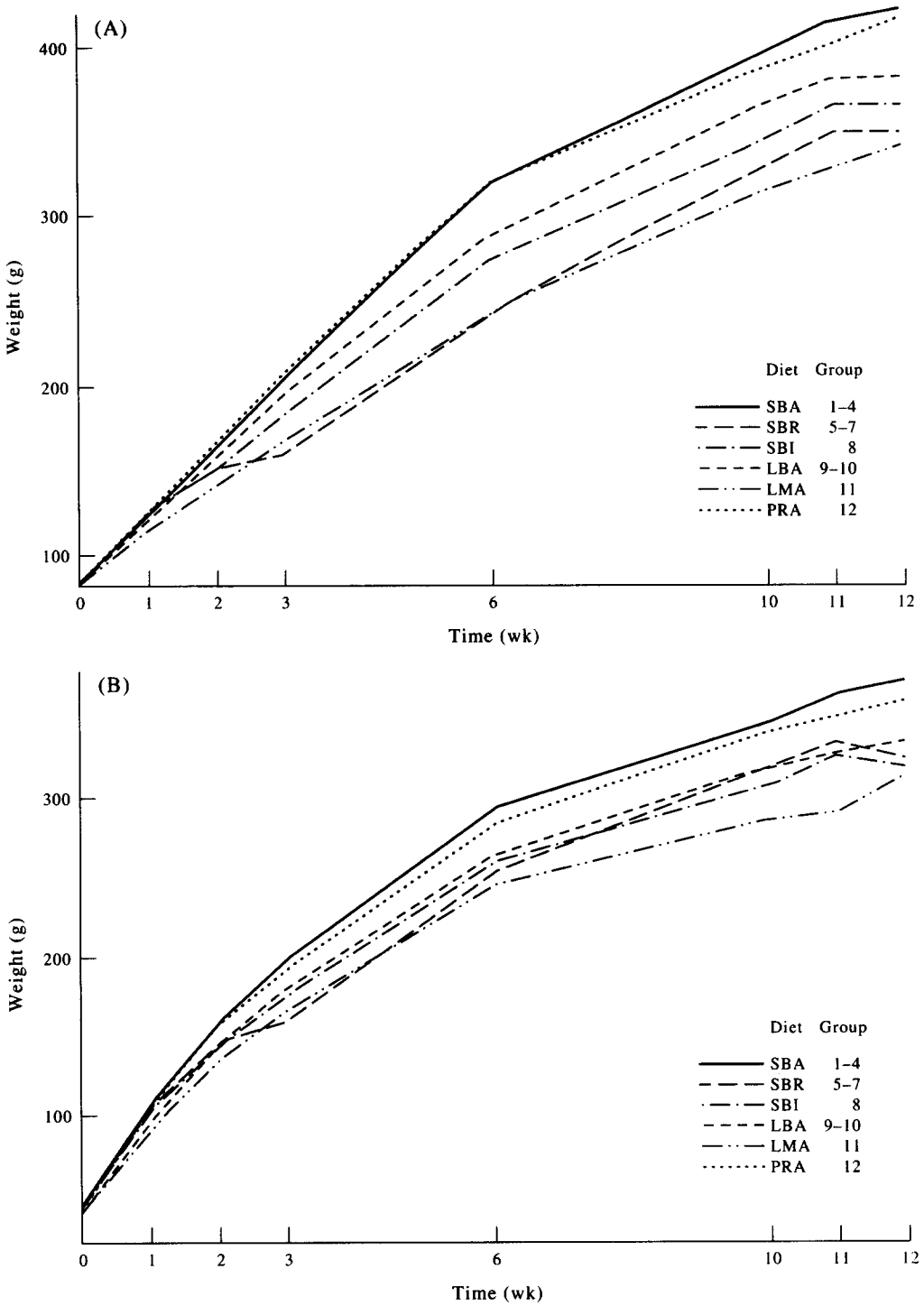

Fig. 1. Body weight—effects of diet during first phase of study: (A) males, (B) females.

by increasing food intake, the intake of ME per 100 g body weight generally was similar to that in the rats fed the SM diet *ad lib.*, suggesting little difference in the utilization of energy.

Finally, the animals fed the PR diet *ad lib.* appeared to require slightly more ME per 100 g body weight than those fed the SM diet. While one can only speculate as to the reason, it is possible that the higher protein content of the PR diet (*c.* 20% *v.* 14%) would lead to a higher protein turnover (though not deposition) which is metabolically very expensive.

At 28 weeks energy consumption per 100 g body weight was lower in SMR than SMA groups but at 60 weeks there was little difference.

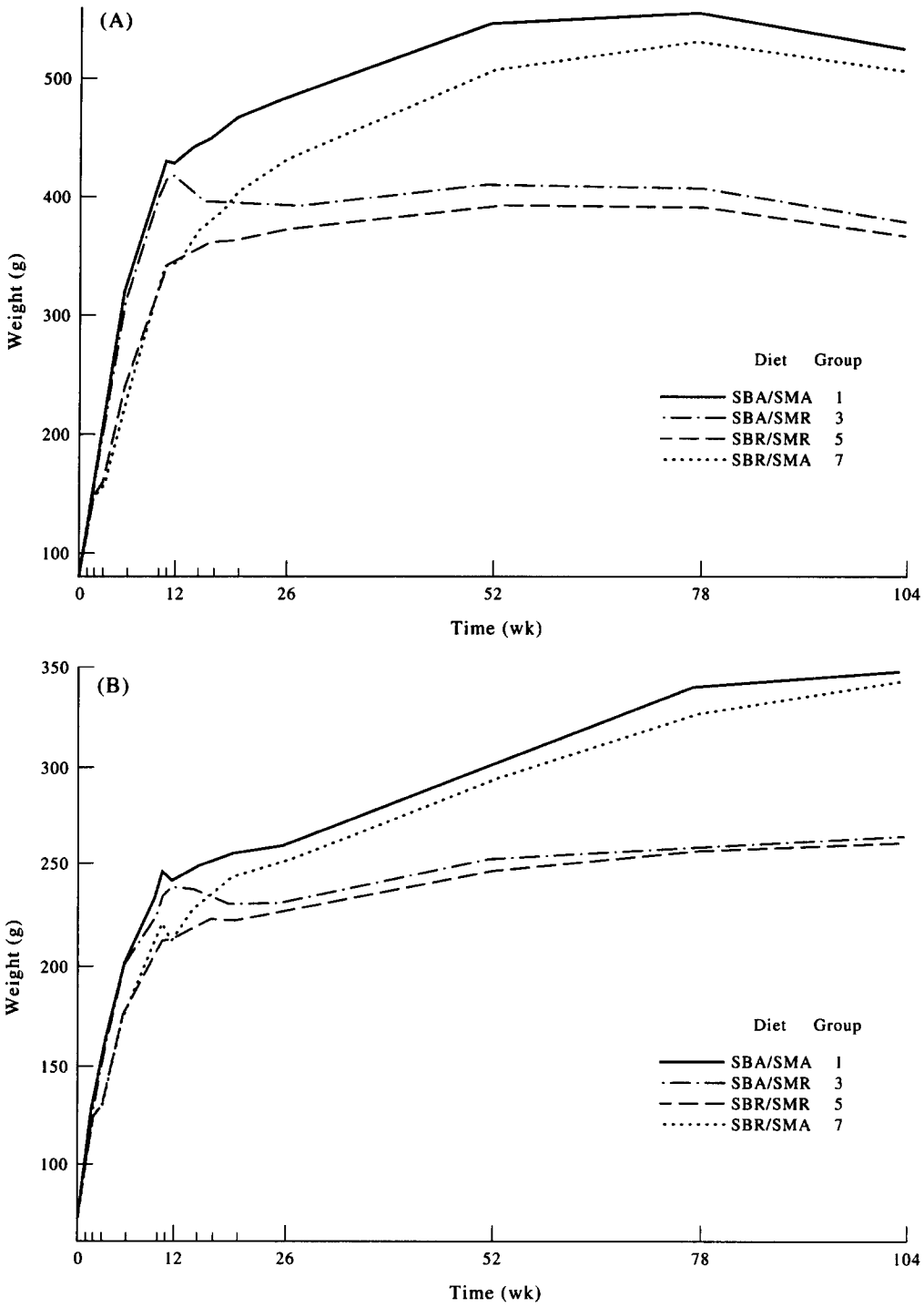

Fig. 2. Body weight—effects of dietary restriction: (A) males, (B) females.

Protein consumption

During the first 13 weeks of the study protein consumption was significantly (usually $P < 0.001$) lower in males of the SBR, SMI, LBA and LMA groups than in males of SBA groups. In females the difference was much less marked for LBA and LMA compared with SBA. In both sexes, PRA rats consistently consumed significantly ($P < 0.001$) more protein than SBA rats.

During the second part of the study SMR rats of both sexes consistently consumed less protein than SMA rats ($P < 0.001$). Whereas LMA and PRA rats consistently consumed more protein than SMA rats ($P < 0.001$), SMI males tended to eat more protein than SMA males, but there was no significant

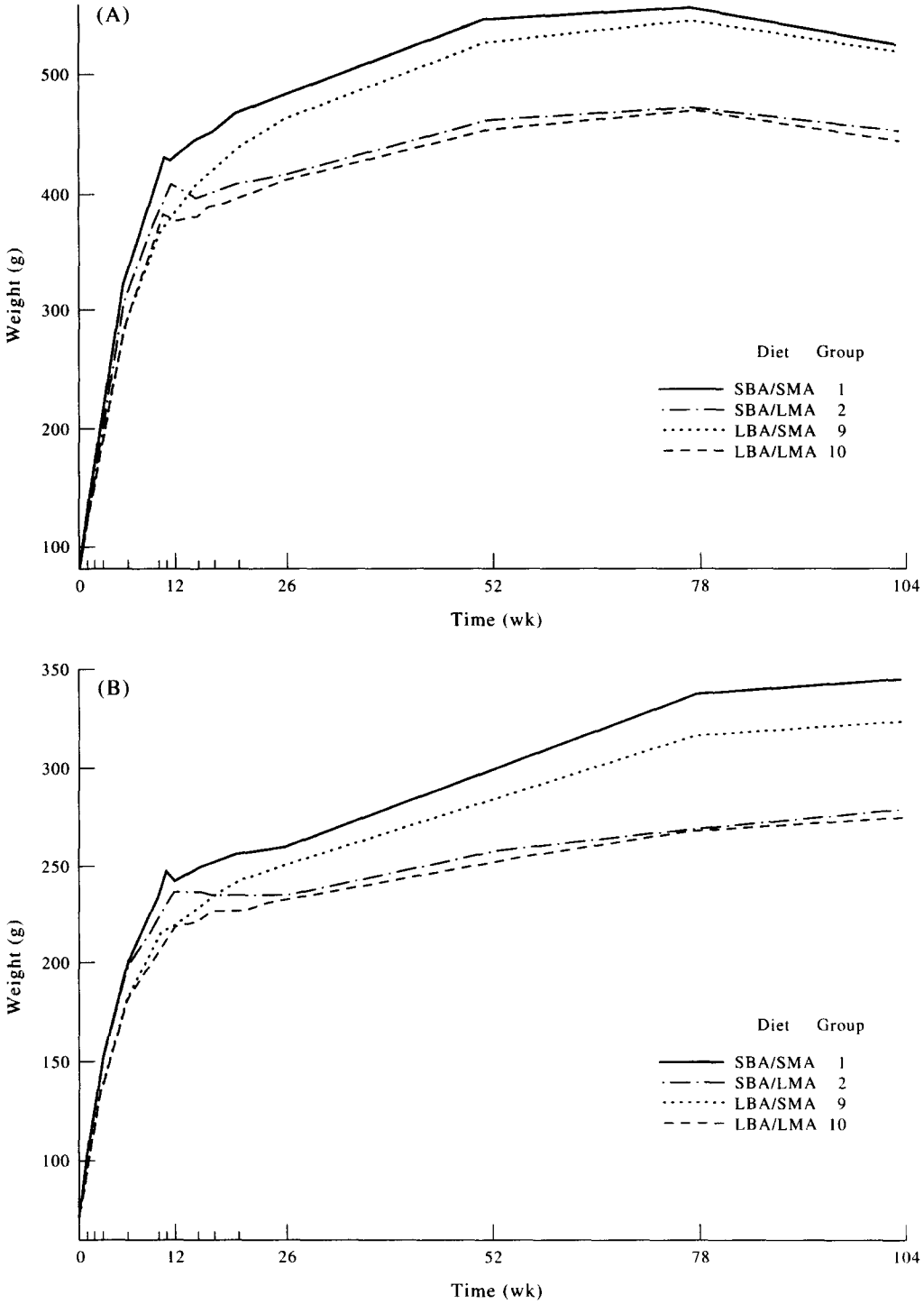

Fig. 3. Body weight—effects of low energy diet: (A) males, (B) females.

differences between the females in these groups in protein consumption.

The relationships between protein intake and incidence/severity of chronic nephropathy are discussed below.

Body weight

In interpreting live body weights it is important also to appreciate the impact of the weight of gut contents, particularly in a study such as this where the relative contribution of gut contents to body weight will differ with diet and type of feeding regimen (e.g. LMA *v.* SMR). For example, true tissue weight may be 20–30 g or more lower than apparent body weight in rats fed LMA and weighed in the morning after a night's feeding while, for those rats restricted by amount or time, the gut may be almost empty by early morning. Notwithstanding this

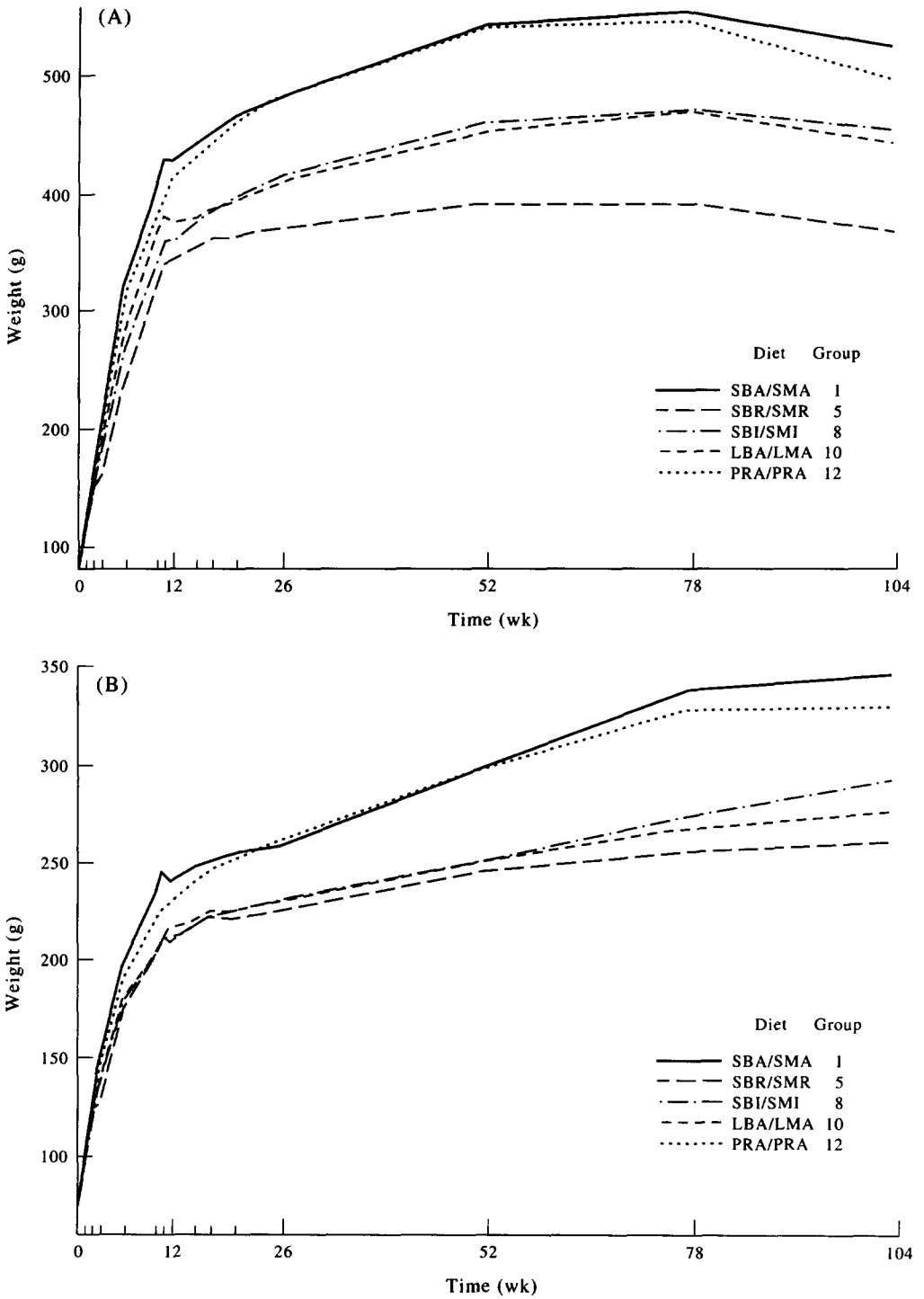

Fig. 4. Body weight—comparison of different dietary regimens: (A) males, (B) females.

difficulty in interpretation, there was generally a close relationship between food consumption and body weight gain during both stages of the study.

Figures 1A and 1B compare the body weights of rats fed SBA, SBR, SBI, LBA, LMA and PRA during the first 13 weeks of the experiment. Animals fed SBR (groups 5, 6 and 7) or SBI (group 8) gained significantly less weight than animals fed SBA (groups 1–4). Groups fed LBA (groups 9 and 10) or LMA (group 11) during this period also gained significantly less weight than those fed SBA. In both sexes, rats fed PRA had a slightly but significantly lower weight gain (up to $P < 0.01$ in males and up to $P < 0.001$ in females) than those fed SBA, which reflected the slightly greater estimated ME intake of the SBA group.

Table 9A. Haematology: effect of diet from week 13 in comparison with SMA (unadjusted for earlier diet)—males

		Median for each diet				
	Month	SMA	SMR	SMI	LMA	PRA
Total white cell count (10^3 mm $^{-3}$)	6	5.10	4.10	4.70$^{N.S.}$	4.90$^{N.S.}$	5.80^{+}
	12	5.80	4.30	4.85	5.80$^{N.S.}$	6.65$^{N.S.}$
	18	6.05	4.10	5.00	5.30	7.30^{+}
	24	7.10	4.45	5.60	6.70$^{N.S.}$	6.40$^{N.S.}$
	30	7.00	4.50	5.10	6.10	6.65$^{N.S.}$
Total red cell count (10^6 mm $^{-3}$)	6	8.68	8.61	8.68$^{N.S.}$	8.78$^{N.S.}$	8.86$^{N.S.}$
	12	8.78	8.40	8.66$^{N.S.}$	8.63$^{(-)}$	8.64$^{N.S.}$
	18	9.00	8.65	8.90	8.90$^{(-)}$	8.95$^{N.S.}$
	24	9.10	8.90$^{N.S.}$	9.30$^{N.S.}$	8.80$^{N.S.}$	8.90$^{N.S.}$
	30	8.10	8.80^{+++}	8.50^{+}	8.40$^{(+)}$	8.25$^{N.S.}$
Haemoglobin (g/dl)	6	15.90	15.65	15.85$^{N.S.}$	16.10^{+}	16.00$^{N.S.}$
	12	15.70	15.40	15.70$^{N.S.}$	15.60$^{N.S.}$	15.75$^{N.S.}$
	18	16.15	15.40	16.00$^{N.S.}$	16.00$^{N.S.}$	16.30$^{N.S.}$
	24	15.70	15.30$^{N.S.}$	15.90$^{(+)}$	15.50$^{N.S.}$	15.95$^{N.S.}$
	30	14.10	15.20^{+++}	15.10^{+++}	14.60^{+}	13.75$^{N.S.}$
Platelets (10^3 mm $^{-3}$)	6	897.00	979.50^{++}	955.00$^{N.S.}$	985.00^{+++}	1013.00^{+++}
	12	861.50	946.00^{+++}	868.00$^{N.S.}$	944.00^{+++}	979.50^{+++}
	18	980.00	1070.00^{+++}	1000.00$^{N.S.}$	1040.00$^{N.S.}$	1205.00^{+++}
	24	1080.00	1135.00$^{N.S.}$	1040.00$^{N.S.}$	1050.00$^{N.S.}$	1195.00$^{N.S.}$
	30	1120.00	1060.00	1085.00$^{N.S.}$	1100.00$^{N.S.}$	1155.00$^{N.S.}$

Key for significance levels as Table 4.

Table 9B. Haematology: effect of diet from week 13 in comparison with SMA (unadjusted for earlier diet)—females

		Median for each diet				
	Month	SMA	SMR	SMI	LMA	PRA
Total white cell count (10^3 mm $^{-3}$)	6	3.80	3.00^{-}	2.90^{--}	4.10$^{N.S.}$	3.30$^{(-)}$
	12	4.00	3.50^{-}	3.40^{-}	3.80$^{N.S.}$	4.10$^{N.S.}$
	18	4.10	3.60	3.70	3.40	3.40
	24	5.15	3.80^{--}	3.70	4.30	3.95
	30	5.10	4.10^{--}	4.00^{-}	4.80$^{N.S.}$	4.30
Total red cell count (10^6 mm $^{-3}$)	6	8.17	7.84	8.10$^{(-)}$	8.09$^{(-)}$	8.13$^{N.S.}$
	12	8.00	7.80	7.80$^{N.S.}$	7.90$^{N.S.}$	8.06$^{N.S.}$
	18	8.20	8.10^{-}	8.0$^{(-)}$	8.10$^{(-)}$	8.20$^{N.S.}$
	24	8.10	7.80	7.80$^{(-)}$	7.80	8.20$^{N.S.}$
	30	7.70	7.60	7.65	7.40	7.60$^{N.S.}$
Haemoglobin (g/dl)	6	15.70	15.30	15.50	15.60	15.80$^{N.S.}$
	12	15.50	15.20	15.30	15.40$^{(-)}$	15.90$^{N.S.}$
	18	15.90	15.70$^{(-)}$	15.60$^{(-)}$	15.70	15.75$^{N.S.}$
	24	15.15	14.70	14.80	15.00$^{(-)}$	15.60$^{N.S.}$
	30	14.40	14.20	14.25$^{(-)}$	13.90	14.70$^{N.S.}$
Platelets (10^3 mm $^{-3}$)	6	945.00	1007.00^{+}	1012.50^{+}	974.50$^{N.S.}$	941.00$^{N.S.}$
	12	848.00	909.50^{+}	907.00^{+}	850.00$^{N.S.}$	866.00$^{N.S.}$
	18	940.00	960.00$^{N.S.}$	1000.00$^{N.S.}$	830.00	940.00$^{N.S.}$
	24	955.00	980.00$^{N.S.}$	1000.00$^{N.S.}$	920.00$^{N.S.}$	960.00$^{N.S.}$
	30	1030.00	1000.00$^{N.S.}$	990.00$^{N.S.}$	1000.00$^{N.S.}$	990.00$^{N.S.}$

Key for significance levels as Table 4.

Between-diet comparisons of body weight gain after week 13 is complicated by the fact that groups that were destined to receive the same diet thereafter differed in mean body weight at the time of the changeover because they had been fed differently during the first stage of the study. Further, average body weight data may have been slightly biased by the premature death of the heavier, faster growing, rats. This phenomenon may account for the apparent fall in body weight towards the end of the study, particularly evident in males in groups 1 and 7. However, the data displayed in Figures 2A and 2B, which compare body weight gain throughout the study in groups 1, 3, 5 and 7 and those in Figures 3A and 3B, which show the same comparison for groups 1, 2, 9 and 10, enable the following conclusions to be drawn. In both sexes, SMR after week 13 (groups 3 and 5) was associated with lower weight gain than SMA after week 13 (groups 1 and 7) and LMA after week 13 (groups 2 and 10) was also associated with less weight gain than SMA after week 13 (groups 1 and 9). However, males of groups 7 and 9 came close to making good the body weight deficits of 85 and 42 g, respectively, that they exhibited at 13 weeks. The same is also true for females of group 7 which had a 20 g body weight deficit at 13 weeks.

Figures 4A and 4B compare the patterns of weight gain throughout life in groups 1, 5, 8, 10 and 12. These groups were selected for comparison because: (i) group 1 (SBA/SMA) and group 12 (PRA/PRA) are the same as or similar to commonly used dietary regimens with the latter involving a higher intake of protein than the former;

Table 10A. Clinical chemistry: effect of diet from week 13 in comparison with SMA (unadjusted for earlier diet)—males

		Median for each diet				
	Month	SMA	SMR	SMI	LMA	PRA
Total plasma protein (g/litre)	6	74.00	68.50	71.00	69.00	77.00^{++}
	12	75.00	69.50	71.50	71.00	76.00$^{N.S.}$
	18	76.00	70.50	73.00	73.00	75.00$^{N.S.}$
	30	69.50	71.00$^{N.S.}$	70.50$^{N.S.}$	69.00$^{N.S.}$	68.00$^{N.S.}$
Albumin (g/litre)	6	38.00	35.00	37.00	36.00	38.00$^{N.S.}$
	12	36.00	34.00	36.00	35.00	35.00
	18	34.00	34.00$^{N.S.}$	34.00$^{N.S.}$	35.00$^{N.S.}$	33.00
	30	31.50	33.00^{+++}	32.00^{+}	31.50$^{N.S.}$	29.50$^{N.S.}$
Blood urea nitrogen (mmol/litre)	6	7.40	6.15	6.70	8.15^{++}	8.70^{+++}
	12	7.20	6.85$^{N.S.}$	6.70$^{(+)}$	6.65	8.60^{+++}
	18	6.45	5.90	6.20$^{N.S.}$	6.20$^{N.S.}$	7.40^{+++}
	30	6.60	7.30$^{N.S.}$	6.90$^{N.S.}$	7.30^{++}	8.05^{+}
Creatinine (μmol/litre)	6	75.00	70.00	73.00	76.00$^{N.S.}$	72.00$^{N.S.}$
	12	70.00	67.00	70.00$^{N.S.}$	71.00$^{N.S.}$	76.00^{+++}
	18	59.00	65.00^{+++}	58.00$^{N.S.}$	60.00$^{(+)}$	67.00^{+++}
	30	62.00	58.00	58.50	61.00$^{N.S.}$	69.50$^{N.S.}$
Glucose (mmol/litre)	6	7.10	5.85	6.90$^{N.S.}$	7.00$^{N.S.}$	8.10$^{N.S.}$
	12	7.45	6.20	6.90	7.00	7.40$^{N.S.}$
	18	7.50	6.25	6.80$^{(-)}$	6.80	7.00$^{N.S.}$
	30	10.15	11.30^{++}	10.45$^{N.S.}$	9.35	9.85$^{N.S.}$
AAT (IU/litre)	30	56.00	70.00^{+++}	73.00^{+++}	118.00^{+++}	65.00^{+}
APC (IU/litre)	30	42.00	51.00^{+++}	47.00$^{(+)}$	54.00^{+++}	54.00^{+}
G6-PD (IU/litre)	30	42.50	55.80$^{(+)}$	59.30$^{N.S.}$	77.10^{+++}	39.20$^{N.S.}$
AATA (IU/litre)	30	130.00	149.00^{+}	144.00$^{N.S.}$	200.00^{+++}	144.00$^{N.S.}$

AAT = alanine aminotransferase activity APC = alkaline phosphatase activity G6-PD = glucose-6-phosphate dehydrogenase AATA = aspartate aminotransferase activity

Key for significance levels as Table 4.

Table 10B. Clinical chemistry: effect of diet from week 13 in comparison with SMA (unadjusted for earlier diet)—females

		Median for each diet				
	Month	SMA	SMR	SMI	LMA	PRA
Total plasma protein (g/litre)	6	75.00	66.00	74.00$^{N.S.}$	69.00	78.50^{++}
	12	79.00	72.00	76.00	72.00	78.00$^{N.S.}$
	18	79.50	73.00	78.00$^{(-)}$	78.00	77.00$^{N.S.}$
	30	72.00	75.00^{++}	74.00$^{N.S.}$	72.00$^{N.S.}$	71.00$^{N.S.}$
Albumin (g/litre)	6	40.00	35.00	40.00$^{N.S.}$	38.00	41.50^{+}
	12	40.00	38.00	40.00$^{N.S.}$	38.00	39.50$^{N.S.}$
	18	38.00	37.00	38.00$^{N.S.}$	39.00^{+}	38.00$^{N.S.}$
	30	34.00	37.00^{+++}	35.00^{++}	35.00^{++}	32.00
Blood urea nitrogen (mmol/litre)	6	8.10	9.90^{+++}	9.00^{++}	8.80^{+}	10.15^{+++}
	12	8.30	10.50^{+++}	9.60$^{(+)}$	8.30$^{N.S.}$	7.65$^{N.S.}$
	18	6.60	8.60^{+++}	7.10^{++}	7.20^{++}	7.40$^{N.S.}$
	30	6.10	8.80^{+++}	7.95^{+++}	7.80^{+++}	6.95^{+}
Creatinine (μmol/litre)	6	78.50	83.00$^{N.S.}$	84.00^{+}	77.00$^{N.S.}$	88.50^{+}
	12	74.00	78.00$^{N.S.}$	69.00	73.00$^{N.S.}$	66.50
	18	59.00	62.00$^{N.S.}$	61.00^{+}	61.00$^{(+)}$	55.00$^{N.S.}$
	30	55.00	58.00^{+}	55.00$^{N.S.}$	56.00$^{N.S.}$	53.00$^{N.S.}$
Glucose (mmol/litre)	6	7.15	4.80	5.20	7.10$^{N.S.}$	7.60$^{(+)}$
	12	7.50	6.20	6.30	7.40$^{N.S.}$	7.30$^{N.S.}$
	18	7.20	6.20	6.60	6.90$^{N.S.}$	8.10^{++}
	30	9.70	9.30$^{N.S.}$	10.20$^{N.S.}$	8.90$^{(-)}$	10.15$^{N.S.}$
AAT (IU/litre)	30	53.50	69.00^{+++}	71.50^{++}	120.00^{+++}	71.00$^{(+)}$
APC (IU/litre)	30	22.00	29.00^{+++}	26.50^{++}	29.00^{+++}	31.00^{++}
G6-PD (IU/litre)	30	51.60	57.50^{+}	57.00$^{(+)}$	106.25^{+++}	42.95$^{N.S.}$
AATA (IU/litre)	30	132.00	195.00^{+++}	175.00^{+++}	188.00^{+++}	130.50$^{N.S.}$

AAT = alanine aminotransferase activity APC = alkaline phosphatase activity G6-PD = glucose-6-phosphate dehydrogenase AATA = aspartate aminotransferase activity

Key for significance levels as Table 4.

(ii) group 5 (SBR/SMR) is similar to group 1 except that animals were restricted to a food intake of 80% *ad lib.* continuously throughout life; (iii) group 8 (SBI/SMI) is also similar to group 1 except that access to food was restricted to a period of 6 hr per day throughout life; and (iv) although group 10 (LBA/LMA) is not directly comparable with any of the other groups, it illustrates what happened in terms of body weight gain in rats that were continuously provided with a non-extreme low nutrient, high fibre diet throughout life.

For the groups fed SMR from week 13, reduction in body weight was correlated closely with the reduction in energy consumed. Similarly, body weights

were significantly lower in LMA than in SMA groups, and this was associated with a lower ME intake with LMA (see Table 7B).

It is interesting that diet restriction by a method which reduced weight gain during the first 13 weeks of the study resulted in lower body weight up to a year or more later, even though free access to the standard diet was offered from 13 weeks onwards.

As will be pointed out later (see below) body weight early in life was a much better predictor of survival and tumour incidence than either food consumption or energy intake.

Haematological findings

Since the first blood sampling time was 6 months after the start of the study, there are no data that relate directly to the diet fed during the first 13 weeks of the study. However, by comparing groups fed according to the same regimen after 13 weeks but differently before the changeover, it was possible to ascertain that diet before week 13 had few or no meaningful lasting effects on any of the haematological parameters measured at 6, 12, 18, 24 or 30 months. At some of these time points, WBC were significantly lower in animals of both sexes fed SBR or LBA during the first 13 week, and in females (but not males) Hb and Hct levels were significantly higher in animals fed SBR during the first part of the study. Mean red cell volume also tended to be higher in rats of both sexes fed SBR during the first part of the study, but this apparent effect was only evident in the blood samples taken at 6 and 12 months. These persistent effects of SBR on Hb and red cell parameters were reflected in differences in derived values such as mean red cell Hb concentration.

Diet after 13 weeks profoundly affected many haematological parameters as shown in Tables 9A and 9B. These tables show the significance values of the differences between dietary regimens after week 13 without correction for diet before week 13.

In both sexes, SMR and SMI were associated with markedly lower total WBC than SMA throughout the study, while LMA rats had lower WBC from 18 months until the end of the study. Except in males at 30 months, total RBC and Hb were also significantly reduced in SMR rats of both sexes throughout the study. In females but not in males LMA was consistently associated with significantly lower RBC and Hb levels than SMA. Platelet counts, on the other hand, were significantly higher in SMR males at 6, 12 and 18 months, in LMA males at 6 and 12 months and in PRA males at 6, 12 and 18 months. Apart from this apparent effect on platelet counts, the blood picture in PRA rats was similar to that in SMA rats throughout the study.

The data for differential WBC and the other parameters listed in the Materials and Methods section showed no very clearly discernible patterns. More detailed haematological data for the 12 groups of animals are stored on computer and can be supplied on request.

Clinical chemistry

Since blood sampling was not begun until 6 months into the study, no data relevant to diet before 13 weeks are available. Tables 10A and 10B compare the effects of different diets at 6, 12, 18 and 30 months on the assumption that diet during the first 13 weeks of the study had no permanent effect on any of the parameters considered. This assumption was warranted in the light of comparisons of the values in groups fed in a similar way after week 13 but in a dissimilar way before week 13. Unfortunately a breakdown in the freezer in which the 24-month samples were stored led to most plasma samples taken at this time being lost.

1. *Plasma protein*

Plasma protein was reduced up to 18 months by all forms of calorific restriction. This reduction was highly significant in both sexes for the SMR diet ($P < 0.001$) and for the LMA diet ($P < 0.01$ or 0.001). It was less marked in SMI-fed rats but still significant in most analyses. In the females fed SMR there was a significant ($P < 0.001$) increase in protein levels at 30 months. PRA increased plasma protein significantly ($P < 0.01$) at 6 months.

2. *Plasma albumin*

Both in the SMR- and LMA-fed males and females earlier blood samples (6 and 12 months) showed highly significantly ($P < 0.001$) reduced plasma albumin levels. At 30 months, however, this effect had reversed, with increased levels in all groups except the LMA-fed males. Since albumin is one of the soluble blood proteins it is not surprising that these results reflect similar effects seen in total protein. The male SMI-fed rats also showed, to a lesser extent, a similar pattern, but the females fed SMI only showed the significant ($P < 0.01$) increase at 30 months.

There was no consistent effect of PRA.

3. *Blood urea nitrogen (BUN)*

The general trend was for PRA to increase BUN throughout the study. Although in the females it was not always significant (12 and 18 months), in the males it was highly significant ($P < 0.001$) at 6, 12 and 18 months and still just significant ($P < 0.05$) at 30 months. This reflects the increased metabolism of the animals on a high protein diet.

In the animals fed SMR, BUN was consistently high in the females ($P < 0.001$). In the males it was reduced significantly at 6 months ($P < 0.001$), but showed no clear difference from SMA subsequently. Differences between SMI- and SMA-fed animals were in the same direction as differences between

SMR- and SMA-fed animals, but the magnitude of the difference was generally smaller.

4. *Creatinine*

There were no consistently different diet-related effects seen in the plasma creatinine levels, although a number of highly significant ($P < 0.001$) differences were seen between the diets.

5. *Glucose*

As expected, SMR significantly ($P < 0.001$) reduced glucose levels in males and females up to 24 months and to a lesser extent so did SMI, particularly in the females. The trend for LMA to reduce plasma glucose slightly was more consistent in the males, but this was not always significant.

PRA-fed animals had similar or slightly higher (females at 18 months) glucose levels than SMA-fed rats.

6. *End-of-test plasma enzymes*

The effect of diet to week 13 had no clear effects on plasma enzymes at the end of test. As with previous parameters, the effects of diets fed after week 13 were large.

(a) *Alanine aminotransferase (ALT).* This enzyme was increased significantly in both males and females ($P < 0.01$ or 0.001) in all groups fed any form of restricted regimen (SMI, LMA, SMR). There was also a smaller increase in the PRA-fed animals, significant in the males ($P < 0.05$) and almost significant in females ($P < 0.1$).

(b) *Alkaline phosphatase (ALP).* All groups of animals, both males and females, had increased alkaline phosphatase activity, when compared with the SMA-fed rats, although this did not reach significance in the SMI-fed males. The most highly significant ($P < 0.001$) increases were in the SMR- and LMA-fed groups, that is those with a low total energy intake.

(c) *Glucose-6-phosphate dehydrogenase (GLDH).* Very highly significant ($P < 0.001$) increases were seen in the LMA-fed group. These animals were fed low fat, low protein but high carbohydrate diet. Some evidence of an increase was also seen in the SMR- and SMI-fed groups.

(d) *Aspartate aminotransferase (AST).* The results in males followed a similar pattern to GLDH, but in the females all groups except PRA-fed animals had increased enzyme activity when compared with the SMA-fed group.

(e) *Overview of effects on clinical chemistry parameters.* Blood albumin levels declined slowly with time in the animals fed SMA. Although the decline was very slight, it showed up as being significant because there was no decline in the restricted-fed animals and there was only a very slight decline in the animals fed a time-restricted regimen or low density diets.

Initially, BUN was reduced significantly in the restricted-fed male animals (6 months) and increased in the low density diets, but as time progressed these differences declined so that by 30 months there were no significant differences between the groups. In the females all groups on any form of restriction had significantly higher BUN levels than the animals fed SMA.

No consistent differences were seen in blood creatinine levels, or in the urine of the selected groups, despite the changes in paracetamol metabolism.

As expected, blood glucose was lower in the restricted- and time-restricted-fed animals, except at 30 months when there were no significant differences in the males and a slight reversal of the values in the females. These results can be explained almost totally by the feeding patterns of the animals and when the blood samples were taken. Routine 'tail bleed' samples were taken between 10.00 and 11.00 hr but the end-of-test samples were taken throughout the day at autopsy. Higher glucose levels would be expected in animals that consumed food shortly before sampling.

Almost all the changes seen in blood clinical chemistry of the PRA- and SMA-fed animals can be accounted for by the increased metabolism of high energy/high protein diets fed to these animals.

Increased enzyme GLDH activity seen at the end of test in animals fed LMA can be accounted for by the reduced energy and protein and the higher carbohydrate in this diet.

Urinalysis findings

The results of urinalysis at 6, 12, 18, 24 and 30 months are relevant to two aspects of the histopathological findings. First, the different dietary regimens were associated with differences in incidence of two forms of nephrocalcinosis, namely corticomedullary nephrocalcinosis and pelvic nephrocalcinosis (see below). In this case, it is clear that diet during the first 13 weeks of the study was an important determining factor. Secondly, the incidence and severity of chronic progressive nephropathy seen in late decedents and terminally killed rats were significantly lower in animals on calorie-restricted regimens during the second phase of the study. One would expect this lower incidence/severity to be associated with lower levels of protein in the urine.

Since the first sampling time was not until 3 months after the start of the second phase of the study, there are no data that relate directly to diet during the first 13 weeks of the study. However, by comparing groups fed similarly after 13 weeks but differently before 13 weeks, it was possible to detect certain apparently persistent effects of earlier diet as follows (results not shown in detail):

(i) Compared with SBA, SBR during the first 13 weeks was, in males, associated with significantly reduced urinary protein levels at 6

Table 11A. Urinalysis: effect of diet from week 13 in comparison with SMA (unadjusted for earlier diet)—males

		Diet				
	Month	SMA	SMR	SMI	LMA	PRA
Volume (ml)	6	1.00	2.20^{+++}	2.00^{+++}	$1.50^{(+)}$	2.00^{++}
median	12	1.60	2.00^{+}	2.00^{+}	1.50	2.00^{+}
	18	1.50	2.10^{+++}	2.40^{+++}	1.60	2.60^{++}
	24	2.00	2.50^{+}	2.30^{+}	1.65	4.05^{+++}
	30	2.60	3.10	2.40	2.60	3.55^{+}
pH	6	5.88	7.48^{+++}	7.18^{+++}	6.45^{+++}	6.00
mean	12	6.16	7.95^{+++}	7.70^{+++}	7.03^{+++}	6.05
	18	6.15	8.18^{+++}	7.64^{+++}	6.67^{+++}	6.24
	24	6.26	8.24^{+++}	7.33^{+++}	6.64^{+}	6.00
	30	6.30	7.35^{+++}	7.06^{+++}	6.26	6.45
Protein	6	10.3	0.0^{--}	0.0	1.3^{-}	35.0^{+++}
% with score 2 or 3	12	44.8	0.0^{---}	17.5^{-}	10.8^{---}	95.0^{+++}
	18	86.5	5.1^{---}	53.9^{---}	34.3^{---}	100.0
	24	92.1	6.1^{---}	66.7^{-}	50.0^{---}	91.7
	30	89.4	15.0^{---}	77.1	41.2^{---}	90.9
Ketones	6	24.1	7.5^{-}	25.0	41.0^{+}	$45.0^{(+)}$
% with	12	20.7	27.5	40.0^{+}	67.6^{+++}	$40.0^{(+)}$
	18	55.8	$74.4^{(+)}$	76.9^{+}	68.6	41.2
	24	76.3	$57.6^{(-)}$	88.9	80.4	33.3^{--}
	30	46.8	50.0	$68.6^{(+)}$	69.1^{+}	$18.8^{(-)}$
Blood	6	15.5	2.5^{-}	5.0	5.1^{-}	15.0
% with	12	13.8	7.5	$2.5^{(-)}$	9.5	45.0^{++}
	18	40.4	5.1^{---}	5.1^{---}	18.6^{-}	41.2
	24	57.9	3.0^{---}	19.4^{--}	19.6^{---}	75.0
	30	38.3	22.5	25.7	$55.9^{(+)}$	63.6

Key for significance levels as Table 4.

Table 11B. Urinalysis: effect of diet from week 13 in comparison with SMA (unadjusted for earlier diet)—females

		Diet				
	Month	SMA	SMR	SMI	LMA	PRA
Volume (ml)	6	1.35	2.00^{+++}	2.00^{+++}	1.20	1.30
median	12	1.30	2.00^{+++}	2.00^{+++}	1.30	1.35
	18	2.00	1.80	2.80^{+++}	1.80	2.00
	24	2.20	2.20	3.00^{+++}	1.80	3.10^{++}
	30	3.10	2.75	3.65	3.05	3.80
pH	6	5.70	7.18^{+++}	6.88^{+++}	6.12^{++}	5.53
mean	12	5.79	7.66^{+++}	7.21^{+++}	6.12^{+}	5.65
	18	6.05	7.68^{+++}	7.26^{+++}	5.95	5.84^{-}
	24	6.00	7.80^{+++}	6.89^{+++}	6.11	5.71^{-}
	30	5.98	7.18^{+++}	6.58^{+++}	5.87	5.78^{-}
Protein	6	0.0	$0.0^{(-)}$	0.0	0.0	5.3
% with score 2 or 3	12	31.0	0.0^{---}	7.7^{---}	0.0^{---}	55.0^{+}
	18	77.2	13.2^{---}	29.0^{---}	14.5^{---}	$94.7^{(+)}$
	24	95.1	20.0	50.0^{-}	18.5^{---}	100.0
	30	85.1	22.5	60.0	26.5^{---}	83.3
Ketones	6	1.7	0.0	2.5	15.6^{++}	15.8^{+}
% with	12	27.6	26.3	64.1^{+++}	61.3^{+++}	25.0
	18	49.1	89.5^{+++}	89.5^{+++}	84.2^{+++}	$26.3^{(-)}$
	24	43.9	62.9	82.1^{++}	81.5^{+++}	58.8
	30	23.4	62.5^{+++}	65.0^{+++}	61.8^{+++}	22.2
Blood	6	3.3	2.5	0.0	3.9	10.5
% with	12	0.0	0.0	2.6	0.0	0.0
	18	0.0	7.9^{+}	7.9^{+}	$5.3^{(+)}$	$5.3^{(+)}$
	24	14.6	25.7	17.9	15.4	23.5
	30	10.6	35.0^{++}	15.0	29.4^{+}	11.1

Key for significance levels as Table 4.

($P < 0.05$), 18 ($P < 0.001$), 24 ($P < 0.01$) and 30 months ($P < 0.01$). This effect was not evident in females.

(ii) Urinary pH was significantly lower in male rats fed LBA during the first 13 weeks at 6 ($P < 0.01$) and 12 months ($P < 0.01$) compared with male rats fed SBA. Again, this effect was not evident in females. No other effects of diet during the first 13 weeks that were not most plausibly attributable to chance were observed.

The effects of diet after 13 weeks on urinalysis parameters are summarized in Tables 11A and 11B.

In these tables, no adjustment has been made for earlier diet. The following conclusions may be drawn:

(i) At most of the five sampling times, except the last at 30 months, urinary volume was significantly higher in SMR and SMI groups than in SMA groups. The same is true for males but not females fed PRA.
(ii) At all five sampling times, urinary pH was significantly more alkaline in SMR and SMI groups than in SMA groups. The same is true for LMA males up to 24 months and females up to 12 months. Urinary pH was generally similar in PRA and SMA groups.
(iii) From 12 months onwards, urinary protein was significantly lower in SMR, SMI and LMA groups than in SMA groups.
(iv) At many sampling times, urinary ketone levels were significantly higher in SMR, SMI and LMA groups than in SMA groups, particularly in females.
(v) At 18 and 24 months, there was significantly less blood in the urine of SMR, SMI and LMA group males than in SMA males. By contrast, in females, significant differences in the opposite direction were seen at 18 and 30 months.

The increase in urine volume in the restricted- and time-restricted-fed animals was almost certainly related to the times when the animals were fed and when the urine samples were collected, since in these animals water consumption was reduced (see above). Food was offered to these animals at 09.00 hr each day. The food offered was in dry pellet form and when rats eat this type of diet they drink. Urine samples from these animals were collected after the food had been consumed or removed, whereas the *ad lib.*-fed animals only eat in the dark and therefore had been sleeping most of the day and not drinking. Urine samples from these animals were collected at the same time thus causing an apparent reduction in urinary output. This effect was also seen by Pickering and Pickering (1984b) as was an increased urinary pH in restricted-fed animals.

Urinary pH proved to be one of the best early indicators of survival (see below) and if this finding were to hold true for other experiments, urinary pH might prove to be a useful early-life predictor of survival in long-term toxicity studies. Thus animals in the high survival groups had urine with high pH (alkaline) and animals in the low survival groups had urine with low pH (acidic). This effect could not be attributed to the relation between the time of urine collection and time of feeding, because the SMI-fed animals were fed at the same time as the SMR-fed rats. The urinary pH of the SMI animals was closer to that of the LMA- and SMA-fed animals than that of the SMR group. The lower urine pH seen in the SMI-, LMA- and SMA-fed groups was proportional to food consumption/energy intake.

The effect of early restricted feeding in reducing the protein levels seen in the urine of rats correlates with the delay in the onset of chronic renal disease (nephropathy) (see below).

The influence of diets fed after week 13 had a highly significant ($P < 0.001$) effect on urinary protein. The high energy, high protein (PRA) diets greatly increased urinary protein and predisposed the animals to renal disease (see below).

The only animals showing consistent increases in ketone levels were animals fed LMA. This diet had a higher fibre concentration than the other formulation. The fibre was in the form of oat and wheat feed, that is cellulose and non-metabolizable carbohydrate. Since ketones are products of fatty acid oxidation in the mitochondria of the liver cells (hepatocytes), this showed a departure from the metabolic pathways seen in the SMA-fed animals. Increased ketone bodies are produced not only under conditions of starvation (catabolic metabolism), but also when dietary carbohydrate is low and then only when there is sufficient dietary fat. Under these conditions gluconeogenesis from glycerophosphate takes place and the remaining triglyceride molecules (i.e. the fatty acids) are broken down to carbon dioxide, water and ketones. Although none of the animals had very high levels of ketone bodies (i.e. ketosis), there were more noticeable increases in the LMA-fed animals than in the SMR-fed animals, despite both groups having low plasma glucose levels, a trigger for ketogenesis. This is probably because the restricted-fed animals could not increase their fat intake.

In-life observations on health and behaviour

In general the animals in the diet-restricted groups (SBA/SMR and SBR/SMR) were more active and had better coats throughout the study and/or lived to older ages than the groups fed SMA or PRA from week 13 of the study onwards. As in all long-term rat studies, some rats developed skin lesions including areas of hair loss, sores and local swellings. Some of these lesions disappeared and some persisted or grew

Table 12. Ringtail: incidence in relation to diet from week 13

	Males					Females				
	Diet									
	SMA	LMA	SMR	SMI	PRA	SMA	LMA	SMR	SMI	PRA
No. of rats	150	200	100	100	50	150	200	100	100	50
No. with tail lesions	5	9	15	2	0	0	0	4	0	0
%	3.3	4.5	15.0	2.0	0	0	0	4.0	0	0

Table 13A. Signs of radiculoneuropathy: lifelong percentage incidence in relation to diet from week 13

	Males					Females				
	Diet									
	SMA	SMR	SMI	LMA	PRA	SMA	SMR	SMI	LMA	PRA
Any sign	50.7	55	64	57	40	26.7	18	19	24.5	18
Reduced muscle tone and/or wasting in hind legs	37.3	36	45	49	34	6.7	4	5	2.5	6
Clenched hind paws	37.3	45	56	42.5	30	22	15	18	23.5	12
Dragging and/or not using one or both hind legs	6	4	5	5	2	0.7	2	0	0	2
Hopping gait and/or walking on heels	2	2	3	1	0	0	0	0	1.5	0

Table 13B. Signs of radiculoneuropathy: influence of diet from week 13—significance of differences from SMA after taking age of onset into account

	Males				Females			
	Diet							
	SMR	SMI	LMA	PRA	SMR	SMI	LMA	PRA
Any sign	(−)	N.S.	N.S.	N.S.	− − −	−	− − −	N.S.
Reduced muscle tone and/or wasting in hind legs	−	N.S.	N.S.	N.S.	−	N.S.	− −	N.S.
Clenched hind paws	N.S.	N.S.	N.S.	N.S.	− − −	N.S.	− −	(−)
Dragging and/or not using one or both hind paws	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Hopping gait and/or walking on heels	N.S.	N.S.	N.S.	N.S.	N.S.'	N.S.	N.S.	N.S.

Key for significance levels as Table 4.

to the point where sacrifice on humane grounds was necessary. Between-group differences in three kinds of clinically evident health effects merit special consideration: (i) tail lesions; (ii) lack of muscle tone in the hind legs; and (iii) gaseous distension of the gastro-intestinal tract associated with respiratory distress. In-life data with respect to the development of masses is considered in the section on Autopsy and histopathological findings.

1. *Tail lesions*

During the rat study some of the animals, particularly males in the restricted- and low-energy-fed groups, developed tail sores that ulcerated. In some cases parts of the tail fell off and/or were eaten. Swabs revealed the presence only of *Staphylococcus aureus*, an ubiquitous, opportunistic pathogen, which was not considered to be the cause of the problem.

From the literature (Flynn, 1967; Njaa *et al.*, 1957; Totton, 1958) it appears that if the RH is maintained at 40% or less, even for a short period, there is a risk that young rats will develop 'ringtail', a vascular disorder of the tail which often progresses to gangrene.

Although in the present study the animal rooms were usually maintained at 21 ± 2°C and the RH at 40–65%, during the period 6–12 January 1986, a sudden fall in the outside air temperature caused the incoming air to be at 0% RH—a level at which the re-humidifiers were unable to cope. The RH in the animal rooms consequently dropped to as low as RH 35–40% during this period. The following winter was an even more severe one, with nighttime outside temperatures sometimes falling below −16°C. As a consequence, the RH in the animal rooms again fell below 40% on six different days during January and February 1987. On three of these occasions the RH fell to below 10%.

The tail lesions encountered in the study fitted the description of ringtail. Most of these occurred late in the study after the periods of low RH which occurred during the winter of 1987.

Interestingly, the incidence of lesions differed according to the diet. In both sexes the incidence was highest in the SMR-fed groups. The incidences were as shown in Table 12.

A plausible explanation of the higher incidence of ringtail in the diet-restricted (SMR) group is that these animals went for longer periods each day when they were not eating and, therefore, not drinking. Their reduced fluid intake may have predisposed them to reduce fluid loss by shutting down blood supply to the tail. The fact that the

Table 14. Gaseous distension of the gastro-intestinal tract and respiratory distress: lifelong percentage incidence of the syndrome in relation to diet from week 13

	Males					Females				
	Diet									
	SMA	SMR	SMI	LMA	PRA	SMA	SMR	SMI	LMA	PRA
% Incidence	22	9^{--}	22	27	24	17	3^{---}	12	9^{-}	18

Key for significance levels as Table 4.

animals were housed in wire-bottomed cages increased the risk of ringtail. In solid-bottomed cages, animals can protect themselves from excessive water loss by creating their own more humid microenvironment. Weindruch and Walford (1988) reported that diet-restricted animals produce less heat than *ad lib.*-fed animals. By shutting down the blood supply to the tail they reduce the rate of heat loss from the tail (Hruza and Hlavackova, 1969).

2. *Signs of radiculoneuropathy*

Towards the end of the study many animals, particularly males, exhibited severe muscle wastage and lack of locomotor activity in their hind legs. This combination of signs is indicative of radiculoneuropathy, a degenerative disease of the myelin of the white matter of the lumbar spinal cord and the nerves of the cauda equina, which leads to partial paralysis and muscle degeneration of the hind legs (Berg, 1967; Burek, 1978; Burek *et al.*, 1976). Since the lumbar spinal cord and cauda equina were not sectioned routinely in this study, there is no unequivocal evidence that radiculoneuropathy caused the muscle wastage etc. However, no other explanation of the clinical signs is plausible or likely. As shown in Table 13A, the lifelong cumulative incidence of clinical signs was fairly similar in rats fed the different diets from week 13 onwards, with far fewer females than males being affected. However, when time of onset of signs was taken into account (see Table 13B) compared with SMA, SMR was found to be marginally ($P < 0.1$) protective in males and SMR ($P < 0.001$), LMA ($P < 0.001$) and SMI ($P < 0.05$) significantly so in females. The reduction in age-standardized incidence of any sign of radiculoneuropathy in *group 5* (SBR/SMR) animals was particularly striking (males, $P < 0.01$; females, $P < 0.05$) compared with that in *group 1* (SBA/SMA). This suggests that calorific restriction during the first 13 weeks of the study was of special value in protecting against the development of radiculoneuropathy later in life. These findings confirm the work of Simms and Berg (1962) that overnutrition can contribute importantly to the risk of development of the disease and are consistent with the possibility that overnutrition during the actively growing period of life is especially important.

3. *Gaseous distension of the gastro-intestinal tract associated with respiratory distress*

Towards the end of the study several of the rats, particularly the interrupted- and *ad lib.*-fed animals, developed what appeared to be clinical signs of respiratory disease, that is dyspnoea followed by a rapid loss of body weight.

Associated with this at autopsy, it was found that many of these animals had gaseous distension of the gastro-intestinal tract, fistulation of the palate adjacent to the upper molars, and erosion of the gum round the lower molars which were compacted with food residues. In addition to these lesions, enlargement of submaxillary and mandibular lymph nodes, and abscesses were observed. The abscesses extended into the cheek and bone of the jaw and in very severe cases extended up to the eye socket. Further, in several cases squamous cell carcinomas were found in association with such abscesses. The mechanism underlying these changes probably starts with the

Table 15. Circulating hormone levels (ng/ml serum) in rats surviving to end of study: effect of diet from week 13 in comparison with SMA (unadjusted for earlier diet)

		Median for each diet				
Sex	Hormone	SMA	SMR	SMI	LMA	PRA
Male	Growth hormone	31.0	7.5	19.3$^{(-)}$	) 22.0$^{(-)}$	61.0$^{(+)}$
	Prolactin	23.5	19.0$^{N.S.}$	25.2$^{N.S.}$	15.8	27.0$^{N.S.}$
	Progesterone	4.9	4.7$^{N.S.}$	6.1$^{N.S.}$	10.0^{++}	9.7^{++}
	Luteinizing hormone	96.0	94.0$^{N.S.}$	94.0$^{N.S.}$	90.0$^{N.S.}$	90.0$^{N.S.}$
	Testosterone	0.49	0.55$^{N.S.}$	0.53$^{N.S.}$	0.56$^{N.S.}$	0.27$^{N.S.}$
	No. of animals	61	67	62	85	13
Female	Growth hormone	46.5	40.3$^{N.S.}$	34.0$^{N.S.}$	37.5$^{(-)}$	64.5$^{(+)}$
	Prolactin	114	48.0	100$^{N.S.}$	48.5	81.0$^{N.S.}$
	Progesterone	30.5	41.5$^{N.S.}$	42.0$^{N.S.}$	24.9$^{N.S.}$	30.5$^{N.S.}$
	Luteinizing hormone	118	109$^{(-)}$	97.5$^{N.S.}$	113$^{N.S.}$	123$^{N.S.}$
	17β-oestradiol	41.0	51.0^{+++}	46.0^{+}	43.0$^{N.S.}$	41.0$^{N.S.}$
	17β-oestradiol/ progesterone ratio	1.47	1.20$^{N.S.}$	1.11$^{N.S.}$	1.43$^{N.S.}$	1.04$^{N.S.}$
	No. of animals	47	68	37	110	21

Key for significance levels as Table 4.

Table 16. Mortality by time of death

		Treatment group												
		1	2	3	4	5	6	7	8	9	10	11	12	Total
		Diet before wk 13/diet from wk 13												
	Period of death	SBA/ SMA	SBA/ LMA	SBA/ SMR	SBA/ SMI	SBR/ SMR	SBR/ LMA	SBR/ SMA	SBI/ SMI	LBA/ SMA	LBA/ LMA	LMA/ LMA	PRA/ PRA	
Male	1–52	0	0	0	0	0	1	1	0	1	1	2	1	7
	53–80	5	4	0	2	1	0	5	1	3	3	4	3	31
	81–94	3	5	3	2	3	2	3	3	6	4	2	5	41
	95–104	7	3	4	2	2	5	4	4	2	5	4	9	51
	105–112	4	4	2	6	2	2	4	0	7	4	1	7	43
	113–118	2	5	4	2	4	5	4	4	3	2	4	2	41
	119–124	5	1	2	4	3	5	2	3	4	6	4	3	42
	> 124	3	4	0	1	1	3	7	3	4	3	5	6	40
	Terminal	21	24	35	31	34	27	20	32	20	22	24	14	304
Female	1–52	1	1	2	1	0	1	3	3	0	0	3	1	16
	53–80	2	1	2	5	0	1	2	3	8	2	0	2	28
	81–94	3	1	0	9	3	4	4	4	5	2	2	7	44
	95–104	3	2	1	4	3	2	5	3	4	4	4	2	37
	105–112	7	2	2	0	2	4	6	7	1	5	2	5	43
	113–118	6	4	2	3	0	4	4	6	9	3	5	4	50
	119–124	6	4	2	1	1	2	5	0	2	4	4	6	37
	> 124	2	5	1	4	3	2	7	2	2	0	4	2	34
	Terminal	20	30	38	23	38	30	14	22	19	30	26	21	311

lodgement of particles of food in the gums. This leads to persistent inflammation which is apt to progress to abscess formation and carcinoma (see Madsen, 1989; Robinson, 1985). If these inflammatory processes result in fistulation between the nasal and oral cavities, then feeding is impaired and excessive amounts of air are inadvertently swallowed. The result is gaseous distension of the stomach with resultant rapid weight loss and death.

The percentage of animals on the different diets during the second part of the study which exhibited this syndrome is summarized in Table 14. Once again

Table 17A. Incidence of death during the second year of the study: effects of diet after week 13 in comparison with SMA (unadjusted for earlier diet)

		Diet					
Sex		SMA	SMR	SMI	LMA	PRA	Total
Male	n	38	13	14	40	17	122
	E	29.10	21.54	21.60	40.23	9.53	122.00
	P		–	–	N.S.	N.S.	
Female	n	35	9	28	25	11	108
	E	25.85	19.19	16.46	37.70	8.80	108.00
	P		– –	N.S.	– –	N.S.	
Total	n	73	22	42	65	28	230
	E	54.95	40.73	38.06	77.93	18.34	230.00
	P		– – –	N.S.	– – –	N.S.	

Key: n number of animals dying during second year of study; E expected number (adjusted for age) if no dietary effect; *P* probability value—see Table 4 for key to significance levels.

Table 17B. Incidence of death before the termination of the study: effects of diet after week 13 in comparison with SMA (unadjusted for earlier diet)

		Diet					
Sex		SMA	SMR	SMI	LMA	PRA	Total
Male	n	89	31	37	103	36	296
	E	67.60	56.00	54.75	97.67	19.98	296.00
	P		– – –	– – –	N.S.	N.S.	
Female	n	97	24	55	84	29	289
	E	63.93	56.87	42.56	103.26	22.38	289.00
	P		– – –	N.S.	– – –	N.S.	
Total	n	186	55	92	187	65	585
	E	131.53	112.88	97.31	220.93	42.35	585.00
	P		– – –	– –	– – –	N.S.	

Key: n number of animals dying before terminal kill; E expected number (adjusted for age) if no dietary effect; *P* probability value—see Table 4 for key to significance levels.

the protective effect of calorie restriction (SMR) is clearly evident in both sexes.

It is relevant to point out that all the diets used in the study contained wheat and/or oat feed. These feeds consist of the remains of whole grain after most of the germ and ludosperm have been removed. This 'chaff'-like material, if not ground finely enough, splits longitudinally into splinters with sharp ends which can pierce the soft lining epithelium of the buccal cavity. It is probable that this is how the sequence of pathological changes begins. The only real puzzle relates to how dietary restriction acts to reduce the incidence of the syndrome.

Circulating hormones

As will be seen from the autopsy and histopathological findings (see below), the incidence of hyperplasia and neoplasia of several endocrine glands and of the mammary glands was different in different groups. In so far as these differences were expected in the light of previous experiments involving calorific restriction, it was an important aspect of the present study that provision was made for measuring the levels of circulating hormones at various time points (6, 12, 18 and 24 months) during the study in satellite subgroups of five key groups (groups 1, 2, 5, 6 and 7) and in all animals killed terminally.

Unfortunately the samples taken at 24 months from the subgroups were lost because of a freezer breakdown.

The findings in satellite rats (six animals per group on each occasion) at 6, 12 and 18 months provided no clear evidence of between-diet differences for growth hormone, progesterone, luteinizing hormone, testosterone or 17β-oestradiol. At 6 months prolactin levels were significantly ($P < 0.01$) lower in males of

Table 18A. Body weight and absolute organ weights in rats surviving to end of the study: effect of diet from week 13 in comparison with SMA (unadjusted for earlier diet)

		Median weight (g) for each diet				
Sex		SMA	SMR	SMI	LMA	PRA
Male	Body	449	399^{--}	412^{--}	386^{--}	403^{-}
	Heart	1.48	1.37^{--}	1.34^{--}	1.33^{--}	1.44$^{N.S.}$
	Liver	16.0	12.6^{--}	14.0^{---}	15.2^{--}	17.6$^{N.S.}$
	Kidney	3.48	2.84^{---}	3.14^{---}	3.04^{---}	3.82^{+++}
	Adrenals	0.086	0.071^{---}	0.085$^{N.S.}$	0.079^{--}	0.103$^{N.S.}$
	Testes	5.23	5.57^{++}	5.42^{+}	5.82^{++}	5.27$^{N.S.}$
	Prostate	0.60	0.71$^{N.S.}$	0.68$^{N.S.}$	0.48^{--}	0.51$^{N.S.}$
	Seminal vesicle	1.03	1.34^{++}	1.14$^{N.S.}$	0.95$^{(-)}$	0.65$^{(-)}$
	Brain	2.38	2.38$^{N.S.}$	2.35$^{N.S.}$	2.37$^{N.S.}$	2.41$^{N.S.}$
	Pituitary	0.017	0.013^{---}	0.015$^{N.S.}$	0.012^{---}	0.014$^{N.S.}$
	No. of animals*	61	69	63	97	14
Female	Body	305	254^{--}	268^{--}	262^{--}	309$^{N.S.}$
	Heart	1.21	1.00^{-}	1.08^{-}	1.10^{-}	1.24$^{N.S.}$
	Liver	12.8	9.04^{---}	10.5^{--}	11.8^{--}	14.4^{++}
	Kidney	2.68	2.12^{---}	2.41^{--}	2.38^{-}	2.74$^{N.S.}$
	Adrenals	0.107	0.082	0.089	0.093	0.104$^{N.S.}$
	Ovaries	0.151	0.138$^{N.S.}$	0.153$^{N.S.}$	0.140$^{N.S.}$	0.159$^{N.S.}$
	Brain	2.12	2.13$^{N.S.}$	2.18$^{N.S.}$	2.15$^{N.S.}$	2.15$^{N.S.}$
	Pituitary	0.025	0.016^{-}	0.019^{-}	0.018^{-}	0.022$^{N.S.}$
	No. of animals*	53	76	44	115	21

Key for significance levels as Table 4.
*For some tissues nun.oers of animals were slightly less than stated.

Table 18B. Organ weights as a percentage of body weight in rats surviving to end of the study: effect of diet from week 13 in comparison with SMA (unadjusted for earlier diet)

		Median percentage for each diet				
Sex		SMA	SMR	SMI	LMA	PRA
Male	Heart	0.33	0.34$^{(+)}$	0.32$^{N.S.}$	0.36^{++}	0.33$^{N.S.}$
	Liver	3.57	3.24	3.28	3.80^{+}	4.10^{++}
	Kidney	0.75	0.72	0.77$^{N.S.}$	0.80$^{(+)}$	0.98^{+++}
	Adrenals	0.020	0.018	0.020$^{N.S.}$	0.020$^{N.S.}$	0.023^{+}
	Testes	1.13	1.41^{+++}	1.35^{+++}	1.48^{+++}	1.23$^{N.S.}$
	Prostate	0.14	0.18^{++}	0.16$^{N.S.}$	0.13$^{N.S.}$	0.12$^{N.S.}$
	Seminal vesicle	0.22	0.33^{+++}	0.26^{+}	0.24$^{N.S.}$	0.15$^{(-)}$
	Brain	0.52	0.61^{+++}	0.57^{+++}	0.62^{+++}	0.58^{++}
	Pituitary	0.004	0.003	0.004$^{N.S.}$	0.003	0.003
Female	Heart	0.40	0.40$^{N.S.}$	0.39$^{N.S.}$	0.41$^{N.S.}$	0.40$^{N.S.}$
	Liver	4.20	3.56	3.80	4.36^{++}	4.43^{+}
	Kidney	0.88	0.83	0.87$^{N.S.}$	0.90$^{(+)}$	0.90$^{N.S.}$
	Adrenals	0.035	0.032$^{(-)}$	0.034$^{N.S.}$	0.035$^{N.S.}$	0.034$^{N.S.}$
	Ovaries	0.048	0.053$^{(+)}$	0.054$^{N.S.}$	0.055$^{(+)}$	0.055$^{N.S.}$
	Brain	0.70	0.84^{+++}	0.78^{+++}	0.82^{+++}	0.68$^{N.S.}$
	Pituitary	0.009	0.006	0.007$^{(-)}$	0.007	0.007$^{N.S.}$

Key for significance levels as Table 4.
Numbers of animals given in Table 18A.

group 6 (SBR/LMA) than in those of *group 1* (SBA/SMA) and in females of *group* 6 (SBR/LMA) ($P < 0.05$) and in females of *group* 7 (SBR/SMA) than in those of *group 1* (SBA/SMA). No between-group differences in prolactin levels were seen at 12 months in either sex, but at 18 months levels in *group* 6 (SBR/LMA) males were significantly lower than those in *group* 1 (SBA/SMA) ($P < 0.05$) and levels in *group* 2 females (SBA/LMA) were similarly lower than those in group 1 (SBA/SMA). Levels of prolactin in *group 5* (SBR/SMR) females and *group 6* (SBR/LMA) females were marginally ($P < 0.1$) lower than those in *group 1* (SBA/SMA) at 18 months.

The findings in rats that survived until the end of the experiment proved to be of greater interest (see Table 15). In males fed the low energy diet (LMA) from week 13 of the study onwards, the median progesterone level was significantly ($P < 0.01$) higher than that in groups fed the standard diet *ad lib.* (SMA) or the standard diet restricted to 80% *ad lib.* (SMR) ($P < 0.01$) or the standard diet restricted by daily period of access (SMI). Males fed the PR diet *ad lib.* (PRA) also had higher progesterone levels than those fed the standard diet *ad lib.* (SMA). The only other between-group difference in males was a lower ($P < 0.05$) serum prolactin level in animals fed LMA than animals fed SMA.

SMR or LMA from week 13 was associated with reduced end-of-test serum prolactin levels compared with SMA, although this was not significant for SMR males. SMI had no such effect. End-of-test levels of progesterone and 17β-oestradiol were higher in the calorie-restricted rats of the groups fed SMR or SMI from week 13. However, this was only statistically significant for 17β-oestradiol (SMR, $P < 0.001$; SMI, $P < 0.05$). There was no significant difference between groups in the ratio of 17β-oestradiol to progesterone.

Survival

As shown in Table 16, there were marked between-group differences in survival between the two groups that were diet restricted either just during the second period of the experiment (*group 3*—SBA/SMR) or throughout the experiment (*group 5*—SBR/SMR). The difference reached statistical significance ($P < 0.05$) for the sexes combined during the second year of the study and the significance increased to $P < 0.001$ during the third year of the study. The beneficial effect of calorie restriction was more marked in females than in males.

If dietary difference during the first part of the study was ignored, analysis of survival data for rats on the five different diets during the second part of the study showed that the low energy diet (LMA) also significantly ($P < 0.01$) reduced the incidence of premature death during the second year of the study (Table 17A). The interrupted dietary regimen (*groups 4 and 8*—SMI) increased survival as compared with the *ad lib.*-feeding regimen (*groups 1, 7 and 9*—SMA), the effects being more evident in males than in females.

During the third year of the study SMR, SMI and LMA all exhibited better survival than SMA or PRA, the effect being more marked in females than males. Combining the effect on survival throughout the study SMR was associated with highly significantly better survival ($P < 0.001$) in both sexes, SMI significantly improved ($P < 0.001$) survival in males but not in females whereas LMA did so in females ($P < 0.001$) but not in males (see Table 17B).

Body and organ weights at the end of the experiment

As compared with the SBA, dietary restriction (SBR) before week 13 (after adjustment for later diet) was associated with significantly reduced mean absolute body weight ($P < 0.05$), liver weight ($P < 0.05$) and pituitary weight and with a significantly higher mean brain:body weight ratio ($P < 0.05$) in male rats. In females, SBR before week 13 was associated with marginally lower body weight ($P < 0.1$), with highly significantly lower absolute liver weight ($P < 0.001$) and less significantly lower absolute kidney weight ($P < 0.05$) than SBA control. Also, compared with SBA, LBA was associated with marginally lower body weight ($P < 0.1$) and liver weight ($P < 0.1$) and with significantly lower heart weight ($P < 0.05$) and kidney weight ($P < 0.01$). Liver weight relative to body weight was significantly lower in SBR ($P < 0.01$).

The effects of diet after week 13 (unadjusted for earlier diet) on body weight and organ weights are summarized in Tables 18A and 18B.

All forms of reduction in calorie intake were associated in both sexes with highly significantly lower end-of-test body weight and absolute weights of heart, liver, kidneys and adrenals compared with SMA. However, the effects of SMR were generally more marked than those of LMA or SMI. Absolute pituitary weights were also significantly lower in the calorie-restricted groups except in SMI males. Absolute testicular weight was significantly higher than in SMA males in SMR and LMA males, but not in SMI males. Absolute prostate weight was significantly lower in LMA males than in SMA males, whereas absolute seminal vesicle weight was significantly higher in SMR males than in SMA males. Diet from week 13 was associated with no observed effect on absolute brain weight in either sex and no effect on absolute ovary weight.

The picture is somewhat different for organ weights relative to body weight. Significant reductions were seen for liver in both sexes for SMR and SMI in comparison with SMA. By contrast, a higher liver:body weight ratio was seen for LMA compared with SMA in both sexes. Relative kidney weights were lower for SMR than for SMA in both sexes and relative adrenal weight was marginally lower for SMR than for SMA. End-of-test testis, prostate and seminal vesicle weights relative to body weights were

Table 19A. Incidence of tumours (non-age-standardized) by group—males

Group . . .		1	2	3	4	5	6	7	8	9	10	11	12
Diet to/from wk 13 . . .		SBA/ SMA	SBA/ LMA	SBA/ SMR	SBA/ SMI	SBR/ SMR	SBR/ LMA	SBR/ SMA	SBI/ SMI	LBA/ SMA	LBA/ LMA	LMA/ LMA	PRA/ PRA
No. of animals		50	50	50	50	50	50	50	50	50	50	50	50
Survivors at week 52		50	50	50	50	50	49	49	50	49	49	48	49
at week 80		45	46	50	48	49	49	44	49	46	46	44	46
at week 104		35	38	43	44	44	42	37	42	38	37	38	32
end of test		21	24	35	31	34	27	20	32	20	22	24	14
Protocol tissues													
Adrenal cortex													
adenoma	b	0	0	1	0	0	1	0	1	0	0	0	1
Adrenal medulla													
benign phaeochromocytoma	b	1	0	3	4	0	6	1	3	2	2	2	4
malignant phaeochromocytoma	m	1	0	0	0	1	1	0	0	1	1	0	0
Epididymis													
haemangioma	b	0	0	0	0	0	0	0	0	0	0	0	1
Heart													
sarcoma	m	0	0	0	0	0	0	0	0	1	0	0	0
Kidney													
adenoma	b	0	0	0	0	1	0	0	0	0	0	0	0
Liver bile-duct													
cholangiocarcinoma	m	1	0	0	0	0	0	0	0	1	0	0	0
Liver cell													
benign hepatoma	b	0	0	0	0	0	1	0	0	0	0	0	0
hepatocellular carcinoma	m	1	0	0	0	0	2	0	0	0	0	3	0
Lung													
adenoma	b	2	3	0	1	0	2	2	1	1	1	1	1
adenocarcinoma	m	0	1	0	0	0	0	2	0	0	0	0	0
Mammary gland													
fibroma	b	0	0	0	1	0	0	0	0	0	0	0	0
fibroadenoma	b	0	0	0	0	0	0	0	0	0	0	1	1
Pancreas exocrine													
adenoma	b	3	0	0	0	0	2	2	0	4	0	0	2
Pancreas islet-cell													
adenoma	b	5	0	0	1	0	3	3	1	4	1	1	2
adenocarcinoma	m	0	0	1	1	0	0	2	0	0	0	0	0
Parathyroid													
adenoma	b	2	1	0	1	1	3	3	1	2	2	3	2
Pituitary pars anterior													
adenoma	b	13	8	9	10	5	10	15	15	14	9	9	11
possibly malignant	d	1	0	0	0	0	1	0	2	0	1	0	0
adenocarcinoma	m	0	1	0	2	0	0	1	0	0	2	0	1
Pituitary pars intermedia													
adenoma	b	8	2	7	4	2	2	5	3	7	1	4	4
possibly malignant	d	0	0	0	1	0	0	0	0	0	1	0	0
adenocarcinoma	m	0	0	0	0	0	0	0	0	1	1	0	0
Prostate													
adenoma	b	1	0	0	0	0	0	0	0	0	0	0	0
adenocarcinoma	b	0	0	0	0	0	0	1	0	0	0	1	0
sarcoma	m	0	0	0	0	0	0	0	0	1	0	0	0
Skin epidermal													
sebaceous adenoma	b	0	0	0	0	0	0	0	0	1	0	0	0
basal cell tumour	b	0	0	1	0	0	0	1	0	0	0	0	0
Spleen													
sarcoma	m	0	0	0	1	0	0	0	0	0	0	0	0
Testis Leydig-cell													
adenoma	b	12	12	12	15	18	23	11	14	13	23	27	18
Testis													
mesothelioma	m	0	0	0	0	0	1	0	0	0	0	0	0
Thyroid C-cell													
adenoma	b	0	6	1	5	1	2	2	3	1	3	3	1
adenocarcinoma	m	0	0	0	0	0	1	0	0	1	0	0	0
Thyroid follicular													
adenoma	b	1	1	1	0	0	1	0	1	1	0	2	1
adenocarcinoma	m	0	0	0	0	0	0	0	0	0	0	0	1

[*contd*]

Table 19A. *Continued*

Group ...		1	2	3	4	5	6	7	8	9	10	11	12
Diet to/from wk 13 ...		SBA/ SMA	SBA/ LMA	SBA/ SMR	SBA/ SMI	SBR/ SMR	SBR/ LMA	SBR/ SMA	SBI/ SMI	LBA/ SMA	LBA/ LMA	LMA/ LMA	PRA/ PRA
Non-protocol tissues													
Brain/meninges													
glioma	m	0	1	0	0	0	1	0	1	0	0	0	1
meningioma	m	0	1	0	0	0	2	1	0	0	1	0	1
Buccal cavity													
squamous papilloma	b	0	0	0	0	0	0	0	0	0	0	0	1
Cervical lymph node													
haemangioma	b	0	0	1	0	0	0	0	0	0	0	0	0
Lip													
squamous papilloma	b	0	0	0	0	0	0	0	0	0	1	1	0
Lymph node (other than cervical & mesenteric)													
haemangioma	b	1	0	0	0	0	1	0	1	0	0	0	0
Mass epidermal													
squamous papilloma	b	1	2	1	1	1	1	3	1	1	1	1	0
keratoacanthoma	b	0	0	2	0	0	0	1	0	0	0	1	0
sebaceous adenoma	b	2	0	0	0	0	0	0	0	0	0	0	0
squamous carcinoma	m	3	1	1	0	0	0	0	1	1	0	0	0
trichoepithelioma	m	0	0	0	0	1	0	0	0	2	1	1	0
basal-cell carcinoma	m	0	2	0	0	0	0	2	0	0	0	1	0
mixed squamous/ adenocarcinoma	m	0	0	0	1	0	0	0	0	0	0	0	0
Mass subcutaneous													
lipoma	b	1	0	0	0	0	0	2	0	0	0	2	1
fibroma	b	2	2	1	2	1	1	9	3	1	2	2	0
adenocarcinoma	m	0	0	0	0	0	0	0	0	1	1	0	0
sarcoma	m	3	5	3	3	0	2	2	2	3	1	3	7
anaplastic sarcoma	m	1	0	0	0	0	0	0	0	0	0	0	0
myxosarcoma	m	0	0	0	0	1	1	2	0	1	0	0	0
anaplastic adenocarcinoma	m	0	0	0	0	0	1	0	0	0	0	0	0
Mass bone													
osteosarcoma	m	0	1	0	0	0	0	1	1	0	0	0	0
Mass vascular													
haemangioma	b	0	0	0	0	0	0	0	0	1	0	0	0
haemangiosarcoma	m	1	0	0	0	0	1	0	0	1	0	0	0
Mass nervous system													
malignant schwannoma	m	1	0	0	0	0	0	0	0	0	0	0	0
Mass other tumours													
chondroma	b	0	0	0	0	0	0	0	1	0	0	0	0
leiomyoma	b	0	1	0	0	0	0	0	0	0	0	0	0
fibromyxoma	b	0	0	0	1	0	0	0	0	0	0	0	0
adenocarcinoma in mediastinum	m	1	0	0	0	0	0	0	0	0	0	0	0
Mesenteric lymph node													
haemangioma	b	3	10	4	1	4	6	3	4	5	10	7	3
possibly malignant	d	2	2	0	0	0	1	3	1	0	4	2	2
haemangiosarcoma	m	1	5	0	4	0	11	4	2	7	7	12	1
Rectum/colon/caecum													
fibromatous polyp	b	0	0	0	0	0	0	0	0	0	0	0	1
spindle-cell sarcoma	m	0	0	0	0	0	0	0	0	0	0	0	1
Salivary gland													
sarcoma	m	0	0	0	1	0	0	0	1	0	0	0	1
Stomach glandular													
adenomatous polyp	b	0	0	0	0	0	0	0	1	0	0	0	0
adenocarcinoma	m	1	0	0	1	0	0	0	0	0	0	1	0
Stomach fore													
squamous papilloma	b	0	0	1	0	0	1	0	2	2	0	1	0
Tail													
haemangioma	b	0	0	0	0	1	0	0	0	1	0	0	0
squamous/sebaceous papilloma	b	0	0	0	0	0	1	0	0	0	0	0	0
Thymus													
thymoma	b	0	1	0	0	0	0	0	0	0	0	0	0
sarcoma	m	0	0	0	0	0	0	0	1	0	0	0	0
Tongue													
squamous papilloma	b	0	0	0	0	0	0	0	1	0	0	0	0
squamous carcinoma	m	0	0	0	0	1	0	0	0	0	1	0	0

[*contd*]

Table 19A. *Continued*

Group ...		1	2	3	4	5	6	7	8	9	10	11	12
Diet to/from wk 13 ...		SBA/ SMA	SBA/ LMA	SBA/ SMR	SBA/ SMI	SBR/ SMR	SBR/ LMA	SBR/ SMA	SBI/ SMI	LBA/ SMA	LBA/ LMA	LMA/ LMA	PRA/ PRA
Duodenum/jejunum/ileum													
adenocarcinoma	m	0	0	0	0	0	0	0	1	0	0	0	0
Preputial gland													
adenoma	b	0	0	0	0	0	0	0	0	1	0	0	0
Skull													
osteoma	b	0	0	0	0	1	0	0	0	0	0	0	0
Unknown primary													
carcinoma	m	0	0	0	0	0	0	0	0	0	1	0	0
Whole animal													
Malignant lymphoma													
present	m	2	5	0	7	2	3	1	4	2	3	0	3
Overall primary tumour incidence													
benign tumour		18	19	32	22	25	22	26	26	22	21	21	22
doubtfully malignant tumour		3	1	0	0	0	1	2	3	0	6	1	2
malignant tumour		15	21	5	18	6	22	15	13	21	16	22	17
*Multiple primary tumour incidence***													
benign tumour		24	20	11	15	11	26	21	20	23	22	31	21
doubtfully malignant tumour		0	1	0	1	0	1	1	0	0	0	1	0
malignant tumour		2	2	0	3	0	3	3	1	3	4	0	0

Table 19B. Incidence of tumours (non-age-standardized) by group—females

Group ...		1	2	3	4	5	6	7	8	9	10	11	12
Diet to/from wk 13 ...		SBA/ SMA	SBA/ LMA	SBA/ SMR	SBA/ SMI	SBR/ SMR	SBR/ LMA	SBR/ SMA	SBI/ SMI	LBA/ SMA	LBA/ LMA	LMA/ LMA	PRA/ PRA
No. of animals		50	50	50	50	50	50	50	50	50	50	50	50
Survivors at week 52		49	49	48	49	50	49	47	47	50	50	47	49
at week 80		47	48	46	44	50	48	45	44	42	48	47	47
at week 104		41	45	45	31	44	42	36	37	33	42	41	38
end of test		20	30	38	23	38	30	14	22	19	30	26	21
Protocol tissues													
Adrenal cortex													
adenoma	b	0	0	2	1	1	1	1	1	1	0	0	0
adenocarcinoma	m	1	0	0	0	0	0	0	0	1	0	0	1
Adrenal medulla													
benign phaeochromocytoma	b	1	0	0	1	1	0	0	2	0	0	0	2
malignant phaeochromocytoma	m	2	1	0	1	0	0	0	0	1	0	2	3
Kidney													
lipoma	b	0	0	0	0	0	0	1	0	0	0	0	0
Liver cell													
benign hepatoma	b	0	0	0	0	0	0	0	1	1	0	0	1
hepatocellular carcinoma	m	0	0	0	0	1	1	0	0	0	1	0	0
Lung													
adenoma	b	2	1	0	0	0	0	0	0	2	0	0	1
adenocarcinoma	m	0	0	0	0	0	1	2	0	0	0	0	0
Mammary													
fibroma	b	1	1	0	1	1	0	1	0	1	2	2	2
fibroadenoma	b	17	3	3	5	4	1	16	6	6	4	4	9
adenoma	b	2	0	0	2	0	0	2	0	1	1	0	0
adenocarcinoma	m	3	0	1	2	0	2	3	3	3	1	1	2
Ovary													
granulosa-cell tumour	b	1	0	0	0	1	0	0	0	0	0	0	0
granulosa-theca tumour	m	1	0	1	0	0	0	1	0	1	1	0	0
tubular adenoma	b	0	2	0	0	1	1	0	0	1	1	0	0
Pancreas islet-cell													
adenoma	b	2	0	1	0	0	0	0	0	0	1	0	1
Parathyroid													
adenoma	b	1	2	0	3	1	4	1	1	0	0	1	1
Pituitary pars anterior													
adenoma	b	33	17	24	19	20	25	19	22	29	22	23	25
possibly malignant	d	3	0	0	0	0	0	0	0	0	0	0	0
adenocarcinoma	m	2	0	1	2	1	3	5	3	2	2	1	1
Pituitary pars intermedia													
adenoma	b	3	0	0	3	0	0	3	2	4	1	1	4
adenocarcinoma	m	0	0	0	0	0	0	0	0	0	0	0	1

[*contd*]

Table 19B. *Continued*

Group ...		1	2	3	4	5	6	7	8	9	10	11	12
Diet to/from wk 13 ...		SBA/ SMA	SBA/ LMA	SBA/ SMR	SBA/ SMI	SBR/ SMR	SBR/ LMA	SBR/ SMA	SBI/ SMI	LBA/ SMA	LBA/ LMA	LMA/ LMA	PRA/ PRA
Spleen													
sarcoma	m	0	1	0	0	0	1	0	0	0	0	0	0
Thyroid C-cell													
adenoma	b	4	2	2	1	2	0	4	1	1	2	3	0
Thyroid follicular													
adenoma	b	1	0	0	0	0	0	1	1	0	1	0	1
adenocarcinoma	m	0	0	0	1	0	0	0	0	0	0	0	1
Thyroid													
ganglioneuroma	b	0	0	0	0	0	0	0	1	0	0	0	0
*Uterus/horn**													
keratinizing squamous polyp	b	0	1	0	0	0	1	0	0	0	0	0	0
adenomatous polyp	b	2	4	2	2	0	0	0	3	0	2	3	1
papillary adenoma	b	0	0	0	0	1	0	0	0	0	0	0	0
fibromyoma	b	0	0	0	0	0	1	0	0	1	0	1	0
squamous carcinoma	m	0	0	0	0	0	0	0	0	0	1	0	0
adenocarcinoma	m	0	3	0	1	0	0	0	0	2	7	3	0
Non-protocol tissues													
Brain/meninges													
glioma	m	0	1	0	0	0	1	0	0	0	0	0	0
meningioma	m	0	0	0	1	0	0	0	1	0	0	1	0
Buccal cavity													
squamous carcinoma	m	0	1	0	1	0	0	0	0	0	0	0	0
Cervical lymph node													
haemangioma	b	0	0	0	0	0	0	0	1	0	0	0	0
Lip													
squamous papilloma	b	0	0	0	0	0	0	0	0	0	1	0	0
Mass epidermal													
sebaceous adenoma	b	0	0	0	0	0	0	1	0	0	0	0	0
squamous carcinoma	m	0	1	0	0	0	0	1	0	0	0	0	0
basal-cell carcinoma	m	0	0	0	0	0	1	0	0	0	0	0	0
Mass salivary gland													
adenocarcinoma	m	1	0	0	0	0	0	0	0	0	0	0	0
Mass subcutaneous													
lipoma	b	0	0	0	0	0	0	0	1	1	0	0	1
fibroma	b	0	1	0	0	0	0	0	0	0	0	0	0
adenocarcinoma	m	0	0	0	1	0	0	0	0	0	0	0	0
sarcoma	m	0	1	3	3	0	1	1	0	2	1	0	4
anaplastic sarcoma	m	0	0	0	1	0	0	0	0	0	0	0	0
myxosarcoma	m	0	0	0	1	0	0	0	0	0	0	0	0
Mass bone													
osteosarcoma	m	0	0	0	0	0	0	0	0	0	0	0	2
Mass vascular													
haemangioma	b	0	0	0	0	0	0	0	0	0	1	0	0
haemangiosarcoma	m	0	0	0	0	0	0	0	0	0	0	0	1
Mass nervous system tumour													
malignant schwannoma	m	0	0	0	0	0	0	0	0	1	0	0	0
Mesenteric lymph node													
haemangioma	b	5	2	1	1	1	1	2	1	0	6	3	0
possibly malignant	d	0	0	0	0	0	0	0	0	0	0	3	0
haemangiosarcoma	m	1	3	1	1	0	8	1	0	1	3	2	0
Salivary gland													
adenocarcinoma	m	0	0	0	0	0	0	0	0	0	0	0	1
Stomach/fore													
squamous papilloma	b	0	0	0	0	0	1	0	0	0	0	0	0
Thymus													
thymoma	b	0	0	0	0	2	0	0	0	1	0	1	0
adenocarcinoma	m	0	0	0	0	0	0	1	0	0	0	0	0
sarcoma	m	0	0	0	0	0	0	0	0	1	0	0	0
anaplastic carcinoma	m	0	0	0	0	0	0	0	0	0	0	1	0
Tongue													
squamous papilloma	b	0	0	0	0	0	0	1	0	0	0	0	0
anaplastic carcinoma	m	0	0	0	0	1	0	0	0	0	0	0	0
Urinary bladder													
adenoma	b	0	0	0	0	0	0	0	0	0	1	0	0

[*contd*]

Table 19B. *Continued*

Group ...		1	2	3	4	5	6	7	8	9	10	11	12
Diet to/from wk 13 ...		SBA/ SMA	SBA/ LMA	SBA/ SMR	SBA/ SMI	SBR/ SMR	SBR/ LMA	SBR/ SMA	SBI/ SMI	LBA/ SMA	LBA/ LMA	LMA/ LMA	PRA/ PRA
*Uterus/body**													
adenocarcinoma	m	2	6	4	5	2	2	1	1	1	2	2	0
anaplastic carcinoma	m	0	3	1	0	0	4	1	0	0	0	2	0
haemangiosarcoma	m	0	1	0	0	0	0	0	0	0	0	0	0
sarcoma	m	1	0	0	2	0	4	0	3	0	0	2	1
myxosarcoma	m	0	0	0	1	1	0	0	0	0	0	0	0
adenomatous polyp	b	0	0	0	0	0	1	0	0	0	0	0	0
Whole animal													
Malignant lymphoma													
present	m	1	0	0	1	2	0	2	2	1	2	1	1
Histiocytic sarcoma													
present	m	1	0	0	0	0	0	0	0	0	0	0	0
Overall primary tumour incidence													
benign tumour		29	21	26	18	22	19	21	24	27	21	21	23
doubtfully malignant tumour		3	0	0	0	0	0	0	0	0	0	2	0
malignant tumour		13	20	11	22	8	27	18	11	14	17	16	16
*Multiple primary tumour incidence***													
benign tumour		31	12	7	18	9	15	20	16	21	17	16	21
doubtfully malignant tumour		0	0	0	0	0	0	0	0	0	0	1	0
malignant tumour		2	2	1	3	0	2	1	2	3	3	2	3

b = benign tumour d = doubtfully malignant tumour m = malignant tumour

Where an animal has a benign and a malignant tumour in a particular category it is counted only under malignant.

*Uterine tumours seen in both horn (protocol tissue) and body (non-protocol tissue).

**Counts for multiple tumour incidence are of animals with two or more tumours of at least the malignancy specified (an animal with a benign and a malignant tumour would count under benign as it did not have two malignant tumours).

Table 20A. Incidence of selected non-neoplastic lesions (non-age-standardized) by group—males

Group ...	1	2	3	4	5	6	7	8	9	10	11	12
Diet to/from wk 13 ...	SBA/ SMA	SBA/ LMA	SBA/ SMR	SBA/ SMI	SBR/ SMR	SBR/ LMA	SBR/ SMA	SBI/ SMI	LBA/ SMA	LBA/ LMA	LMA/ LMA	PRA/ PRA
Protocol tissues												
Adrenal cortex												
haemorrhage and/or degeneration												
any grade	25	36	32	30	28	36	25	29	30	26	31	32
focal hyperplasia												
any grade	0	2	1	0	2	0	1	0	0	1	1	3
Adrenal medulla												
hyperplasia												
any grade	3	1	3	2	1	6	3	4	1	1	1	6
Epididymis												
atrophy												
any grade	19	17	22	19	20	26	16	16	21	19	29	22
Heart												
chronic inflammation												
minimal or slight	13	13	20	22	16	13	18	22	14	14	9	27
moderate or severe	2	2	1	1	1	0	1	0	3	2	2	6
fibrosis												
minimal or slight	12	5	3	10	2	4	11	2	4	6	1	21
moderate	0	2	0	2	1	0	0	0	1	0	1	1
Kidney												
chronic progressive nephropathy												
minimal or slight	19	27	26	33	13	26	28	32	20	35	23	8
moderate	14	11	3	6	0	7	13	10	15	8	4	18
severe or very severe	15	1	0	5	0	0	6	0	11	1	1	23
lymphocyte aggregates in cortex												
minimal or slight	14	28	35	22	34	35	30	36	24	33	36	8
moderate	21	9	3	12	0	5	13	7	16	12	6	27
severe or very severe	5	0	0	2	0	1	3	0	4	1	1	13
corticomedullary mineralization												
minimal or slight	0	0	0	0	10	9	12	6	0	0	0	0
moderate	0	0	0	0	2	3	2	1	0	0	0	0
severe	0	0	0	0	0	0	1	0	0	0	0	0
pelvic mineralization												
minimal	1	3	2	2	0	3	2	3	0	0	0	3
slight	3	0	1	2	2	2	3	0	2	0	0	4
moderate	0	0	1	0	1	0	0	0	0	1	1	5

[*contd*]

Table 20A. *Continued*

Group ...	1	2	3	4	5	6	7	8	9	10	11	12
Diet to/from wk 13 ...	SBA/ SMA	SBA/ LMA	SBA/ SMR	SBA/ SMI	SBR/ SMR	SBR/ LMA	SBR/ SMA	SBI/ SMI	LBA/ SMA	LBA/ LMA	LMA/ LMA	PRA/ PRA
Liver												
bile-duct hyperplasia												
minimal or slight	13	14	15	9	15	20	19	10	13	21	23	20
moderate, severe or very severe	1	11	2	0	0	9	5	0	2	7	4	3
Fatty degeneration												
minimal or slight	3	1	0	3	1	1	3	2	0	2	4	0
moderate or severe	0	2	0	0	0	2	1	0	0	3	1	3
Lung												
lymphocytic aggregates around airways												
minimal or slight	39	45	45	39	46	40	44	43	45	40	45	40
moderate or severe	9	2	3	6	1	7	6	3	4	9	5	7
interstitial pneumonitis												
minimal or slight	43	30	44	39	46	39	40	42	46	36	44	30
moderate	4	17	4	5	0	7	7	4	3	11	2	17
severe or very severe	0	2	0	0	1	1	0	0	0	0	0	1
Mammary gland/skin												
acinal hyperplasia												
minimal	14	8	9	6	9	5	6	11	9	9	11	9
slight	6	7	5	4	11	0	3	2	5	6	6	10
moderate	3	2	2	1	1	4	0	3	0	2	0	4
secretory activity												
minimal	5	1	0	3	4	0	6	2	6	1	2	4
slight	2	1	0	1	0	2	2	3	3	1	0	2
moderate or severe	4	1	0	3	0	0	1	5	0	1	0	0
galactocoele(s)												
present	0	0	0	0	0	0	0	2	0	1	0	0
Pancreas												
chronic atrophic pancreatitis												
minimal or slight	8	2	2	4	3	3	4	1	5	1	4	6
moderate, severe or very severe	0	0	0	0	0	0	0	0	1	1	0	1
polyarteritis												
minimal or slight	3	2	1	0	2	1	0	2	1	1	3	2
moderate	1	3	0	0	1	2	2	0	1	0	0	3
severe or very severe	1	2	0	0	0	0	1	0	1	2	0	9
basophil foci												
present	0	0	0	3	2	0	0	2	4	2	0	0
Parathyroid												
focal hyperplasia												
minimal or slight	8	14	9	5	7	8	8	11	7	2	4	7
moderate or severe	0	0	1	0	0	0	0	0	1	0	0	4
Pituitary pars anterior												
focal hyperplasia												
minimal	5	4	5	1	5	4	0	5	4	1	2	4
slight	0	3	0	0	1	2	1	3	2	1	1	0
moderate	0	0	1	0	0	0	0	1	0	0	0	0
Pituitary pars intermedia												
minimal	1	4	1	4	0	1	1	4	2	3	4	2
slight	1	2	1	0	1	1	1	2	2	1	3	3
moderate or severe	1	1	1	1	0	1	0	2	5	2	3	3
Prostate												
acute inflammation												
minimal or slight	3	2	1	1	1	7	0	1	1	2	4	5
moderate	5	1	0	1	0	0	2	1	2	0	3	0
severe or very severe	4	0	2	3	0	1	5	3	5	1	3	3
chronic inflammation												
minimal or slight	5	4	1	0	1	6	0	3	5	5	12	5
moderate	2	0	0	3	1	3	0	1	6	3	4	1
severe or very severe	2	1	0	1	0	0	1	0	0	0	1	0
Spleen												
atrophy												
any grade	0	1	3	1	0	0	0	0	3	0	0	0
generalized hyperplasia												
minimal or slight	0	0	0	2	3	0	0	0	0	0	3	3
moderate	1	0	2	0	0	1	0	0	1	0	0	1
severe or very severe	0	0	2	0	0	1	1	1	0	0	0	1
Testis												
focal mineralization												
minimal	12	4	7	6	6	10	6	5	8	7	15	10
slight	4	5	9	8	7	11	5	6	5	4	3	5
moderate	0	1	5	1	1	2	1	3	3	2	4	1

[*contd*]

Table 20A. Incidence of selected non-neoplastic lesions (non-age-standardized) by group—males

Group ...	1	2	3	4	5	6	7	8	9	10	11	12
Diet to/from wk 13 ...	SBA/ SMA	SBA/ LMA	SBA/ SMR	SBA/ SMI	SBR/ SMR	SBR/ LMA	SBR/ SMA	SBI/ SMI	LBA/ SMA	LBA/ LMA	LMA/ LMA	PRA/ PRA
Testis contd												
atrophy												
minimal or slight	0	4	4	5	2	8	1	3	4	7	4	7
moderate	4	1	4	1	6	1	2	0	1	3	2	1
severe or very severe	16	15	18	17	15	23	15	16	19	20	27	20
oedema												
any grade	0	2	0	0	2	1	0	2	0	0	1	0
arteriolitis												
minimal or slight	0	0	0	0	0	0	1	1	1	0	4	0
moderate	3	0	0	1	0	0	0	0	2	0	0	4
severe or very severe	0	0	0	0	0	0	0	0	0	0	0	3
Leydig-cell hyperplasia												
minimal or slight	0	5	6	3	8	5	4	5	0	3	4	1
moderate or severe	0	0	0	0	0	1	0	0	0	0	1	0
Thyroid												
C-cell hyperplasia												
any grade	0	0	0	0	1	0	0	2	2	0	1	0
follicular cell hyperplasia												
any grade	0	0	0	0	0	0	0	0	0	1	0	0
Mesenteric artery												
marked polyarteritis	0	2	0	1	1	1	1	0	0	1	0	9
Sciatic nerve												
degeneration/inflammation	17	16	23	23	10	20	19	21	16	17	20	15
Seminal vesicle												
reduced distension	8	14	8	13	11	19	9	10	19	15	21	15
increased distension	1	0	5	4	8	0	1	4	3	0	3	3
Tail												
ulceration and cellulitis	3	1	1	1	8	3	1	1	0	1	4	0
Voluntary muscle												
degeneration/atrophy/ chronic inflammation	19	19	25	24	11	22	22	27	16	21	24	15

Table 20B. Incidence of selected non-neoplastic lesions (non-age-standardized) by group—females

Group ...	1	2	3	4	5	6	7	8	9	10	11	12
Diet to/from wk 13 ...	SBA/ SMA	SBA/ LMA	SBA/ SMR	SBA/ SMI	SBR/ SMR	SBR/ LMA	SBR/ SMA	SBI/ SMI	LBA/ SMA	LBA/ LMA	LMA/ LMA	PRA/ PRA
Protocol tissues												
Adrenal cortex												
haemorrhage and/or degeneration												
any grade	38	41	28	32	30	38	41	32	35	38	40	39
focal hyperplasia												
any grade	3	1	2	0	4	1	2	1	0	0	0	2
Adrenal medulla												
hyperplasia												
any grade	0	0	0	2	0	1	0	0	0	1	1	1
Heart												
chronic inflammation												
minimal or slight	22	5	10	5	15	5	20	13	19	4	8	25
moderate or severe	1	2	0	1	0	0	3	0	4	1	0	4
fibrosis												
any grade	13	1	1	2	0	2	6	1	5	1	2	14
Kidney												
chronic progressive nephropathy												
minimal or slight	21	21	21	24	19	26	18	26	24	30	23	16
moderate	21	6	1	5	1	2	18	8	12	4	3	26
severe or very severe	8	0	0	3	0	0	7	0	7	0	0	8
lymphocyte aggregates in cortex												
minimal or slight	27	28	29	23	30	36	19	31	25	39	38	24
moderate	19	4	1	7	1	5	16	3	16	2	3	22
severe	2	0	0	2	0	0	8	0	3	0	0	1
corticomedullary mineralization												
minimal or slight	10	16	11	20	24	27	21	25	26	22	6	15
moderate	32	20	29	19	13	8	11	15	6	5	0	1
severe or very severe	8	12	9	11	6	2	2	3	0	0	0	0
pelvic mineralization												
minimal	0	0	0	1	0	0	0	2	1	0	0	6
slight	0	0	0	0	0	0	1	0	2	0	0	6
moderate	0	0	0	0	0	0	0	0	0	0	0	2

[*contd*]

Table 20B. *Continued*

Group ...	1	2	3	4	5	6	7	8	9	10	11	12
Diet to/from wk 13 ...	SBA/ SMA	SBA/ LMA	SBA/ SMR	SBA/ SMI	SBR/ SMR	SBR/ LMA	SBR/ SMA	SBI/ SMI	LBA/ SMA	LBA/ LMA	LMA/ LMA	PRA/ PRA
Liver												
bile-duct hyperplasia												
minimal or slight	13	17	16	7	14	10	13	11	26	22	23	23
moderate or severe	1	3	1	0	0	2	4	1	0	4	4	3
fatty degeneration												
minimal or slight	2	3	0	2	1	0	1	2	1	0	3	1
moderate, severe or very severe	2	2	0	0	1	2	1	0	1	4	3	0
Lung												
lymphocytic aggregates around airways												
minimal or slight	41	47	48	45	44	46	41	47	42	47	41	46
moderate or very severe	4	2	0	1	4	2	6	2	5	2	5	2
interstitial pneumonitis												
minimal or slight	40	37	44	34	47	41	39	36	42	45	41	34
moderate	5	9	4	12	1	5	7	12	5	4	3	14
severe	0	2	1	1	0	2	0	0	0	0	4	0
Mammary gland/skin												
acinal hyperplasia												
minimal	3	10	7	7	7	9	4	9	4	6	9	7
slight	4	9	13	16	11	9	11	11	10	11	8	11
moderate	20	10	7	14	9	12	15	18	18	7	7	13
severe or very severe	18	0	1	3	2	5	10	5	7	3	3	12
secretory activity												
minimal	5	8	4	7	8	9	6	10	5	8	6	5
slight	13	9	11	8	8	5	12	13	13	10	10	9
moderate	14	9	14	14	6	9	11	8	11	7	8	17
severe or very severe	12	1	2	5	0	4	7	8	7	4	3	7
galactoceole(s)												
present	6	2	0	2	0	2	7	3	7	3	2	4
Ovary												
cyst(s)	2	6	2	3	2	5	2	2	4	4	3	2
Pancreas												
chronic atrophic pancreatitis												
minimal or slight	1	1	1	1	2	3	3	1	4	2	2	3
moderate or severe	0	2	1	0	0	1	0	0	0	0	0	1
polyarteritis												
minimal or slight	4	3	0	0	0	1	0	2	2	0	1	5
moderate	2	0	0	1	0	0	1	0	0	0	1	
severe or very severe	3	0	0	0	0	0	0	0	0	0	0	4
basophil foci												
present	0	0	1	0	2	0	0	1	0	2	0	1
Parathyroid												
focal hyperplasia												
minimal or slight	2	3	3	2	6	1	3	1	5	1	3	4
severe	1	0	0	0	0	0	0	0	0	0	0	0
Pituitary pars anterior												
focal hyperplasia												
minimal	5	5	3	3	2	3	2	2	1	4	5	4
slight	0	2	0	1	0	1	1	1	1	1	2	0
moderate	0	0	0	1	0	0	0	0	0	0	0	0
Pituitary pars intermedia												
focal hyperplasia												
minimal	1	1	0	0	0	1	2	1	2	0	0	1
slight	0	0	0	0	0	0	0	1	1	1	0	0
moderate or severe	0	0	0	3	0	0	0	1	1	0	0	0
Spleen												
atrophy												
any grade	0	0	1	2	1	0	0	3	0	0	0	2
generalized hyperplasia												
minimal or slight	2	1	3	4	2	3	0	2	1	2	1	3
moderate	1	3	1	4	0	0	0	1	1	0	2	3
severe	1	0	1	0	2	1	0	1	1	3	1	1
Thyroid												
C-cell hyperplasia												
any grade	3	0	1	1	2	0	0	0	2	1	0	0
follicular cell hyperplasia												
any grade	1	2	1	1	0	0	0	0	0	0	0	1

[*contd*]

Table 20B. *Continued*

Group ...	1	2	3	4	5	6	7	8	9	10	11	12
Diet to/from wk 13 ...	SBA/ SMA	SBA/ LMA	SBA/ SMR	SBA/ SMI	SBR/ SMR	SBR/ LMA	SBR/ SMA	SBI/ SMI	LBA/ SMA	LBA/ LMA	LMA/ LMA	PRA/ PRA
Uterus/horn												
distension												
minimal or slight	4	13	13	4	5	13	5	14	8	10	8	6
moderate	4	8	3	6	6	3	5	6	3	11	8	5
severe or very severe	1	5	4	5	4	4	1	0	1	5	5	7
polyp(s)												
present	3	10	8	3	5	6	5	3	4	9	5	8
haemorrhage												
minimal or slight	1	0	1	0	0	2	1	1	0	0	1	0
moderate	0	0	2	1	0	1	0	0	0	1	0	1
severe or very severe	1	0	0	1	1	0	1	0	1	3	0	0
endometrial hyperplasia												
minimal or slight	0	2	0	5	4	1	0	2	0	3	0	3
moderate or severe	0	2	2	1	1	0	0	0	1	0	1	1
pyometra												
any grade	0	2	0	1	1	0	0	1	0	1	2	0
Non-protocol tissues												
Mesenteric artery												
marked polyarteritis	1	1	0	0	0	0	0	0	0	0	0	1
Sciatic nerve												
degeneration/inflammation	5	3	4	2	3	2	6	1	4	2	2	4
Tail												
ulceration and cellulitis	0	0	0	1	3	0	0	0	1	0	0	0
Uterus/body												
marked hypertrophy of wall of cervix	0	5	0	0	3	4	0	3	1	5	3	1
polyp	1	2	1	0	0	1	0	0	1	0	1	1
Voluntary muscle												
degeneration/atrophy/chronic inflammation	5	3	4	2	3	2	6	2	7	2	1	7

markedly higher for SMR than for SMA and so were relative testis weights for SMI and LMA. Relative brain weights were high in both sexes in SMR, SMI and LMA groups and relative pituitary weights were low in SMI and LMA groups.

Histopathological findings

As stated in the Materials and Methods section, only a limited list of tissues (i.e. adrenals, heart, kidneys, liver, lungs, mammary gland, mesenteric lymph node, ovaries, pancreas, pituitary, prostate, seminal vesicle, spleen, testes and uterus) were routinely examined histologically. These are referred to below as 'protocol tissues'. However, sections were also taken from all 'non-protocol' organs/tissues considered to be abnormal during the course of a careful standard autopsy, which included exploration of the contents of the cranial cavity, but not of the spinal cord. The failure to include the lumbar spinal cord and cauda equina in the standard list of sections to be prepared was a pity in so far as disturbances of gait and wasting of the hind quarters, almost certainly attributable to radiculoneuropathy affecting the cauda equina, was encountered in high incidence in the study. As it is, the effects of the different diets on the incidence of radiculoneuropathy could only be inferred from the histopathological findings in the sciatic nerves and thigh muscles of animals where changes in these tissues were observed at autopsy.

Non-age-standardized incidences, by group and sex, for neoplastic and non-neoplastic findings are given in Tables 19 and 20, respectively. In the sections that follow we first describe the findings for the protocol tissues, then those for the non-protocol tissues, before giving results for radiculoneuropathy and polyarteritis (conditions which affect multiple tissues) and for overall tumour incidence. Significance tests all take into account differences in mortality between the groups.

Histopathological findings: protocol tissues

1. *Adrenal cortex*

No significant relationships to tumour incidence were seen. However, in the analysis of tumours or hyperplasia, there was a significant ($P < 0.05$) increase in males in relation to PRA diet from week 13 and a significant ($P < 0.05$) reduction in females in relation to LMA diet from week 13. In the absence of corresponding effects in the opposite sex, these marginally significant relationships are not convincing effects of treatment.

There was a significantly ($P < 0.01$) reduced incidence of haemorrhage and/or degeneration in females, but not males, on the SMR diet from week 13. No clear relationship to other diets was seen, marginally significant differences in some analyses, probably reflecting chance and/or small differences between groups in the severity grading of the lesion.

2. *Adrenal medulla*

There was a significant increase in the PRA group in the incidence of tumours ($P < 0.05$) and of tumours or hyperplasia ($P < 0.01$) for the sexes combined compared with the groups fed SMA after week 13. Incidence was similarly increased in both sexes. A reduction in incidence of tumours or hyperplasia for the SMR diet approached significance ($P < 0.1$) for the sexes combined, but otherwise no other effects were evident.

3. *Epididymis*

A haemangioma is included in 'other tumours' (see Table 28).

No effect of diet from week 13 on the incidence of atrophy was seen, but there was a significant ($P < 0.01$) increase in relation to the LMA diet to week 13.

4. *Heart*

A sarcoma is included in 'other tumours' (see Table 28).

In females on the SMR, SMI and LMA diets from week 13 there was a very highly significant ($P < 0.001$) reduction in the incidence of chronic inflammation, but no such effect was seen in males. In contrast, the PRA diet was associated with a significant ($P < 0.001$) increase in males, which was also to some extent evident in females ($P < 0.05$ for grades of at least slight).

A similar pattern was evident for fibrosis, where highly significant reductions in incidence were seen in relation to diet from week 13 in SMR females ($P < 0.001$), SMI females ($P < 0.01$) and LMA females ($P < 0.001$), and a significant increase was seen in PRA males ($P < 0.001$). In this case, however, some reduction was also evident in SMR males ($P < 0.01$) and LMA males ($P < 0.05$). There was also some evidence of a reduction in incidence in relation to early diet, with incidence reduced for the SBR, SBI and LBA diets, and significantly ($P < 0.05$ for the sexes combined) for SBI and LBA.

No relationship to the incidence of distension was seen.

5. *Kidney*

An adenoma and a lipoma are included under 'other tumours' (see Table 28).

The incidence and severity of chronic progressive nephropathy was affected massively by diet. This is best illustrated by considering the results for grades of at least moderate, where for the sexes combined the incidence was around 50% in the SMA groups. It was virtually eliminated, to 2.5%, in the SMR groups, substantially reduced, to less than 20%, in the SMI and LMA groups, and substantially increased, to 75%, in the PRA group. All these comparisons were highly significant ($P < 0.001$) in each sex, with the exception of PRA in females ($P < 0.05$). Incidence of nephropathy was also reduced significantly ($P < 0.001$) in males in relation to SBR diet to week 13, and, in higher grade analysis, in relation to LBA diet in females and SBI diet in males ($P < 0.05$).

The pattern of incidence for lymphocyte aggregates in the cortex in relation to diet from week 13 closely followed that for chronic progressive nephropathy, with clear reductions in relation to SMI, LMA and particularly SMR and increases in relation to PRA, although the last finding was only evident in males. Unlike for nephropathy, however, there was evidence of an increase in incidence in relation to LBA and LMA diet to week 13 ($P < 0.01$ for sexes combined) and the reduction in incidence for the SBR diet was far less clear, being significant ($P < 0.05$) only in males in one analysis (grades of at least moderate).

The incidence of cortical cysts for the sexes combined was reduced significantly ($P < 0.01$) in the SMR groups and reduced almost significantly ($P < 0.1$) in the LMA groups.

The effect of diet on corticomedullary mineralization was strikingly different from that seen for other lesions in that it was apparently only affected by diet to week 13 and hardly, if at all, by diet from week 13. Also the effects were strikingly different for the two sexes. In males, of 46 cases seen, 39 were the SBR diet (26%) and seven on the SBI diet (14%) as against none on the SBA, LBA, LMA or PRA diets. The increases in the SBR and SBI groups were highly significant ($P < 0.001$). In contrast, in females, there was a highly significant ($P < 0.001$) reduction in incidence and severity compared with SBA for all the other diets. The reduction was huge in the LMA and PRA diets, next most marked for the LBA diet and least marked for the SBR and SBI diets.

The incidence of pelvic mineralization was increased in both sexes ($P < 0.001$ for the sexes combined) in relation to the PRA diet, but was unaffected by diet otherwise.

Cortical mineralization, hydronephrosis and acute pyelitis were not affected significantly by diet.

Medullary mineralization was reduced marginally significantly ($P < 0.05$ for sexes combined) for the LMA diet from week 13, but no other relationships were seen.

6. *Liver*

The incidence of hepatocellular tumours was not significantly related to diet, although there was an indication of an increase on the LMA diets in males, where six of the seven tumours occurred.

Two cholangiocarcinomas are included under 'other tumours' (see Table 28).

Bile-duct hyperplasia was much more affected by diet to week 13 than by later diet. Thus there was a highly significant ($P < 0.001$) increase in relation to LBA, LMA and PRA diets to week 13, and much less evidence of an effect of SBR and SBI diets. After adjusting for early diet, the only significant effect of

diet from week 13 was an increase in males in the LMA groups ($P < 0.01$).

The incidence of fatty degeneration was somewhat increased in relation to LMA diet from week 13 ($P < 0.05$ for sexes combined for grades of at least moderate) but was otherwise unaffected by diet.

Fibrosis around bile ductules was more affected by diet to week 13 than by later diet. Compared with animals fed SBA to week 13, highly significant ($P < 0.001$) increases in incidence, more evident in females than males, were seen in rats fed LMA and PRA. A significant ($P < 0.05$) increase was also seen on the LBA diet. After adjustment for earlier diet, the only significant ($P < 0.05$) difference seen in relation to later diet was a reduction in incidence in SMR males.

Eosinophilic foci/areas were increased significantly ($P < 0.05$) in PRA males, but not affected otherwise by diet.

Foci/areas of margination of cytoplasm were highly significantly ($P < 0.001$) increased in females in relation to LMA diet to week 13 but otherwise were not affected significantly by early or later diet.

Although five out of seven cases of cystic foci/areas were seen in rats fed LMA from week 13 this did not represent a significant treatment effect.

The incidence of foci/areas of fatty degeneration was increased significantly ($P < 0.01$ for sexes combined) in relation to LMA diet from week 13.

No clear relationship of diet to the incidence of basophilic foci/areas was seen.

The incidence of foci/areas of telangiectasis was reduced in relation to LMA and SMR diets from week 13 ($P < 0.05$ for sexes combined).

The incidence of widespread degeneration/necrosis was increased marginally significantly ($P < 0.05$) in females in relation to LMA diet from week 13.

There was no clear treatment relationship of diet to the incidence of focal haemorrhage/degeneration or focal coagulative necrosis.

7. *Lung*

Lung tumours were seen in 13 out of 300 rats fed SMA after week 13. In contrast, no tumours were seen in 200 SMR rats ($P < 0.01$) and only two in 200 SMI rats ($P < 0.05$). No significant relationship to LMA and PRA diet was seen.

The incidence of higher grades of lymphocytic aggregates around the airways was reduced significantly ($P < 0.01$) in relation to SMR diet from week 13. No other clear relationships to diet were seen.

The incidence of at least moderate interstitial pneumonitis was increased highly significantly ($P < 0.001$) in group 12 (PRA/PRA) and in group 2 (SBA/LMA), the increases being evident in both sexes. The group 2 increase is particularly difficult to interpret as no such increase was seen in other rats fed SBA early in life or LMA late in life.

8. *Mammary gland*

Diet from week 13 had a large effect on the incidence of mammary tumours. Incidence was most affected by the SMR and LMA diets where highly significant ($P < 0.001$) reductions were seen for overall tumour incidence and significant ($P < 0.05$) reductions were also seen for malignant tumour incidence. Overall tumour incidence was also reduced on the SMI diet but to a lesser extent ($P < 0.05$). All but three of the tumours were in females.

In females, the incidence of acinal hyperplasia was reduced highly significantly ($P < 0.001$) in relation to SMR and LMA diet from week 13. For higher grades of hyperplasia a reduction ($P < 0.01$) was also seen for SMI diet from week 13. In males no very clear pattern was seen, except for an increase in grades of at least slight hyperplasia for the PRA diet (compared with the SMA diet).

In both sexes the incidence of secretory activity was reduced highly significantly ($P < 0.001$) in relation to SMR and LMA diet from week 13. After adjustment for later diet, there was also a reduced incidence ($P < 0.05$) in relation to SBR diet to week 13.

In females, the incidence of galactocoele(s) was reduced in relation to SMR diet from week 13 ($P < 0.001$) and to a lesser extent in relation to LMA ($P < 0.01$) and SMI ($P < 0.1$).

9. *Ovary*

Neither the incidence of granulosa cell tumours nor the incidence of tubular tumours or hyperplasia was affected significantly by diet.

The incidence of cysts was not related to diet.

10. *Pancreas*

All 13 exocrine tumours were seen in males. Incidence was reduced significantly ($P < 0.05$) in rats fed the SMR, SMI and LMA diets from week 13.

The incidence of islet-cell tumours, much more common in males than females, was again reduced significantly on the SMR ($P < 0.01$), SMI ($P < 0.05$) and LMA ($P < 0.01$) diets from week 13.

The incidence of chronic atrophic pancreatitis was reduced in relation to LMA diet in males ($P < 0.01$) and in relation to SMI diet in the sexes combined ($P < 0.05$).

The incidence of polyarteritis of the pancreatic artery is discussed in a later section.

No clear relationship of basophil foci to treatment was seen.

11. *Parathyroid*

No relationship of diet was seen to the incidence of tumour or hyperplasia.

12. *Pituitary*

The incidence of tumours, anterior or intermediate lobe, was affected markedly by diet from week 13.

Highly significant ($P < 0.001$) reductions were seen on the SMR and LMA diets, with a less significant ($P < 0.05$) reduction in the SMI group. Incidence was unaffected by the PRA diet. The significance levels cited are for overall tumour incidence for the sexes combined, with reductions being evident in both sexes.

Anterior lobe cysts were unaffected by diet. However, the incidence of intermediate lobe cysts was reduced significantly ($P < 0.01$) in relation to the SMR diet from week 13 for females and for the sexes combined.

13. *Prostate*

No relationship of diet to the incidence of adenomas or adenocarcinomas was seen.

A sarcoma is included in 'other tumours' (see Table 28).

A highly significant ($P < 0.001$) reduction in incidence of acute inflammation was seen in relation to SMR diet from week 13 and, where grades of at least moderate were analysed, in relation to LMA diet from week 13. A lesser reduction was seen for SMI diet ($P < 0.05$).

The incidence of chronic inflammation was affected strongly by diet to week 13, but not by later diet. The main effects of early diet were a highly significant ($P < 0.001$) increase in the LMA diet, and a less significant ($P < 0.05$) increase in the LBA diet.

14. *Spleen*

No relationship of diet was seen to the incidence of sarcoma of the spleen.

Generalized hyperplasia of the spleen was increased highly significantly ($P < 0.001$) in relation to SMR and PRA diet from week 13 but was not affected otherwise by diet.

The incidence of atrophy of the spleen was unaffected by diet.

15. *Testis*

There was no significant relationship of any of the diets to the incidence of testis Leydig-cell tumours or hyperplasia, with the exception of the LMA diet. Incidence was increased markedly in the group fed LMA throughout life and also increased in two of the three groups fed LMA from week 13. The detailed statistical analyses showed that both the effect of diet to week 13, adjusted for diet from week 13, and the effect of diet from week 13, adjusted for diet to week 13, were significant ($P < 0.01$).

A mesothelioma is included in 'other tumours' (see Table 28).

There was some indication that the incidence of focal mineralization was increased by LMA diet up to week 13, the incidence in group 11 (LMA/LMA) significantly ($P < 0.01$) exceeding that in group 2 (SBA/LMA). However, the group 2 incidence was rather low compared with other groups fed SMA early in life and the group 11 incidence was rather high compared with the other group fed LMA late in life, so this may be a chance finding.

The incidence of atrophy appeared to be related to diet to week 13 and not to subsequent diet. Compared with SBA to week 13, incidence was increased in relation to LMA ($P < 0.01$), PRA ($P < 0.05$) and LBA ($P < 0.1$).

The incidence of arteriolitis showed significant ($P < 0.001$) between-group variation mainly due to a high incidence in group 12 (PRA/PRA).

The incidence of oedema was unrelated to diet.

16. *Thyroid*

No significant relationship of diet to the incidence of C-cell tumours or hyperplasia was seen, although a reduction in the PRA group for the sexes combined approached significance.

The incidence of follicular tumours or hyperplasia was not apparently affected by diet.

A ganglioneuroma is included in 'other tumours' (see Table 28).

17. *Uterus*

Uterine tumours were classified into five categories for the purposes of analysis (see Table 27). The incidence of the most commonly occurring category, glandular tumours of the uterine horn or body (adenocarcinoma, anaplastic carcinoma, adenomatous polyp and papillary adenoma), was increased significantly ($P < 0.01$) in relation to LMA diet from week 13, where the incidence was 20.5% compared with 6% in SMA-fed rats. Although not significant when considered in isolation, it was noticeable that all three squamous tumours of the uterine horn, two of the three fibromyomas of the uterine horn and the single haemangiosarcoma were also seen in LMA-fed rats. The incidence of sarcomas or myxosarcomas was also somewhat above expected in the LMA group, although not significantly so. For this group of tumours, a marginally significant ($P < 0.05$) increase in relation to SMI diet was seen.

The incidence of distension was increased highly significantly ($P < 0.001$) in relation to LMA diet from week 13. A lesser increase was also seen in relation to SMI ($P < 0.05$).

The incidence of endometrial hyperplasia was increased in relation to diet from week 13 for SMR ($P < 0.05$), SMI ($P < 0.05$), LMA ($P < 0.1$) and PRA ($P < 0.05$).

The incidence of marked hypertrophy of the uterine body in the region of the cervix was increased highly significantly ($P < 0.01$) in relation to LMA diet from week 13.

The incidence of polyps, haemorrhage and pyometra of the uterine horn was unrelated to diet as were polyps of the uterine body.

Histopathological findings: non-protocol tissues

18. *Abdominal fat*

The incidence of nodules of necrotic fat was unaffected by diet.

19. *Brain*

Neither the incidence of gliomas nor meningiomas were affected significantly by diet, though it was notable that for both tumour types incidence was highest on the LMA diet and lowest on the SMR diet.

20. *Cervical lymph node*

The incidence of cystic nodes was reduced significantly in relation to SMR ($P < 0.01$) and LMA ($P < 0.05$) diet from week 13. The reductions were evident in both sexes.

In females, but not in males, the incidence of lymphoid hyperplasia was reduced significantly in relation to SMR ($P < 0.01$) and LMA ($P < 0.001$) diet from week 13.

21. *Duodenum/jejunum/ileum*

An adenocarcinoma is included in 'other tumours' (see Table 28).

22. *Eye*

There was no relationship of diet to incidence of acute keratitis with pus in the anterior chamber.

23. *Jaw and buccal cavity*

There was a significant ($P < 0.01$) variation between the groups in the incidence of squamous or anaplastic carcinoma of the jaw. Incidence was highest (9%) in the group (12) on the PRA diet, incidence averaging 3% in the other groups. Because the PRA diet was only tested throughout life, and because of the relatively low incidences, it was difficult to be certain from the pattern of results obtained to distinguish effects of early and late diets on the incidence of these jaw tumours. However, the results seemed more indicative of an early diet effect. Compared with rats fed SBA to week 13, incidence was highly significant ($P < 0.001$ for sexes combined, $P < 0.01$ for each sex individually) in PRA-fed rats, with increases also seen in all the other groups [SBI $P < 0.05$, LBA $P < 0.1$, SBR and LBA not significant (N.S.)]. In contrast, where comparison was made with rats fed SMA from week 13, the increase in the PRA group was less marked ($P < 0.1$ for sexes combined, $P < 0.01$ for males, N.S. for females) and there were no significant differences for the other groups, although a reduction in the SMR group approaches significance.

A squamous papilloma and two squamous carcinomas of the buccal cavity are included in 'other tumours' (see Table 28).

Diet did not affect the incidence of abscess/ulceration of the jaw.

24. *Lip*

Three squamous papillomas are included in 'other tumours' (see Table 28).

25. *Lymph nodes (other than mesenteric, cervical or thymic)*

No relationship of diet to tumour incidence was seen.

SMR diet from week 13 reduced significantly ($P < 0.05$) the incidence of cystic lymph nodes.

No relationship of diet was seen to the incidence of lymphoid hyperplasia.

26. *Cutaneous and subcutaneous masses*

The incidence of epidermal tumours, predominantly occurring in males, was reduced significantly ($P < 0.05$) in relation to SMR, SMI and LMA diet from week 13, and was reduced almost significantly in relation to PRA diet.

The incidence of subcutaneous tumours was not apparently affected by diet in females. In males, however, there was a significant ($P < 0.01$) reduction in SMR-fed rats and an almost significant ($P < 0.1$) reduction in SMI-fed rats.

The incidence of bone tumours and of vascular tumours was not affected significantly by diet.

A variety of other tumour types each occurring in just one or two rats are included in 'other tumours' (see Table 28).

The incidence of subcutaneous keratin-filled cysts was reduced marginally significantly ($P < 0.05$) in relation to LMA diet from week 13.

27. *Mesenteric lymph node*

The incidence of mesenteric lymph node tumours was increased very highly significantly ($P < 0.001$) in rats fed LMA from week 13, with 27% of rats having haemangiomas or haemangiosarcomas on the LMA diets as against 13% on SMA. In contrast, incidence was reduced significantly ($P < 0.01$) on SMR, to 4%, and reduced almost significantly ($P < 0.1$) on SMI.

The incidence of cystic nodes was lower on the SMR, SMI and LMA diets from week 13 than on the SMA and PRA diets. The only significant difference was a reduction in LMA in males ($P < 0.05$).

The incidence of haemorrhagic nodes was unaffected by diet.

28. *Preputial gland*

An adenoma is included in 'other tumours' (see Table 28).

29. *Rectum/colon/caecum*

A spindle-cell sarcoma and a fibromatous polyp are included in 'other tumours' (see Table 28).

30. *Salivary glands*

The incidence of sarcomas was not affected by diet.

Table 21. Corticomedullary mineralization of the kidney: effects of diet to week 13 (unadjusted for later diet)

Sex		Diet					
		SBA	LBA	LMA	PRA	SBI	SBR
Males	n	← 0 →				7	39
	E	← 30.87 →				3.79	11.35
	n/E	← 0 →				1.85	3.44
	P					+++	+++
Females	n	195	59	6	16	43	114
	E	144.5	71.9	35.9	36.2	36.4	108.1
	n/E	1.35	0.82	0.17	0.44	1.18	1.05
	P	---	---	---	---	--	---
% (any grade)		97.5	59	12	32	86	76
% (grades 2–5)		89.5	27	2	6	58	53
% (grades 4–5)		20	0	0	0	6	7

n number of cases E expected number (adjusted for age) if no dietary effect *P* probability value—see Table 4 for key to significance level

31. *Sciatic nerve*

The incidence of small mineral deposits was not clearly affected by treatment.

The incidence of degeneration/inflammation is discussed under radiculoneuropathy (see below).

32. *Seminal vesicles*

There was a tendency for rats fed SMR from week 13 to have increased distension ($P < 0.05$) compared with SMA-fed rats.

33. *Stomach*

The incidence of tumours of the forestomach and glandular stomach were both unaffected by diet.

34. *Tail*

Ulceration and cellulitis was increased ($P < 0.05$) in relation to SMR diet from week 13.

35. *Thymus*

The incidence of thymomas was not affected by diet.

A variety of other tumour types were seen in the thymus. These are included under 'other tumours' (see Table 28).

The incidence of cystic thymic lymph nodes was unrelated to diet.

36. *Tongue*

No relationship of diet to the incidence of tongue tumours was seen.

37. *Urinary bladder*

An adenoma is included in the 'other tumours' (see Table 28).

38. *Voluntary muscle*

See section on radiculoneuropathy below.

39. *Neoplasms: primary unknown*

A carcinoma with an unknown primary site is included in 'other tumours' (see Table 28).

40. *Multicentric tumours*

No relationship of diet to the incidence of malignant lymphoma was seen.

A histiocytic sarcoma is included in 'other tumours' (see Table 28).

41. *Other tumours*

A variety of occasionally occurring tumours were combined into an 'other tumours' category (see Table 28). The incidence was reduced significantly ($P < 0.01$) on the SMR diet for the sexes combined, the reduction being evident in both sexes. There was also a significant ($P < 0.05$) reduction in the incidence on the LMA diet, in the case of malignant tumours were analysed.

Radiculoneuropathy

Degeneration/inflammation in either the sciatic nerve or voluntary muscle was reduced in both sexes in relation to SMR diet from week 13 ($P < 0.001$ for

Table 22. Squamous carcinoma of the jaw: effects of diet to week 13 in comparison with SBA (unadjusted for earlier diet)

Sex		Diet					
		SBA	SBR	SBI	LBA	LMA	PRA
Males	n	6	4	5	1	3	6
Females	n	0	4	2	6	2	3
Total	n	6	8	7	7	5	9
	E	14.46	10.85	3.66	6.55	3.56	2.92
	P		N.S.	+	N.S.	(+)	+++

Key as Table 21.

Table 23. Histopathology: effect of diet before week 13 in comparison with SBA (adjusted where possible for diet from week 13)

Tissue/finding	Sex	Diet				
		SBR	SBI	LBA	LMA	PRA
Epididymis						
—atrophy	M	N.S.	N.S.	N.S.	++	N.S.
Heart						
—myocardial fibrosis	M/F	N.S.	–	–	N.S.	N.S.
Kidney						
—lymphocyte aggregates in cortex	M/F	N.S.	N.S.	+++	++	+++
—chronic progressive nephropathy	M/F	– –	N.S.	(–)	–	N.S.
Liver						
—bile-duct hyperplasia	M/F	N.S.	N.S.	+++	+++	+++
—periductular fibrosis	M/F	N.S.	N.S.	+	+++	+++
—foci/areas of margination of cytoplasm	M/F	N.S.	N.S.	N.S.	+	(+)
Mammary gland						
—secretory activity	F	–	N.S.	– –	N.S.	N.S.
Pancreatic/mesenteric artery						
—polyarteritis	M/F	–	N.S.	– –	N.S.	N.S.
Prostate						
—chronic prostatitis	M	N.S.	N.S.	+	+++	N.S.
Testis						
—atrophy	M	N.S.	N.S.	(+)	++	+
—Leydig-cell tumour or hyperplasia	M	N.S.	N.S.	N.S.	+++	N.S.

Key for significance levels as Table 4.

sexes combined) and in females in relation to SMI ($P < 0.05$) and LMA ($P < 0.001$) from week 13. The pattern was similar if incidence in either tissue alone was considered.

Polyarteritis

Polyarteritis in either the pancreatic or mesenteric artery was affected strongly by diet from week 13. It was reduced significantly on the SMR diet ($P < 0.01$ for females and sexes combined), on SMI ($P < 0.05$ for sexes combined) and on LMA ($P < 0.05$ for females). In contrast, it was increased highly significantly ($P < 0.001$) in males and to a lesser extent in females ($P < 0.05$) on PRA. After adjustment for diet from week 13, there was also some evidence of a reduction in rats fed SBR ($P < 0.05$) and LBA ($P < 0.01$) to week 13. Most cases reported were in the pancreatic artery.

Overall tumour incidence

After adjustment for diet from week 13 there was no evidence of an effect of diet to week 13 on overall tumour incidence. Compared with the SMA diet from week 13, there was a massive reduction ($P < 0.001$), clearly evident in both sexes, in the incidence of malignant tumours (16% *v.* 37%) and in the incidence of benign or malignant tumours (68% *v.* 83%) in SMR rats. The incidence of malignant tumours was not affected significantly by the SMI or LMA diet but, in males but not in females, was increased significantly ($P < 0.05$) on the PRA diet. The incidence of benign or malignant tumours was reduced significantly ($P < 0.001$) by LMA in females but was not affected by LMA in males. It was reduced slightly by SMI in both sexes ($P < 0.01$ for both sexes combined). For PRA diet, conclusions were similar to those for malignant tumour incidence.

The incidence of tumours of multiple sites was reduced highly significantly ($P < 0.001$) by SMR, SMI and LMA diets, particularly by SMR. The reduction was clearly evident in both sexes for SMR and SMI, but was only seen in females for LMA. The incidence of multiple malignant tumours was affected only significantly by SMR diet, where it was reduced massively, with only one case out of 200 for the sexes combined, as against 20 out of 300 for SMA ($P < 0.001$).

Effects of diet to week 13 on histopathological findings (Tables 21–23)

Effects of diet to week 13 could be classed into four main categories:

(1) Corticomedullary mineralization of the kidney;
(2) Squamous carcinoma of the jaw;
(3) Lesions showing increased incidences on the LMA diet (and also in some cases on the LBA and PRA diets);
(4) Lesions showing reduced incidences on the SBR and SBI diets (and also in some cases on the LBA and LMA diets).

Effects in categories 1, 2 and 3 related, with minor exceptions, to lesions where diet from week 13 had no apparent effect. Effects in category 4 were typically weak versions of the reduction seen in relation to the corresponding diets from week 13.

1. *Corticomedullary mineralization of the kidney*

The striking effect of diet to week 13 is illustrated in Table 21. In males 39 out of 46 cases were in the three groups on SBR diet to week 13, with the

Table 24. Histopathology: main effects of PRA diet in comparison with SMA from week 13 (unadjusted for earlier diet)

Tissue/finding	Sex		
	Male	Female	Combined
Adrenal cortex			
—focal hyperplasia and/or neoplasia	+	N.S.	N.S.
Adrenal medulla			
—tumour or focal hyperplasia	(+)	+	++
—malignant tumour	+	N.S.	N.S.
Heart			
—myocarditis	+++	N.S.	+++
—myocardial fibrosis	+++	N.S.	+++
Kidney			
—chronic progressive nephropathy (grades 3–5)	+++	+	+++
—pelvic mineralization	++	+++	+++
Lung			
—interstitial pneumonitis	+	+	+++
Mammary gland			
—acinal hyperplasia (grades 2–5)	++	N.S.	N.S.
Pancreatic/mesenteric artery			
—polyarteritis	+++	(+)	+++
Spleen			
—generalized hyperplasia	+	+	+++
Testis			
—arteriolitis (grades 2–5)	++		
Uterus/uterine horns			
—distension (grades 4–5)		++	
—endometrial hyperplasia		+	

Key for significance levels as Table 4.

Table 25. Histopathology: main effects of LMA diet in comparison with SMA from week 13 (unadjusted for earlier diet)

Tissue/finding	Sex		
	Male	Female	Combined
Adrenal cortex			
—haemorrhage/degeneration (grades 2–5)	++	N.S.	+
Heart			
—myocarditis	N.S.	– – –	– – –
—myocardial fibrosis	–	– – –	– – –
Kidney			
—chronic progressive nephropathy (grades 3–5)	– – –	– – –	– – –
Liver			
—bile-duct hyperplasia (grades 2–5)	+++	N.S.	++
—fatty degeneration (grades 3–5)	N.S.	N.S.	+
Lymph node—cervical			
—cystic	N.S.	– –	–
—lymphoid hyperplasia	N.S.	– – –	(–)
Mammary gland			
—acinal hyperplasia (grades 4–5)	N.S.	– – –	– – –
—secretory activity	– – –	– – –	– – –
—galactocoele formation	N.S.	– –	–
—neoplasm	N.S.	– – –	– – –
Mesenteric lymph node			
—haemangioma/haemangiosarcoma	+++	N.S.	+++
Pancreas			
—exocrine adenoma	–	N.S.	–
—islet-cell tumour	– –	N.S.	– –
Pituitary gland			
—anterior lobe adenoma/adenocarcinoma	–	– – –	– – –
—intermediate lobe adenoma/adenocarcinoma	– – –	– – –	– – –
Prostate			
—acute inflammation	(–)		
Radiculoneuropathy			
—affecting muscle and sciatic nerve	N.S.	– – –	(–)
Subcutaneous tissue			
—keratin-filled cyst	N.S.	N.S.	–
—tumour	N.S.	N.S.	(–)
Testis			
—Leydig-cell tumour	+++		
Uterus			
—adenoma/adenocarcinoma		++	
—adenocarcinoma		+	
—distension		+++	
—hypertrophy of cervical wall		++	
Benign or malignant tumour at any site	N.S.	– – –	–
Benign or malignant tumour at any site except uterus or mesenteric lymph node	N.S.	– – –	– – –
Tumours of two or more sites	N.S.	– – –	– – –

Key for significance levels as Table 4.

remaining seven cases in the group on SBI. In females, where the condition was seen much more commonly, there is a hugh difference in distribution of severity according to diet to week 13. In decreasing order of severity the diets could be placed in the order SBA, SBI, SBR, LBA, PRA, LMA. The contrast between the extremes was striking with animals on SBA, virtually all (97.5%) having the condition and 69% having grades of at least moderate, compared with animals on LMA, where only 12% had the condition and none had grades of at least moderate. After adjustment for diet to week 13 there was really no clear evidence of an effect of subsequent diet whatsoever.

2. *Squamous carcinoma of the jaw*

As shown in Table 22, the incidence of squamous carcinoma of the jaw was lowest for SBA diet to week 13, with only 6/400 (1.5%) developing the tumour. It was increased in the SBI and PRA groups, where incidence was respectively 7 and 9%, but was also elevated in all other groups. There was no real

Table 26. Histopathology: main effects of SMR and SMI diet in comparison with SMA from week 13 (unadjusted for earlier diet)

	Diet/sex					
	SMR			SMI		
Tissue/finding	M	F	M+F	M	F	M+F
Adrenal cortex						
—haemorrhage/degeneration (grades 3–5)	N.S.	– –	– – –	N.S.	N.S.	N.S.
Heart						
—myocarditis	N.S.	– – –	– – –	N.S.	– – –	– – –
—myocardial fibrosis	– –	– – –	– – –	N.S.	– –	– – –
Kidney						
—chronic progressive nephropathy (grades 3–5)	– – –	– – –	– – –	– – –	– – –	– – –
Liver						
—periductule fibrosis	– –	N.S.	– –	–	N.S.	(–)
Lung						
—lymphoid aggregates round airways (grades 2–5)	(–)	–	– –	N.S.	(–)	(–)
—adenoma/adenocarcinoma	–	(–)	– –	N.S.	N.S.	–
Lymph nodes—cervical						
—cyst	–	–	– –	N.S.	(–)	N.S.
—lymphoid hyperplasia	N.S.	– –	(–)	N.S.	N.S.	N.S.
Mammary gland						
—acinal hyperplasia (grades 4–5)	N.S.	– – –	– – –	N.S.	– –	– –
—secretory activity	– – –	– – –	– – –	N.S.	N.S.	N.S.
—galactocoeles	N.S.	– – –	– – –	N.S.	(–)	N.S.
—fibroma/fibroadenoma/adenoma/adenocarcinoma	N.S.	– – –	– – –	N.S.	–	–
Mesenteric lymph node						
—haemangioma/haemangiosarcoma	–	N.S.	– –	N.S.	N.S.	(–)
Pancreas						
—exocrine adenoma	–	N.S.	–	–	N.S.	–
—islet-cell adenoma/adenocarcinoma	–	N.S.	– –	(–)	N.S.	–
Pituitary gland						
—anterior lobe						
—adenoma/adenocarcinoma	– – –	– – –	– – –	N.S.	–	–
—intermediate lobe						
—cyst	N.S.	– –	– –	N.S.	N.S.	N.S.
—adenoma/adenocarcinoma	(–)	– –	– – –	–	N.S.	–
Polyarteritis affecting mesenteric and/or pancreatic artery	N.S.	– –	– –	N.S.	N.S.	–
Prostate						
—acute inflammation	– – –			–		
Radiculoneuropathy						
—affecting muscle and sciatic nerve	–	–	– – –	N.S.	–	N.S.
Skin						
—epidermal tumour	(–)	N.S.	–	–	N.S.	–
Subcutaneous						
—tumour	– –	N.S.	– –	(–)	N.S.	N.S.
Malignant tumour						
—any site	– – –	– – –	– – –	N.S.	N.S.	N.S.
—any site except uterus or mesenteric lymph node	– – –	– – –	– – –	N.S.	N.S.	N.S.
—two or more sites	– –	–	– – –	N.S.	N.S.	N.S.
—'other' tumours (see Table 26)	(–)	(–)	– –	N.S.	N.S.	N.S.
Benign or malignant tumour						
—any site	– – –	– – –	– – –	N.S.	N.S.	–
—any site except uterus or mesenteric lymph node	– – –	– – –	– – –	N.S.	–	–
—two or more sites	– – –	– – –	– – –	– –	– –	– – –
—'other' tumours (see Table 26)	(–)	(–)	– –	N.S.	N.S.	N.S.

Key for significance levels as Table 4.

Table 27. Groupings used to analyse incidence of tumours and associated hyperplasia

Grouping		Lesions included		
No.	Name (total cases)	Focal hyperplasia	Benign tumour	Malignant tumour
1.	Adrenal cortex (42)	+	Adenoma	Adenocarcinoma
2.	Adrenal medulla (86)	+	Benign phaeochromocytoma	Malignant phaeochromocytoma
3.	Liver hepatocellular (13)		Benign hepatoma	Hepatocellular carcinoma
4.	Lung (27)		Adenoma	Adenocarcinoma
5.	Mammary gland (109)		Mammary fibroma, fibroadenoma or adenoma	Mammary adenocarcinoma
6.	Ovary granulosa-cell (7)		Benign tumour	Malignant tumour
7.	Ovary tubular (9)	+	Tubular adenoma	
8.	Pancreas exocrine (13)		Adenoma	
9.	Pancreas islet-cell (30)		Adenoma	Adenocarcinoma
10.	Parathyroid (165)	+	Adenoma	
11.	Pituitary anterior (538)	+	Benign tumour	Malignant (or probably malig.) tumour
12.	Pituitary intermed. (150)	+	Benign tumour	Malignant (or probably malig.) tumour
13.	Prostate (3)		Adenoma	Adenocarcinoma
14.	Spleen (3)			Sarcoma
15.	Testis Leydig-cell (223)	+	Benign tumour	
16.	Thyroid C-cell (67)	+	Adenoma	Adenocarcinoma
17.	Thyroid follicular (23)	+	Adenoma	Adenocarcinoma
18.	Uterus horn squamous (3)		Squamous polyp	Squamous carcinoma
19.	Uterus horn fibromyoma (3)		Fibromyoma	
20.	Uterus horn or body glandular (73)		Adenomatous polyp papillary adenoma	Adenocarcinoma or anaplastic carcinoma
21.	Uterus body sarcoma/ myxosarcoma (15)			Sarcoma or myxosarcoma
22.	Uterus body haemangiosarcoma (1)			Haemangiosarcoma
23.	Brain glioma (6)			Glioma
24.	Brain meningioma (9)			Meningioma
25.	Jaw carcinoma (43)			Squamous or anaplastic carcinoma
26.	Lymph nodes (other than mesenteric) (5)		Haemangioma	
27.	Mass epidermal (includes tumours of mammary gland, skin and skin and tail) (42)		Squamous papilloma, keratoacantoma, sebaceous adenoma, benign basal cell tumour	Squamous carcinoma, malignant trichoepithelioma, basal cell carcinoma, anaplastic carcinoma, mixed squamous/adenocarcinoma
28.	Mass subcutaneous (95)		Lipoma or fibroma	Adenocarcinoma, sarcoma, anaplastic sarcoma, myxosarcoma or anaplastic carcinoma
29.	Mass bone (includes skull) (8)		Osteoma	Osteosarcoma
30.	Mass vascular (includes) tail (8)		Haemangioma	Haemangiosarcoma
31.	Mesenteric lymph node (178)		Benign tumour	Malignant (or probably malignant) tumour
32.	Salivary gland sarcoma (3)			Sarcoma
33.	Stomach/fore (8)		Squamous papilloma	
34.	Stomach/glandular (4)		Adenomatous polyp	Adenocarcinoma
35.	Thymus (5)		Benign thymoma	
36.	Tongue (5)		Squamous papilloma	Squamous carcinoma, anaplastic carcinoma
37.	Malignant lymphoma (44)			Malignant lymphoma
38.	Other tumours (34)		(See Table 26)	(see Table 26)
39.	Tumours of any site (969)		Benign	Malignant
40.	Tumours of multiple sites (514)		Benign	Malignant

Key: +indicates focal hyperplasia was used in that grouping.

evidence of an effect of later diet where early diet had been adjusted for.

3. *Lesions showing increased incidences on LMA (and also LBA and PRA) diet*

Table 23 gives results for eight lesions. They fall into a number of groups. First, lesions in the male sex organs; Leydig-cell tumours or hyperplasia of the testis; atrophy of the epididymis and of the testis; and chronic inflammation of the prostate. In all cases incidence on the LMA diet was clearly increased ($P < 0.01$), but although incidences on the LBA and PRA diets to week 13 also tended to be increased, this was less marked and often not significant.

The second group were three lesions: lymphocyte aggregates in the cortex in the kidney; bile-duct hyperplasia; and fibrosis round bile ductules in the liver, where a significant increase (usually $P < 0.001$) was evident in the LMA, LBA and PRA groups.

The final group was the single lesion, foci/areas of margination of the cytoplasm of the liver, where only the increase on the LMA diet was significant ($P < 0.05$).

Two points should be noted in relation to Table 23. First, results are presented unadjusted for diet from

week 13. This was because, for virtually all these lesions, there was no evidence of an effect of diet from week 13. Also adjustment for diet from week 13 would have meant elimination of the PRA diet from the analysis when it seemed fairly likely that the relatively high incidence in group 12 (PRA/PRA) was indeed a result of the early diet. In fact, where diet from week 13 had been shown to have an effect, adjustment for it did not materially affect conclusions, merely reducing the significance of the LMA reduction for testis Leydig-cell tumours and hyperplasia and the LBA reduction for lymphocyte aggregates of the cortex in the kidney from $P < 0.001$ to $P < 0.01$ and not affecting the significance of the LMA reduction for the latter condition.

The second point to be noted is that, for the kidney and liver lesions, results are presented for sexes combined. This is because patterns were generally similar in the two sexes. A possible exception is foci/areas of margination of cytoplasm of the liver, where in males no significant difference was seen between the LMA and SBA groups whereas in females there was a highly significant $P < 0.001$) difference. However, even here, incidences were low and significance of results for the sexes combined ($P < 0.05$) may be considered more reliable.

Table 28. Benign and malignant neoplasms that occurred in low incidence and that are referred to in the text as 'Other tumours'

Tissue/tumour type	Sex	Group
Benign tumours		
Epididymis—haemangioma	M	12
Kidney—adenoma	M	5
Kidney—lipoma	F	7*
Thyroid—ganglioneuroma	F	8
Buccal cavity—squamous papilloma	M	12
Lip—squamous papilloma	M	10, 11
	F	10
Mass—chondroma	M	8
Mass—leiomyoma	M	2
Mass—fibromyxoma	M	4
Preputial gland—adenoma	M	9
Rectum—fibromatous polyp	M	12
Urinary bladder—adenoma	F	10
Malignant tumours		
Heart—sarcoma	M	9
Liver—cholangiocarcinoma	M	1, 9
Prostate—sarcoma	M	9
Testis—mesothelioma	M	6
Buccal cavity—squamous carcinoma	F	2, 4
Duodenum/jejunum/ileum —adenocarcinoma	M	8
Jaw—sarcoma	F	8
Mass—malignant schwannoma	M	1
	F	9
Mass—adenocarcinoma in mediastinum	M	1
Rectum/colon/caecum —spindle-cell sarcoma	M	12
Salivary gland—adenocarcinoma	F	1, 12
Thymus—adenocarcinoma	F	7*
Thymus—sarcoma	M	8
	F	9
Thymus—anaplastic carcinoma	F	11
Unknown primary—carcinoma	M	10
Histiocytic sarcoma	F	1

*Tumours were in the same animal.

4. *Lesions showing reduced incidences on SBR and SBI (and also LBA and LMA) diets*

All of the four lesions in question (fibrosis of the heart, chronic progressive nephropathy, secretory activity and polyarteritis) were clearly affected more strongly by diet from week 13, so the results shown in Table 23 are adjusted for diet from week 13. As can be seen, evidence of a reduction is most evident for the SBR and LBA diets, but less clear or even non-existent in some cases for the SBI and LMA diets. The results are presented for the sexes combined for all lesions (except secretory activity). This was because the trend was in the same direction in both sexes, although it should be noted that the effects on nephropathy were rather more marked in males than females whereas those on polyarteritis were rather more marked in females than males.

Effects of diet from week 13

The results summarized in Tables 24–26 are for diet from week 13 unadjusted for earlier diet. Most of the lesions considered were not apparently affected at all by diet to week 13, and those that were, were generally much less affected by early than by late diet and adjustment for early diet really served only to lose power to detect differences.

Effects of diet from week 13 could be divided into four main categories:

(1) Increases in incidence in relation to PRA diet;
(2) Increases in incidence in relation to LMA diet;
(3) Other increases in incidence;
(4) Reductions in incidence in relation to SMR, SMI and LMA diets.

1. *Increased incidences in rats on PRA diet (Table 24)*

Since the PRA diet was only given in a single group (12) which received it throughout life, it is not possible, rigorously, to determine whether any increased incidence in this group is an effect of early or late diet. However, one can in fact come to some tentative conclusions based first on knowledge from other diets as to whether a lesion is affected by early or late diet and secondly on observing which showed a bigger difference, PRA *v.* SBA or PRA *v.* SMA. Effects of PRA diet on corticomedullary mineralization of the kidney, squamous carcinoma of the jaw, atrophy of the testis, bile-duct hyperplasia, and fibrosis around bile ductules of the liver appear to be early diet effects (Tables 21–23). Effects that appear to be related to PRA feeding from week 13 are summarized in Table 24.

Although PRA feeding was associated with higher incidences of adrenal cortex tumour or hyperplasia in males, and with increased adrenal medullary tumour in both sexes than SMA feeding, the differences are not very striking. Otherwise it is clear that the PRA and SMA diets did not produce very different tumour profiles. An explanation of the adrenal medullary effects of PRA feeding is discussed below.

There are, however, a number of non-neoplastic lesions where a marked increase in incidence is associated with PRA diet from week 13. Thus Table 24 demonstrates highly significant ($P < 0.001$) increases in chronic inflammation and fibrosis of the heart, in chronic progressive nephropathy and pelvic mineralization of the kidney, in interstitial pneumonitis (though this may be dubious—see below), in polyarteritis, and in generalized hyperplasia of the spleen. There are also a range of other lesions with less significant increases—mammary gland acinal hyperplasia, arteriolitis of the testis, and uterine distension and endometrial hyperplasia.

It is striking that there is no evidence that, compared with the SMA diet, the PRA diet reduced incidence of any lesion.

2. *Increased incidences in rats on LMA diet*

As shown in Table 25, LMA diet from week 13 resulted in a marked increase in the incidence of three tumour types: Leydig-cell tumours of the testis ($P < 0.001$); glandular tumours of the uterus ($P < 0.01$); and mesenteric lymph node haemangiomas and haemangiosarcomas ($P < 0.001$ for males and for sexes combined).

LMA diet was also associated with an increased incidence of non-neoplastic lesions: uterine distension; marked hypertrophy of the wall of the cervix; fatty degeneration and foci/areas of fatty degeneration of the liver; and adrenal cortex haemorrhage and/or degeneration.

As will be seen below, the LMA diet also resulted in a large number of decreases in incidence, such lesions also being reduced in the SMR and sometimes also in the SMI groups. The effect of these decreases on tumour incidence outweighed the adverse effects on testis, uterus and mesenteric lymph node tumours.

3. *Other diet-related increased incidences*

The following four conditions were seen in higher incidence in rats fed SMR compared with rats fed SMA: generalized hyperplasia of the spleen; endometrial hyperplasia; increased distension of seminal vesicles; and ulceration and cellulitis of the tail. Some of the differences were of only marginal significance ($P < 0.05$) and some could easily be chance findings bearing in mind the number of lesions studied. Endometrial hyperplasia was also increased on the SMI diet as was uterine distension. For all these five endpoints the comparison was with all rats fed SMA after week 13. For the final endpoint, interstitial pneumonitis, incidence (of higher grades) was particularly increased in two groups, group 12 (PRA/PRA) and group 2 (SBA/LMA). Both increases were highly significant ($P < 0.001$), but it seems particularly difficult to explain the latter increase since other groups fed SBA to week 13 or LMA from week 13 showed no increase. It is either an unusual interaction of the two diets or perhaps it is a result of inconsistent severity grading.

4. *Reduced incidences in rats on SMR, SMI and LMA diets*

Tables 25 and 26 summarize results for the very large number of conditions which were reduced by SMR, SMI and/or LMA diets.

The SMR diet from week 13 caused a reduction in the incidence of a whole range of tumour types, with highly significant ($P < 0.01$ or $P < 0.001$) reductions noted for lung, mammary, pancreas islet-cell, pituitary, subcutaneous tissue masses and mesenteric lymph node tumours as well as for the 'other tumours' group. There was also evidence of an effect on pancreas exocrine tumours ($P < 0.05$) and on epidermal tissue (mass) tumours ($P < 0.5$). As a result, the overall incidence of malignant tumours, benign or malignant tumours and multiple tumours was reduced highly significantly ($P < 0.001$). Thus, for example, only 16% rats fed SMR had malignant tumours against 37% fed SMA, despite the longer survival of the SMR rats.

For most of these sites there was evidence of some reduction in incidence in SMI rats, but the reductions were much smaller and at best of moderate significance.

The reduction in incidence of mammary, pancreas islet-cell and pituitary tumours for LMA rats was of the same significance and of almost the same magnitude as that for SMR rats. Significant ($P < 0.05$) reductions were also noted for pancreas exocrine tumours, epidermal tissue mass tumours and 'other tumours'. However, because of the marked increase in mesenteric lymph node and uterine tumours (see Table 25), no reduction in overall tumour incidence was seen in males and, although the reduction in females was highly significant ($P < 0.001$), it was much smaller than that for SMR rats. The results of an additional analysis of overall tumour incidence except that of the uterus or mesenteric lymph node are included in Tables 25 and 26. As would be expected, these increased the difference between the LMA and SMA diets, the reduction (for the sexes combined) becoming significant at $P < 0.01$ for malignant tumours and at $P < 0.001$ for benign or malignant tumours.

A wide range of non-neoplastic findings were reduced on the SMR diet, with many of the reductions evident on the LMA and SMI diets. The most notable reductions were in chronic inflammation and fibrosis of the heart, acinal hyperplasia and secretory activity of the mammary gland, acute inflammation of the prostate and radiculoneuropathy. Where reductions were seen they were usually most marked in the SMR group.

Various interrelationships

The large size of the database enabled us to look for possible interrelationships between different variables, for instance between different variables measured at the same time, between the same

variables measured at different times, and between variables measured at one time and other variables measured at an earlier or later time. So far we have confined our interest to a limited number of what we considered to be potentially the most interesting and/or important relationships. Our intention was not so much to come up with firm answers, but to highlight specific relationships which require further more detailed study. For this reason some of the analyses we have conducted are rather superficial in nature. Further in-depth analyses may be undertaken and reported at a later date.

Correlates of survival, tumour incidence, and of certain common non-neoplastic conditions

1. *Introduction: comparisons of levels of predictors in animals which did or did not develop certain conditions*

As a first step, correlates of survival, tumour incidence, and of certain common non-neoplastic conditions were considered. Of particular interest were variables measured early in life which were strong predictors of later disease, although we also studied variables measured late in life (or even at death) when the correlation might have resulted from an effect of the disease rather than being a cause of it. For the purposes of this exercise, 12 key *conditions* were considered:

1. week of death (1–78, 79–104, 105–118, >118, terminal kill)
2. mammary tumours (none, benign, malignant)
3. pituitary (anterior) tumour/hyperplasia (none, hyperplasia, benign, malignant)
4. testis Leydig-cell tumour/hyperplasia (none, hyperplasia, benign, malignant)
5. uterus glandular tumours (none, benign, malignant)
6. neoplasms/subcutaneous (none, benign, malignant)
7. mesenteric lymph node tumours (none, benign, malignant)
8. tumours at any site (none, benign, malignant)
9. tumours of multiple sites (none, benign, malignant)
10. radiculoneuropathy (none, minimal, fatal)
11. polyarteritis (none, minimal, fatal)
12. chronic progressive nephropathy (none, minimal, slight, moderate, severe to fatal)

For each of the *predictors*, listed below, a Kruskal–Wallis rank analysis was carried out to determine differences in value of the predictor by level of condition and to see whether there was a trend in value of the predictor over 'level' of the condition (the 'levels' are shown in parentheses in the above list). The predictors tested were:

1. body weight (days 106, 120, 141, 183, 365, 547 and 729)
2. total serum protein (at 6, 12, 18, 24 and 30 months)
3. serum albumin (at 6, 12, 18, 24 and 30 months)
4. blood urea nitrogen (at 6, 12, 18, 24 and 30 months)
5. serum creatinine (at 6, 12, 18, 24 and 30 months and in the urine and plasma at 30 months)
6. blood glucose (at 6, 12, 18, 24 and 30 months)
7. serum alanine aminotransferase activity (at 30 months)
8. serum alkaline phosphatase activity (at 30 months)
9. serum glucose-6-phosphate dehydrogenase activity (at 30 months)
10. serum aspartate aminotransferase activity (at 30 months)
11. white blood cell count (at 6, 12, 18, 24 and 30 months)
12. red blood cell count (at 6, 12, 18, 24 and 30 months)
13. haemoglobin concentration (at 6, 12, 18, 24 and 30 months)
14. haematocrit (at 6, 12, 18, 24 and 30 months)
15. red cell distribution width (at 6, 12, 18, 24 and 30 months)
16. platelet count (at 6, 12, 18, 24 and 30 months)
17. mean platelet volume (at 6, 12, 18, 24 and 30 months)
18. polymorph count (at 6, 12, 18, 24 and 30 months)
19. lymphocyte count (at 6, 12, 18, 24 and 30 months)
20. urine volume (at 6, 12, 18, 24 and 30 months)
21. urinary pH (at 6, 12, 18, 24 and 30 months)
22. urinary protein (at 6, 12, 18, 24 and 30 months)
23. urinary ketone (at 6, 12, 18, 24 and 30 months)

The output from these analyses were studied to identify consistent major relationships and the following were noted:

2. *Survival*

In both sexes, longer-surviving animals consistently had lower weights than earlier decedents. The relationship was evident in both sexes and most

Table 29. Relationship between mean body weight on day 365 and the presence and severity of chronic progressive nephropathy seen at autopsy

	Nephropathy					
Median body weight (g)	None	Minimal	Slight	Moderate	Severe/ fatal	Significance of trend
Males	425.0	456.2	472.4	501.0	539.5	+++
Females	247.1	254.2	266.4	284.2	295.8	+++

Key for significance levels as Table 4.

evident for body weight at days 183, 365 and 547. Longer survivors also had lower urinary protein levels at 6, 12, 18 and 24 months in males, and at 12, 18 and 24 months in females. Total serum protein was lower in longer-surviving females at 6 and 12 months. In males, there was a clear positive relationship between urinary pH and survival.

3. *Mammary tumours*

In females, there was a significant relationship of tumour incidence to increased body weight at all time points, to increased total urinary protein and albumin at 6 months, and to increased urinary protein at 18, 24 and 30 months. At 30 months also, animals which subsequently developed tumours had increased urine volume, increased WBC, and decreased serum alanine and aspartate aminotransferase activity. There were too few males with mammary tumours for worthwhile analysis.

4. *Anterior pituitary tumour/hyperplasia*

Body weight at days 365, 547 and 729 was strongly related to pituitary tumour incidence in females. A weaker relationship was evident in males at this time and in both sexes earlier in life, although this was not always statistically significant. The only other clear correlates with pituitary tumour incidence were at 30 months, increased total and urinary protein, and urine volume in both sexes, and decreased alkaline phosphatase activity, RBC and urinary ketones in males.

5. *Testis Leydig-cell tumour/hyperplasia*

At 30 months, and to some extent at 24 months, animals with tumours had altered haematology (increased RBC, Hb, Hct, and RDW, and decreased PLT and MPV). The only clear relationship with early-life variables was increased PLT at 6 months in animals which were subsequently found to have tumours.

6. *Uterus: endometrial tumours*

Tumour incidence was negatively correlated with body weight from day 183 onwards, and with PLT at 6 months. Other correlations were with variables measured at 30 months (reduced total protein, glucose, RBC, Hb, Hct, and different lymphocytes; increased ALP, WBC, RDW and differential polymorphs).

7. *Subcutaneous neoplasms*

No strong or consistent relationships were seen.

8. *Mesenteric lymph node tumours*

In males, but not females, body weight from day 365 onwards (particularly day 729) was positively related to tumour incidence. With the exception of a tendency in both sexes for a positive relationship with urinary ketone at 12 months, the only clear relationships were with variables measured at 30 months (decreased pH in males and increased ALT and AST activity in both sexes and increased GLDH activity in females).

9. *Tumours of any site*

There was a strong tendency in both sexes for tumour incidence to be positively related to body weight, particularly from day 183 onwards. Tumour incidence was also negatively related with urinary pH whenever measured and positively with urinary protein (at 24 and 30 months in males and at 12, 18 and 24 months in females). Other relationships were with variables measured at 30 months (positively with serum alanine aminotransferase activity in males, with WBC and with RDW in both sexes, and with polymorph count in females; and negatively with serum albumin, blood glucose, RBC, Hb, Hct and MPV in both sexes, and with plasma creatinine in males).

10. *Multiple tumours*

The relationships with body weight, urinary pH and urinary protein were qualitatively similar to those with the incidence of tumours at any site. Additionally, in females, total serum protein at 6 and 12 months, and serum albumin at 12 months were positively related to multiple tumour incidence. Again there were many relationships with variables measured at 30 months, the direction of relationships and the range of variables affected being similar.

11. *Radiculoneuropathy*

In females, animals which subsequently developed radiculoneuropathy had increased body weights at all times throughout the study. In males, this relationship was evident only at day 729. Other relationships noted were positively with RDW at 6 months and with MPV at 18 months, and negatively with albumin at 30 months and with urinary ketone at 18 months. These relationships, each evident in both males and females, were never highly significant.

Table 30. Relationship of tumour incidence and premature death to grouped body weight at day 183—trends and departures from trend

	Benign (B) or Malignant (M)	Total incidence[1]	Trend		Departure from trend	
			Chi-squared	*P*	Chi-squared	*P*
Premature deaths						
Males		294	28.61	+++	1.43	N.S.
Females		286	16.70	+++	1.99	N.S.
Tumour type (sexes combined)						
Any site	M	444	25.98	+++	5.04	N.S.
	B or M	968	35.46	+++	2.26	N.S.
Multiple sites	M	58	9.18	++	1.27	N.S.
	B or M	514	56.14	+++	1.77	N.S.
Adrenal cortex	B or M	15	0.00	N.S.	1.87	N.S.
Adrenal medulla	M	15	2.11	N.S.	1.14	N.S.
	B or M	50	2.46	N.S.	1.25	N.S.
Liver hepatocellular	B or M	13	0.26	N.S.	2.26	N.S.
Lung	B or M	27	4.10	+	1.74	N.S.
Mammary gland	M	21	1.30	N.S.	0.65	N.S.
	B or M	109	24.46	+++	1.68	N.S.
Pancreas exocrine	B or M	13	7.68	++	4.06	N.S.
Pancreas islet-cell	B or M	30	3.57	(+)	1.14	N.S.
Parathyroid	B or M	36	0.16	N.S.	0.17	N.S.
Pituitary	M	38	8.73	++	10.61	**
(anterior)	B or M	443	11.27	+++	1.95	N.S.
Pituitary (intermediate)	B or M	75	9.64	++	0.86	N.S.
Testis Leydig-cell	B or M	198	1.05	N.S.	3.35	N.S.
Thyroid C-cell	B or M	52	0.47	N.S.	2.31	N.S.
Thyroid follicular	B or M	17	3.73	(+)	1.71	N.S.
Uterus glandular	M	54	0.93	N.S.	6.37	(*)
	B or M	73	2.23	N.S.	8.15	*
Uterus sarcoma/ myxosarcoma	M	15	5.26	–	4.31	N.S.
Jaw squamous carcinoma	M	37	7.60	++	0.74	N.S.
Mass epidermal	M	20	3.37	(+)	3.15	N.S.
	B or M	42	2.46	N.S.	3.64	N.S.
Mass subcutaneous	M	61	6.66	++	2.52	N.S.
	B or M	95	5.99	+	2.46	N.S.
Mesenteric lymph node	M	95	1.51	N.S.	15.72	**
	B or M	178	2.93	(+)	6.14	N.S.
Malignant lymphoma	M	44	3.74	(+)	4.62	N.S.
'Other' tumours	M	21	13.26	+++	1.56	N.S.
	B or M	13	9.27	++	0.82	N.S.
Any site except uterus or mesenteric lymph node	M	311	37.61	+++	0.71	N.S.
	B or M	897	54.12	+++	1.71	N.S.

[1]Total incidence is number occurring among rats surviving to day 183 with valid weight data.
$^{**}P < 0.01$; $^{*}P < 0.05$; $(^{*})P < 0.1$.
For key for other significance levels see Table 4.

12. *Polyarteritis*

There was a consistent tendency in both sexes for a positive relationship between body weight and polyarteritis. There was also, in males at 12 and 18 and particularly at 6 months, a positive relationship with urinary protein in urine. Other relationships noted in both sexes were a positive relationship with PLT at 24 months and with serum creatinine at 30 months, and a negative relationship with serum albumin at 30 months.

13. *Chronic progressive nephropathy*

There was an absolutely massive positive correlation in both sexes between grade of nephropathy and body weight measured at any time during the study. As illustrated in Table 29, which summarizes data for day 365, the rise in median body weight with increasing grade was remarkably smooth and very highly significant indeed.

Throughout the study in both sexes nephropathy was also strongly positively related to urinary protein and strongly negatively related to urinary pH. Nephropathy was strongly positively related in both sexes to total serum protein at 6, 12 and 18 months, although in males the relationship became negative at 30 months. Serum albumin showed a strong early-life positive correlation and a strong late-life negative correlation with nephropathy in both sexes. BUN generally showed a positive relationship in males and a negative relationship in females. At 12 and 18 months, glucose was positively related, and urinary ketone negatively related, with nephropathy in both sexes. WBC were very strongly positively related at all time points to nephropathy, although only in males. MPV was positively related with nephropathy

Table 31. Relationship of tumour incidence and premature death to grouped body weight at day 183—relative risks and 95% confidence intervals

Endpoint	Sex[1]		Body weight groups VL[2]	L[2]	Mi[2]	H[2]	VH[2]	Significance[3] of trend
No. of animals surviving to 29 weeks	M		120	120	120	120	118	
	F		120	121	118	122	116	
Premature deaths	M	n[4]	42	50	58	66	78	+ + +
		RR[5]	1.00	1.23	1.57	1.84	2.55	
		95%CI[6]		(0.82–1.86)	(1.06–2.34)	(1.25–2.70)	(1.77–3.69)	
	F	n	42	56	51	69	68	+ + +
		RR	1.00	1.50	1.38	1.97	2.12	
		95%CI		(1.00–2.24)	(0.91–2.08)	(1.34–2.88)	(1.45–3.11)	
Benign or malignant tumour of any site	M	n	87	99	103	103	102	+ + +
		RR	1.00	1.68	2.51	2.66	3.19	
		95%CI		(1.06–2.66)	(1.57–4.00)	(1.63–4.34)	(2.02–5.03)	
	F	n	90	94	96	103	91	+ +
		RR	1.00	1.38	1.35	1.96	1.75	
		95%CI		(0.93–2.03)	(0.91–2.00)	(1.33–2.88)	(1.16–2.63)	
Malignant tumours of any site	M&F	n	69	81	97	112	85	+ + +
		RR	1.00	1.31	1.76	2.30	1.89	
		95%CI		(0.94–1.84)	(1.26–2.44)	(1.68–3.15)	(1.35–2.63)	
Malignant tumours of any site except uterine, glandular or mesenteric lymph node	M&F	n	41	52	64	80	74	+ + +
		RR	1.00	1.40	1.90	2.67	2.85	
		95%CI		(0.92–2.14)	(1.27–2.84)	(1.82–3.91)	(1.92–4.22)	
Benign or malignant tumours of two or more sites	M&F	n	79	90	102	128	115	+ + +
		RR	1.00	1.36	1.76	2.71	2.89	
		95%CI		(0.96–1.92)	(1.25–2.46)	(1.94–3.78)	(2.05–4.08)	
Pituitary tumour	M&F	n	86	103	96	107	112	+ + +
		RR	1.00	1.57	1.14	1.83	2.23	
		95%CI		(1.10–2.25)	(1.01–2.07)	(1.27–2.63)	(1.51–3.28)	
Mammary gland tumour	M&F	n	12	14	19	32	32	+ + +
		RR	1.00	1.26	1.82	3.52	4.30	
		95%CI		(0.54–2.90)	(0.85–3.89)	(1.74–7.10)	(2.18–8.49)	
Mass epidermal tumour	M&F	n	8	10	5	8	11	N.S.
		RR	1.00	1.42	0.73	1.46	2.76	
		95%CI		(0.54–3.77)	(0.23–2.33)	(0.51–4.15)	(1.06–7.20)	
Mass subcutaneous tumour	M&F	n	15	15	20	27	18	+
		RR	1.00	1.06	1.59	2.27	1.69	
		95%CI		(0.51–2.22)	(0.81–3.12)	(1.19–4.31)	(0.82–3.48)	
Jaw squamous carcinoma	M&F	n		8	9	10	13	+ + +
		RR	1.00	3.96	5.02	5.91	8.57	
		95%CI		(1.01–15.47)	(1.28–19.60)	(1.51–23.14)	(2.52–29.15)	

[1] M = male, F = female.
[2] VL = very low, L = low, Mi = middle, H = high, V = very high.
[3] Key for significance levels see Table 4.
[4] n = number occurring among rats surviving to day 183 with valid weight data.
[5] RR = relative risk adjusted for survival.
[6] 95%CI = 95% confidence interval.

up to 24 months in both sexes. There were a number of relationships with variables measured at 30 months (positive with creatinine in males; negative with all four enzymes in both sexes; negative with RBC in males; negative with Hb and Hct in males, positive in females; positive with RDW in females; positive with platelet counts in both sexes; negative with MPV in males; positive with polymorph counts in both sexes; negative with lymphocyte counts in females; positive with urine volume in females). Many of these relationships were also evident at 24 months. Generally, the strength of the relationships with nephropathy were much stronger than with the other 11 conditions studied.

The clearest effects of early life parameters were:

Increased body weight:
nephropathy, premature death, overall tumour incidence, and mammary tumour incidence

Increased protein:
nephropathy, premature death and overall tumour incidence

Increased pH:
nephropathy and premature death

Increased glucose and albumin:
nephropathy.

Table 32. Relationship between diet from week 13 and grouped body weight at day 183

Sex	Grouped body weight at day 183	Diet from wk 13					
		SMA	SMR	SMI	LMA	PRA	Total
Males	VL	5	56	6	53	0	120
	L	17	22	28	52	1	120
	M	22	15	33	48	2	120
	H	47	7	18	33	15	120
	VH	59	0	15	12	32	118
	Total	150	100	100	198	50	598
Females	VL	6	33	23	57	1	120
	L	14	31	26	46	4	121
	M	30	14	22	48	4	118
	H	39	18	21	32	12	122
	VH	59	4	8	16	29	116
	Total	148	100	100	199	50	597

Key: VL = Very low; L = Low; M = Middle; H = High; VH = Very high.
The table shows the numbers of animals in the groups specified.

14. *Probability of development of certain conditions in relation to level of predictor*

The above statistical analyses were based on comparing levels of predictors, such as body weight, in animals which did or did not get certain conditions. In the following sections we give some results where we look at the correlations the other way round, that is looking at how the probability of the condition varies by level of the predictor. In these analyses we have typically divided the predictor by quintiles into five groups and have used age-adjusted Peto analyses to compare risks by group and to look for trends over groups.

15. *Body weight at day 183 as a predictor of tumour incidence and of premature death*

Animals of each sex were divided separately by body weight as recorded at day 183 into five approximately equal groups (VL = very low, L = low, M = middle, H = high, VH = very high) and the age-adjusted risk of tumours of various types and of premature death compared over the groups. Table 30 summarizes the results of significance tests for the relationship of grouped body weight on day 183 to overall tumour incidence, to incidence of a range of tumour types (for which there was a total incidence of 10 or more) and to premature death. It shows, for the sexes combined, the trend chi-squared (on 1 degree of freedom) and the departure from trend chi-squared (on 3 degrees of freedom), together with their significance. Where appropriate, results for the tumour types are presented separately for malignant occurrences and for benign or malignant occurrences. For premature death, and for various major tumour categories, Table 31 displays further results of these analyses, giving incidences, relative risks and 95% confidence intervals by body weight group.

As can be seen from Tables 30 and 31, the risk of premature death showed, in each sex, a highly significant ($P < 0.001$) trend with increasing body weight at day 183, rising steadily over the five weight groups, being over twice as high in the VH as in the VL group. A steady rise in incidence over the weight groups was also evident for overall tumour incidence to males, but in females the trend was less smooth, although still significant ($P < 0.01$). Incidence of malignant tumours, while rising steadily over the first four weight groups, also showed some tendency to fall off between the H and the VH group. Risk was again, however, twice as high in the H and VH groups than in the VL group. It was interesting to note, however, that when uterine (glandular) and mesenteric lymph node tumours were excluded (for reasons discussed below), the trend in incidence of malignant tumours became much stronger and smoother, with risk almost three times as high in the VH groups as in the VL group. A similarly strong trend was also evident where incidence of multiple tumours was considered.

Specific tumour types showing a significant ($P < 0.05$) positive trend with increasing body weight were numerous. Tumours showing the strongest trends were of the pituitary (both anterior and intermediate lobes) and mammary gland. For mammary gland tumours, risk rose steadily with weight group, by a factor of over four between the VH and VL groups. Other significant trends were seen for lung, exocrine pancreas, jaw carcinoma, subcutaneous mass and tumours of 'other sites' (see Table 28 for definition). Near-significant positive trends were also seen for pancreas islet-cell, thyroid follicular, epidermal mass and mesenteric lymph node tumours, and for malignant lymphomas.

Most tumour types considered in Table 30 could be divided into two categories:

(i) those where a significant trend was seen, but no departure from trend, that is findings consistent with a simple relationship with body weight; or

(ii) those where neither a significant trend or departure from trend was seen.

There were, however, some exceptions that require comment. The significant departures from trend for mesenteric lymph node and uterine glandular tumours can be explained by the marked positive

relationship of the LMA diet to incidence of those tumours (see above). A relationship to specific diets, rather than to body weight, may also explain the negative trend of body weight with sarcoma/myxosarcoma of the uterus.

The significant departure from trend for malignant tumours of the anterior lobe of the pituitary, with incidences of three, six, five, 17 and seven in the five weight groups, has no obvious explanation. Overall incidence of benign or malignant tumours of the anterior lobe, and incidence of intermediate lobe pituitary tumours showed no such pattern.

Table 32 shows the relationship between diet from week 13 and grouped body weight at day 183. Clearly there is a very strong relationship indeed, with SMA and PRA animals being predominantly in the H and VH groups, and SMR being predominantly in the L and VL groups. For those conditions, apparently most strongly related to body weight at day 183, it is of interest to answer two questions:

Q1 Can the relationship with diet from week 13 be fully explained by body weight differences? and

Q2 Can the relationship with body weight be fully explained by diet?

Q1 can be answered by testing whether diet from week 13 still has a significant relationship after adjustment for body weight whereas Q2 can be answered by testing whether body weight still has a significant relationship after adjustment for diet from week 13 or adjustment for group.

Answers to these questions were first addressed in relation to premature death and it was clear that the answers to both Q1 and Q2 were "No". In all analyses (results not shown) adjustment reduced considerably, but did *not* eliminate the unadjusted associations. Even after adjusting for diet in 12 groups there was a significant ($P < 0.01$) trend for the probability of premature death to increase with increasing body weight group. This is consistent with the findings of Turnbull *et al.* (1985) that, within animals in the same treatment group, heavier animals tend to die before lighter animals. On the other hand, the reduced incidence of premature death in the SMR (and to a lesser extent the LMA and SMI) animals remained significant even after adjustment for body weight. This conclusion was not affected by alternative analyses (results not shown) adjusting for 20 instead of just five levels of body weight at day 183 (to carry out a more precise adjustment), or adjusting for body weight at day 365 instead of day 183. Although a very substantial part of the relationship of diet to premature death could be explained by its effect on body weight, it seems that the SMR diet was still, to some extent, additionally advantageous.

The questions were also addressed for mammary tumour incidence since it seemed to have a strong simple relationship to body weight at day 183. In this case the answer to Q1 was again "NO" but that to Q2 seemed to be essentially "Yes" (results not shown). Thus, adjustment for body weight at day 183 reduced but far from eliminated the low risk of mammary tumours in the SMR and LMA groups. By contrast, given treatment group, there was no significant positive trend of body weight to mammary tumour incidence.

DISCUSSION

I. Previous studies

1. *Earlier studies relating survival, ageing, body weight and tumour incidence to food intake*

The influence of food composition on longevity and tumour incidence in laboratory animals, and particularly the effects of reduced intake in increasing longevity and decreasing tumour incidence, has been known for 60 years (McCay *et al.*, 1935; Robertson *et al.*, 1934).

Tannenbaum (1940, 1942, 1944 and 1945), Tannenbaum and Silverstone (1949a,b and 1950) and Silverstone and Tannenbaum (1951) carried out a classical series of nutritional and dietary studies. Dietary restriction, by reduced food quality (e.g. low calorific value, low protein, low fat) or by reduced food quantity, led to a reduction in the incidence of virus-induced mammary tumours in DBA and C3H mice, and of spontaneous lung tumours in Swiss mice. Similar results were obtained in rats, where dietary restriction was associated with greatly increased lifespan and lower tumour incidence.

Berg and Simms (1960) observed a delayed onset of five types of lesion in rats restricted to 46 or 33% of the food consumed by *ad lib.*-fed controls.

Widdowson and McCance (1960) found that in rats, body weight at the time of weaning is inversely related to litter size and that animals that are relatively large, when aged 21 days, eat more and grow more than animals that are smaller at this age. In other words, animals that are large at the time of weaning become large adults. They pointed out that some developmental events (e.g. ocular vision, eruption of teeth) relate mainly to chronological age, whereas others (e.g. sexual maturity) relate more closely to the age at which a critical body size is attained, and yet others (e.g. chemical maturity and skeletal muscular development) are influenced by both age and body size.

Widdowson and Kennedy (1962), by restricting the food intake during suckling, produced smaller rats, which did not live longer but did have a reduced tumour incidence.

Ross and Bras (1965) found that by lowering the quantity of protein, carbohydrate and total calories in the diet, they could improve the longevity and reduce tumour incidence in rats. The group most severely restricted (i.e. with the lowest energy intake) developed the fewest tumours and lived the longest. The heaviest rats had the highest tumour incidence.

Diet composition also influenced tumour pattern, such that higher protein intake was associated with higher incidences of malignant lymphomas, and lower protein intake with higher incidences of fibromas and fibrosarcomas. Ross and Bras (1971) reported that reduced body weight gain early in life was associated with reduced risk of tumours at all sites.

Nolen (1972) attempted to find a suitable model for long-term rat carcinogenicity studies by using an 'enriched, well-balanced' diet. Animals that received 80% *ad lib.*-fed group were observed to be smaller, healthier and longer lived and, in the case of males, to have drastically lower tumour incidence.

Rowlatt *et al.* (1973), using C3HAvy mice, a strain with a high incidence of spontaneous mammary and liver tumours, produced a lower tumour incidence and a longer lifespan by offering each mouse only 1.5 g of food/day.

Gellatly (1975) found that the incidence of hepatic nodules (he preferred the term nodule to hepatoma) in C57BL mice to be proportional to the percentage of groundnut oil in the diet.

Roe and Tucker (1974) found that dietary restriction was associated with significantly reduced tumour incidence of malignant lymphoma, liver and lung tumours in mice.

Conybeare (1980), in a large 18-month study involving 640 mice, reported that the total tumour incidence was reduced from 44% in *ad lib.*-fed control males to 22.5% in males restricted to 75% intake of the *ad lib.*-fed group. In the females, 31% of the controls and 11% of the restricted group developed tumours. The incidence of malignant tumours of all sites (combined) was also drastically reduced and survival was enhanced markedly.

Cheney *et al.* (1980), using severe (50%) diet restriction in B6 mice, reported that the reduction of tumour incidence was more striking than the prolongation of lifespan. The predominant disease was lymphoma, which occurred less frequently and at a later age in the restricted-fed mice. Extending this work, Cheney *et al.* (1983), using $B6C3F_1$ hybrid mice, studied the effect of pre- and post-weaning diet restriction, as well as late onset restriction (i.e. starting dietary restriction at 14 months of age). Their results pointed to the fact that the longer an animal is on a regimen of dietary restriction, the lower the tumour incidence.

Weindruch *et al.* (1986) confirmed this work, and again found that the incidence of all tumours and not only that of the more commonly occurring tumours (i.e. lymphomas and hepatomas) was reduced greatly in animals fed a restricted feeding regimen. There was a reduction in hepatoma incidence in the restricted-fed group, but no direct correlation between body weight and liver tumour incidence.

Pollard and Wostmann (1985) reported that calorie restriction to 75% *ad lib.* significantly prolonged the survival and significantly and dramatically decreased the incidence of tumours (especially of the liver, adrenal medulla and other endocrine glands) in Lobund Wistar rats maintained under germ-free conditions throughout life.

2. *Reasons why importance of calorific intake has been ignored by many toxicologists*

Despite the abundance of the evidence that excessive calorific intake predisposes to increased tumour incidence, it is not yet common practice to reduce the dietary intake of rodents during long-term toxicity or oncogenicity studies. The main reason for this is simply an unwillingness to depart from the traditional use of 24-hour-per-day *ad lib.* feeding regimens. Despite the clear evidence that modest dietary restriction promotes good health rather than the opposite, some investigators seek to justify *ad lib.* feeding on the grounds that dietary restriction may lead to malnourishment.

Other reasons for persisting in the use of over-nutritive regimens are, first, that a switch to a calorie-restricted regimen would render historical control data from studies involving *ad lib.* feeding useless. Secondly, there is a fear that regulatory bodies may not accept data from carcinogenicity studies conducted in calorie-restricted animals.

Another fact that has contributed to the overall problem is that in the past, at least, diets from laboratory animals tended to be formulated for rapid growth during early life rather than for optimum health throughout life. Thus they are excessively nutritious and contain too much protein and fat. Such diets, which were originally designed to fulfil the requirements of breeding animals and/or their rapidly growing young, are certainly not suitable for mature animals whose nutritional requirements are much less.

The eradication of many parasitic and infectious diseases through the establishment of SPF animal colonies has also influenced the situation, first because the benefits of calorie restriction on longevity are more difficult to see in animals which tend to die early from infectious diseases, secondly because fewer diseased animals live to ages at which neoplastic diseases are most common, and, thirdly, because the nutritional requirements of diseased animals may be greater than those of SPF animals. Animals maintained under conventional conditions, as commonly existed before the general availability of clean facilities, were burdened with parasitic worms, ectoparasites, and a wide variety of debilitating bacterial, fungal and viral infections. The failure of Osborne *et al.* (1917) to show extended lifespan in response to dietary restrictions in his experiment is explained by these considerations. Saxton *et al.* (1944) reported that AK mice showed a lower incidence and later onset of leukaemia when severely under-fed. However, 10–16% of the mice died before 6 months of age from fighting and pneumonia. Saxton (1945) also studied rats from McCay's colony and found that the most common disease was chronic

pneumonia with bronchiectasis. This disease was retarded, but not prevented, by a regimen of dietary restriction. Nowadays, outbreaks of serious life-shortening diseases are rare. Consequently laboratory rats and mice, on average, live much longer. There has been little research aimed at comparing the nutritional requirements of conventionally maintained animals with those of essentially disease-free SPF animals, particularly after the post-weaning period of rapid growth.

In most chronic toxicity/oncogenicity studies as presently conducted, obesity, premature onset of age-related and degenerative diseases, multiple manifestations of major endocrine disturbances and high incidences of neoplasia are exceedingly common. These characteristics of laboratory animals are associated with—indeed they are indicative of—abnormal physiological status. Roe (1981) has argued that it makes no sense to conduct carcinogenicity studies on xenobiotic chemicals using physiologically abnormal animals when, by the use of slightly calorie-restricted animals, testing could be conducted in animals of normal, or of much closer to normal, physiological status.

The influence of dietary composition and calorific intake on the response of laboratory animals to carcinogens was reviewed by Clayson (1975). Surprisingly, however, even though he discussed the influence of calorific restriction on the incidence of tumours in control groups, he did not suggest that carcinogenesis testing should, if possible, be conducted under conditions of isocalorific food intake.

3. *Does calorie restriction influence longevity of genetically short-lived strains of rodents?*

According to Walford (1969) it is well established in the gerontological literature that age-specific tumour incidence for most tumours is secondary to, and related to, physiological ageing whereas tumour incidence itself is not an important determinant of longevity.

Smith *et al.* (1973), in a study in which they found that lifespan and age-specific cancer incidence in F_1 hybrid mice could not be predicted from parental characteristics, concluded that 'maximum' lifespan (mean age of death of the tenth decile) is virtually independent of environmental factors (such as calorie intake) whereas mean lifespan is influenced greatly by such factors. In general, despite similar feeding regimens, F_1 hybrids live longer than the inbred strains from which they are derived and mean age of death with tumour is also higher in the hybrids.

According to Good and Gajjar (1986) even 'genetically' short-lived strains can have their lifespan increased dramatically by a restricted dietary regimen. Many such short-lived strains develop a particular disease at an early age, and diet restriction either delays the onset of the disease in question or prevents its occurrence altogether [examples are the systemic lupus erythematosus-like syndrome of B/WF1 mice (Fernandes *et al.*, 1976 and 1978; Gajjar *et al.*, 1987), the diseases of obesity in the C57B1/6J ob/ob strain of mice (Harrison *et al.*, 1984) and the spontaneous hypertension of a susceptible strain of rats (Lloyd, 1984)]. In the hypertensive rats, fewer of the restricted-fed animals showed changes of the heart (oedema and fatty infiltration), adrenals (vacuole formation and congestion) and kidneys (interstitial fibrosis). Instead, many of the animals showed late-life lesions more typical of rat strains not prone to develop hypertension.

The present view, particularly in the USA, is that all these effects can be attributed solely to calorific intake irrespective of the composition of the diet (Masoro, 1984), since restriction of fat (Birt *et al.*, 1982b; French *et al.*, 1953), protein (Birt *et al.*, 1982a; Feldman *et al.*, 1982; Nakagawa *et al.*, 1974) or carbohydrate (Dalderup and Visser, 1969) do not increase lifespan where there is no associated reduction of total energy intake.

Recently, Harrison and Archer (1987) showed that increasing the amount of food available to previously severely restricted-fed mice, towards the end of a lifespan study, had a beneficial effect of further increasing lifespan. This may, in part, be because in 'older' animals the efficiency in using protein etc. is diminished and the excess of food offered in later life benefits the animals by helping them sustain the minimal maintenance required for continued life.

Tucker (1979) demonstrated a dramatic reduction in pituitary tumours. *Ad lib.*-fed male rats had a 32% incidence of pituitary adenomas, whereas no such tumours were seen in comparable restricted-fed males. The figures for females were 66% for *ad lib.*-fed animals against 38% for the restricted-fed group. In this study the female restricted-fed group was offered 15 g of diet/rat/day. This food was offered every morning at the same time, whereas the control animals were allowed free access to food throughout the 24 hours. It transpired that the females on the *ad lib.*-feeding regimen consumed, on average, the same amount of food as the restricted-fed group, namely 15 g/rat/day. However, whereas the rationed animals gobbled up their food shortly after it was provided to them each day, the *ad lib.*-fed animals spread their food consumption over many hours—eating mainly at night. Thus it seems that how and when rats eat their food may also have some bearing on the incidence of pituitary and other endocrine-related tumours.

In addition to Tucker, other workers have noted high incidences of pituitary tumours in *ad lib.*-fed rats (Ito *et al.*, 1972; Pickering and Pickering, 1984c; Prysor-Jones and Jenkins, 1981), Ross *et al.* (1970) reported there being a direct correlation between the incidence of pituitary chromophobe

adenomas and body weight earlier in life. Almost certainly the tumours described as chromophobe adenomas by Ross and his colleagues were, in reality, prolactinomas. Carlson and Hoelzel (1946) reported that Wistar rats subjected to intermittent fasting with only a very slight reduction in food intake, had five times fewer mammary tumours than the *ad lib.*-fed controls.

Rehm *et al.* (1984, 1985a,b and 1987), selecting sublines of Han:NMRI mice for leanness or obesity, and then feeding them either *ad lib.* or restricted to 80% *ad lib.* intake, noted that the onset of common tumours (e.g. pulmonary, haemopoietic, ovarian) was delayed by dietary restriction, and the incidence of pituitary and mammary tumours was reduced by some two- to four-fold in the restricted-fed animals. However, a serious problem arose: gastric ulceration occurred in high incidence in the restricted-fed mice and was a cause of premature death in 20% of them. The author suggests that these ulcers were stress related and noted that housing the animals in fives rather than singly may have further contributed to increasing the incidence of ulcers.

Pickering and Pickering (1984a) reported that continual breeding protected rats to some extent against the development of pituitary tumours in a similar way as did diet restriction. Presumably the increased calorie requirement associated with pregnancy partly compensated for the effects of overeating.

In any study of the possible association between genetically determined obesity and tumour incidence, it is important to be aware that what is true for one organ may not be true for another. Thus, Heston and Vlahakis (1962) found that obesity as a manifestation of a recessive gene in mice is associated with increased liver tumour incidence but reduced incidences of lung and mammary tumours.

4. *Effects of sexual segregation compared with those of calorie restriction*

A protective effect of breeding has also been reported by Salmon *et al.* (1990) with males benefiting more than females. In this study male rats were allowed to mate with a 'fresh virgin' female rat every 2 weeks, and the female rats were allowed to have one litter. In the males there was a reduction of all types of tumours and age associated diseases, except for Leydig-cell tumours which were increased, compared with the *ad lib.*-fed controls. These benefits were seen despite increased food consumption, suggesting that sexual activity and fulfilment despite high food consumption can have beneficial effects in male rats. However, in females, although there was no evidence that being allowed to litter once led to a reduction in the incidence of pituitary or any other tumours, there was reduced mammary gland hyperplasia. In the same study, the best survival and lowest incidence of non-neoplastic and neoplastic diseases was seen in rats calorie-restricted by giving them access to food during only 6 hr per day, which was associated with a 20% decrease in food consumption and a corresponding reduction of body weight compared with *ad lib.*-fed controls.

5. *Slight versus severe calorie restriction*

Early investigators tended to use drastic methods to restrict the dietary intake of their rats and mice. Some, like White and Andervont (1943), used such severely restricted-fed mice that at 6 months of age the animals weighed just 12 g. This regimen certainly produce a marked reduction in mammary gland tumours. However, the animals were by no means 'normal'. Berg and Simms (1960) reduced the intake to only just over 30% of the intake of the *ad lib.*-fed animals. Such severe restriction, in reality, amounted to 'semi-starvation' and led to marked stunting of growth and, not surprisingly, to reduce survival (Berg and Simms, 1960). Weindruch *et al.* (1980) restricted female mice to a body weight of 15 g. Under these circumstances even brain weights were reduced by 13%. In our opinion restriction that stunts body growth and/or reduces brain weight amounts to malnutrition.

In a large study in which groups of 50 male and 50 female rats were fed the same synthetic diet, either *ad lib.* or restricted to 60 or 80% *ad lib.*, Nolen (1972) found that the 60% *ad lib.* regimen prolonged survival by more than the 80% *ad lib.* regimen. On the other hand, the former gave rise to a persistent stunting of growth (e.g. as determined by femur length) whereas the less stringent restriction did not. Nolen's recommendation for use in toxicological/carcinogenicity studies was that rats should be fed *ad lib.* for the first 12 weeks of life post weaning and thereafter restricted to 80% *ad lib.*

6. *Effects of calorific restriction on non-neoplastic diseases*

In addition to reducing tumour incidence and increasing lifespan, animals fed a regimen of moderate dietary restriction develop fewer non-neoplastic diseases. Yu *et al.* (1982) and Maeda *et al.* (1985), both from Masoro's group in Texas, USA, found chronic nephropathy to be one of the major diseases in *ad lib.*-fed Fischer 344 rats. This disease occurred much later, in fewer animals, in lower severity and with a slower rate of progression in the restricted-fed animals. Masoro *et al.* (1989) also reported that, although a reduction in the proportion of protein in the diet reduces the incidence of severity of the disease in *ad lib.*-fed rats, by far the most effective method of reducing chronic nephropathy is to reduce the calorific intake of the animals by dietary restriction. The same conclusion was reached earlier by Bras and Ross (1964) and by Tapp *et al.* (1989).

The study by Maeda *et al.* (1985) found that, after chronic progressive nephropathy, cardiomyopathy was the main life-shortening non-neoplastic disease in *ad lib.*-fed rats. Food restriction starting either at

6 weeks or at 6 months of age was very effective at reducing and slowing the development of both these diseases. However, the animals which started a regimen of dietary restriction at 6 weeks of age had fewer tumours despite a 13% increase in average lifespan when compared with the animals starting their dietary restriction from 6 months of age. This provides further evidence that the longer animals are on a restricted feeding regimen, the greater is the prolongation of lifespan, and the lower the incidence of age-associated disease.

Mice of the B/WF1 hybrid strain develop an autoimmune, proliferative nephritis associated with a xenotropic virus infection, immune complexes and glomerulosclerosis. Izui *et al.* (1981) found that mice of this strain fed only 10 kcal/day showed only minimal to mild renal changes at 8 months of age, whereas mice fed 20 kcal/day displayed severe changes.

In the light of beneficial effects of a reduced food intake on survival, tumour incidence, hormonal status and incidence and severity of chronic diseases, it must be concluded that *ad lib.* feeding cannot be regarded as 'natural'. Evolution has depended on the principle that the fittest have a survival, and therefore, a procreation, advantage and clearly slightly restricted 'animals' are fitter than over-fed ones. No animals in the wild enjoy the freedom from microbial and parasitic diseases enjoyed by SPF laboratory rodents, and few would enjoy for prolonged periods an excess of food over and above their daily requirements. Direct comparison of the health and longevity of wild and SPF laboratory rodents in respect of ageing associated diseases is, however, virtually impossible because so few wild rats or mice survive beyond the age of 9 months (Berry *et al.*, 1973). Also, selective breeding over numerous generations has changed the genetic pool of laboratory animals such that it is vastly different from that of their wild counterparts.

Most of the very early laboratory work reported did not use commercially prepared laboratory diets as we know them today. Instead, experimentalists tended to feed animals diets made in their own laboratories to their own recipes. The latter included ingredients such as potatoes, cereals, seeds and oil or lard, and these were mixed and made into a mash to be fed daily. Visscher *et al.* (1942), for example, in their diet-restriction studies, used a diet containing 20% lard. The unusual composition of these diets makes it very difficult to compare the results of early studies with those of present-day ones. To make matters worse it was very common in the past for experimentalists not to provide any information concerning the composition of the diets they fed to their animals. Even today results on dietary studies are published without the author providing full details of the diet used. Instead such phrases as " . . . a standard laboratory chow/diet was fed . . . " constitute the only information provided.

7. *Food consumption and disease incidence*

It is easy to over-simplify discussions of the relationships between food consumption and disease incidence. First, there is the problem that animals have different food preferences. Ross and Bras (1974) described a study in which at the time of weaning rats were given a choice of foods. Most of them chose diets which gave them maximum growth and earliest sexual maturity. In 'evolutionary terms' this seems to mean that perpetuation of the species had higher priority than individual longevity or disease-free old age.

As is clear from the study described in the present paper, spillage of food can be a major problem and one that makes it difficult or impossible to measure food consumption accurately. Increased spillage is a sign of lack of palatability. In toxicity tests failure to measure spillage accurately is a common failing even though, as we have shown, the incidence of many neoplastic and non-neoplastic diseases is influenced by food consumption.

The development of radiculoneuropathy by aged rats may impair their ability to eat. For instance, they may have difficulty in obtaining food from the food basket or may get their semi-paralysed hind limbs trapped in grid floors. Caution is therefore needed in the interpretation of the relationships between calorific intake and disaese incidence in elderly rats. Berg (1976) reported that although dietary restriction reduced the incidence of most ageing-related diseases in rats, it did not affect the incidence of radiculoneuropathy.

In the study described here, one of the methods of dietary restriction used was to provide animals with 80% of the food consumed by animals in a comparable *ad lib.*-fed group during the previous period in which food consumption was measured in the latter. This does not take into account the fact that 80% *ad lib.*-fed restricted animals weigh about 20% less than *ad lib.*-fed ones. This means that on a g/kg BW basis the two groups receive approximately the same amount of food each day. However, the restricted animals are clearly more alert and physically active than the overweight *ad lib.*-fed animals. Thus they burn up more energy because of their higher physical activity. Also, it may be that they need to use up more energy in order to maintain their body temperature, since unlike obese animals, their internal organs are not insulated by a thick layer of fat in the body wall.

8. *Water consumption*

Water consumption is also something that is sometimes not very intelligently or accurately measured in toxicity studies. In general, animals drink during the same period of the day in which they eat and animals that eat more, drink more and those that eat less, drink less (Adolph, 1947; Bolles, 1961; Finger & Reid, 1952; Strominger, 1947; Verplank and Hayes, 1953). Cizek and Nocenti (1965) showed a gradual

increase in water-to-food intake ratio as food intake rises. The results in the present study are fully in accord with those of Adolph (1947) and of the other investigators cited above. Failure to drink enough water throughout the day increases the risk of the dehydration-associated disease known as wet-tail in rats and mice. In the present study diet restriction was observed to increase the risk of wet-tail, especially after the relative humidity in animal rooms fell to a low level during periods of very cold weather.

9. *Energy intake*

In rats, fibre is to a limited extent broken down in the large intestine with the consequence that up to 5% of daily energy requirements may be obtained from this source (Jacobs, 1986). The fermentation of the fibre results in the formation of short-chain fatty acids, principally acetate, butyrate, propionate and lactate, and these substances are broken down further to release energy either in the large intestine or in the liver. It was necessary therefore in relation to the present study actually to measure the ME of the different diets used. The results of this preliminary study are shown in Table 6 and the calculations of ME consumed by different groups of animals in the present study are depicted in Tables 7A and 7B (see also Appendix 1).

We recognize that ME density of diet depends not only on its composition, but also on the animal's physiological state and the feeding regimen (e.g. *ad lib.*, restricted). Thus the ME intakes in this study are estimates only, and where we believe they may have been distorted by treatment we have drawn attention to this possibility.

Since the classical works of Adolph (1947 and 1949), which are readily reproducible and consistent, it has been accepted that " . . . within limits animals eat for calories". In experiments reported by Hervey and Tobin (1983) changes in energy density of the diet in the range 0.5 to 2.0 (where that of a pelleted rodent diet was 1.0) were well compensated for by increased food consumption by rats.

The proportion of energy available for utilization by the body (i.e. the ME), or metabolizability, differs with the source of the energy (and the amount eaten); typical values for protein, starch and fat are 74, 93 and 97%, respectively. Fibrous carbohydrate materials, on the other hand, typically have metabolizabilities in the range 30 to 50%. The efficiencies by which these substances can be converted into tissue are even more different. For example, in rats the efficiency of conversion of the ME in protein, available carbohydrate, and fats/fatty acids into body fat energy (net energy) is about 64, 78 and 85%, respectively (Blaxter, 1989). Thus, increasing the fat content of a diet at the expense of protein or carbohydrate (particularly fibrous carbohydrate material) will increase substantially the efficiency with which the gross energy of the diet can be used for growth.

Boutwell *et al.* (1949) proposed that the higher 'net energy value' of a high fat diet accounted for the enhancement of carcinogenesis. This suggestion was based on earlier studies (Forbes *et al.*, 1946) showing that as dietary fat increased, the amount of energy expended as heat was decreased, so that more energy was retained by the carcass.

Pariza (1986) concluded, using data from a rat study, " . . . that the apparent enhancement by dietary fat of mammary cancer . . . , is really a manifestation of the calorie effect". Kraft (1983) concluded similarly.

Sacher (1977) supported the theory of a 'rate of living' and there is certainly a relationship between the maximum lifespan of a species and its metabolic rate. Cutler (1978) showed this with a series of graphs which demonstrated that the lifetime energy intake for each species from mice to elephants is approximately 200 calories per gram body weight (cal/gBW). The exception to this was man, with about 800 cal/gBW.

Totter (1985) proposed that energy for maintaining core body temperature and reproductive capacity is the main source of oxygen radical production, which he assumes is the cause of ageing, whereas energy diverted to food gathering, namely work energy, is not. He suggested that in times of food shortage in the wild, diversion of energy to muscular work (to search for food) and away from basal membrane metabolism and reproduction might lead to a slower rate of ageing. Recently, Ames and Gold (1990) proposed that increased cell production (mitogenesis) leads to increased risk of endogenous DNA adducts being converted into mutations. For it is known that, associated with restricted-feeding, there is a lower level of cellular proliferation in various tissues, including the mammary gland, oesophagus, intestines, urinary bladder and dermis (Lok *et al.*, 1990) and a deceleration of the age-associated increase in ileal villus cellularity (Heller *et al.*, 1990). Or it may be that the risk of developing cancer is increased because higher energy consumption leads to a higher rate of generation of DNA-damaging oxidants.

Even if Totter's reliance on the free radical theory of ageing is not wholly accepted, there are a number of observations on the effects of diet restriction on metabolism, reproductive capabilities and neuro-endocrine and neuro-immune systems that fit into a hypothesis with an evolutionary perspective. Although oxygen complexes definitely play important roles in normal physiology and pathogenesis, there are some eminent scientists who believe that free radicals are a source of disease and cancer, but not of ageing (Pryor, 1987).

10. *Protein consumption*

The findings in the present study (PRA *v.* SMA) are consistent with the conclusion of Masoro *et al.* (1989) that the consumption of diets high in protein only leads to increased incidence/severity of chronic

progressive nephropathy if energy intake is also high. In the absence of a high energy intake, high protein concentration in the diet did not increase the incidence of chronic nephropathy as previously claimed by Yu *et al.* (1982) and Maeda *et al.* (1985).

The present study, however, was not specifically designed to investigate the influence of dietary protein concentration under conditions of isocalorific energy intake.

11. *Effects of diet on haematological parameters*

Kubo *et al.* (1984) found that dietary restriction led to a 50% reduction in WBC in a short-lived autoimmune disease-susceptible strain of mice. Weindruch and Walford (1988), using a longer-lived strain of mice, reported a similar finding. Pickering and Pickering (1984b), who fed weanling Wistar rats of both sexes either *ad lib.* or a restricted regimen, found that the erythrocyte count, Hct and Hb level were higher in the restricted group.

12. *Effects of diet on clinical chemistry parameters*

Sachan and Das (1982) reported that 50% dietary restriction led to reduced levels of circulating glucose, uric acid and fat in Sprague–Dawley rats. However, other parameters including calcium, phosphorus, protein, albumin, total bilirubin and several serum enzymes were not affected. Pickering and Pickering (1984b) reported increased plasma chloride levels in restricted-fed Wistar rats.

In females all forms of dietary restriction were associated with higher BUN levels than SMA. By contrast, in males significantly lower BUN was seen at 6 months. However, later in the study the extent of this difference ceased to be statistically significant.

Ross (1969) compared the effects of five different diets on hepatic enzyme (ATPase, ALP, histidase and catalase) levels at seven times points ranging from 21 to 1000 days of age in rats. He found a close correlation between levels of hepatic enzyme activity and life expectancy and that long-term calorie restrictions led to the persistence into middle and old age of enzyme profiles seen in young rats. Birchenall-Sparks *et al.* (1985) reported that dietary restriction slows the rate of protein synthesis in rats, and Tuchweber *et al.* (1987) described effects of diet restriction on bile formation in ageing rats.

13. *Correction by calorie restriction of adverse affects of over-nutrition on the endocrine system*

In rats many of the beneficial effects of dietary restriction on the incidence of neoplastic diseases involve the endocrine system and hormone-controlled tissues such as the mammary gland. However, in mice the benefits seen are mainly in non-endocrine tissues including the liver, lung and lymphoreticular system.

As rats age, serum prolactin levels increase (Merry *et al.*, 1985; Roe, 1981) while growth hormone and thyroid-stimulating hormone levels remain unchanged (Sarker *et al.*, 1982). Thus it is not unusual to find levels of protein in the range 500 to 1000 ng/ml in 2-yr-old *ad lib.*-fed female rats (Conybeare, personal experience). This is of the order of five times the highest level seen in restricted-fed rats of this age. Elevation of serum prolactin levels is associated initially with general and/or focal hyperplasia of prolactin-producing cells in the anterior pituitary gland (adenohypophysis) and later with the development of pituitary tumours, which are composed mainly of prolactin-producing cells, i.e. prolactinomas.

It has been demonstrated that prolactin can act as a tumour promoter in rats previously exposed to a genotoxic carcinogen in the mammary gland (Rose and Mountjoy, 1983) and liver (Buckley *et al.*, 1985). The strikingly lower incidence of mammary tumours in the restricted-fed rats of both sexes reported by Tucker (1979) can almost certainly be attributed to lower prolactin levels, although no measurements of the circulatory levels of the hormone were made. Enhancement of skin carcinogenesis by prolactin in mice has been reported (Lupulescu, 1985) but the mechanism involved is unclear.

In over-nourished *ad lib.*-fed animals, increasing age is associated not only with a marked increase in serum prolactin levels, but also with a progressive reduction in central dopamine neuronal function (Huang *et al.*, 1976). Dopamine released from the terminals of the hypothalamic tuberoinfundibular dopamine neurones in the median eminence is transported in the hypophysial portal blood to the pituitary (adenohypophysis) and inhibits the release of prolactin from the anterior pituitary (Ben-Jonathon *et al.*, 1977). Not only is the control of lactotrophic function attenuated in ageing rats (Simpkins *et al.*, 1977; Demarest *et al.*, 1982), but there is also a decrease in the striatal dopamine receptor concentrations in the brain, which is decelerated by a regimen of dietary restriction (Roth *et al.*, 1984). The relation between dopaminergic control of pituitary lactotroph function and the slowing of age-related changes in serum prolactin levels by dietary restriction was the subject of a report by Atterwill *et al.* (1989).

Whatever the reason for the dysfunction seen during ageing in over-nourished *ad lib.*-fed rodents, chronic exposure to the dopamine agonist, bromocriptine, appears to correct it and to mimic the effects of diet restriction with serum prolactin and pituitary dopamine concentrations remaining at the levels seen in younger animals (Demarest *et al.*, 1985).

14. *Effects of diet on circulating hormones and endocrine functions*

Many of the differences seen in the uterus, ovaries and mammary glands of rats fed a regimen of diet restriction compared with *ad lib.*-fed animals have been known since 1939 (Pomerantz and Mulinos, 1939) and the similarity between the appearances of

these tissues in severely diet-restricted rats and hypophysectomized tissues from rats led Huseby *et al.* (1945) to introduce the term 'pseudo-hypophysectomy'. However, the notion that a regimen of diet restriction retards ageing through 'nutritional hypophysectomy' (Everitt, 1973; Segall, 1979) has been challenged as being too simplistic (Merry and Holehan, 1985).

As rats age, certain physical and biological changes related to the endocrine system occur at different time points and these can be used as 'biological markers'. Merry and Holehan (1981 and 1985) compared some of these markers in rats fed *ad lib.* with those that were 50% diet restricted. This severe restriction produced the following results:

(1) Delayed vaginal opening in 90% animals from 37 to 147 days of age.

(2) Lowered prepubertal serum FSH and progesterone with increased 17β-oestradiol.

(3) Modification of the oestrous cycle, with LH peaking 6.5 hr earlier and the lower maximum peak of 17β-oestradiol also delayed.

(4) Only *ad lib.*-fed rats had a late-life fall in testosterone.

(5) LH was higher in the restricted-fed animals.

(6) Prolactin was lower in the restricted-fed animals.

Less severe restriction has less marked effects. Thus Conybeare (1988) reported that a regimen of 6 hr per day feeding delayed the onset of oestrous cycling by only 4 days.

Campbell *et al.* (1977), in studies of up to 7 days duration, found that anterior pituitary hormone release falls with severe short-term dietary restriction and that this appears to decrease hypothalamic stimulation of the pituitary. However, the latter remains capable of a normal response.

Earlier, Dilman (1971) postulated that an age-related increase in hypothalamic threshold-feedback regulation caused the changes seen in ageing. Age-related reduction in hypothalamic catacholamines, including noradrenalin, may be instrumental in reproductive decline. Noradrenalin arises normally in the preoptic area just before ovulation, and L-dopa, which increases brain catacholamines, can re-initiate oestrous cycling (Finch, 1979; Meites *et al.*, 1987). However, the limited number of ovarian follicles appears to act as a pacemaker for reproductive senescence while interacting with neuroendocrine ageing changes.

Boutwell (1964) demonstrated that carcinogen-induced skin tumour formation is inhibited by topically or systemically administered cortisone. This is one of the earliest direct reports of a link between hormones (adrenal gland function) and tumour formation.

Lansfield (Finch and Lansfield, 1985; Lansfield, 1978) hypothesized that glucocorticoids may modulate hippocampal ageing by an apparent loss of cells with corticosterone receptors. Sapolsky *et al.* (1986) speculated that stress leads to corticosterone hypersecretion, which eventually leads to a gradual neuronal cell/receptor loss.

Levin *et al.* (1981) found that the age-related loss in striatal dopamine receptors was retarded by a regimen of dietary restriction. The concentration of dopamine receptors in 24-month-old restricted-fed rats was 50% greater than that of the *ad lib.*-fed controls, and resembled that of 3–6-month-old controls. Roth *et al.* (1984) found that restricted-fed rats did not show a decline in dopamine receptors during the first year of life, unlike the controls, and that at 30 months of age the receptor levels were similar to the levels seen in the controls at 24 months. However, preliminary results (Weindruch and Walford, 1988) on striatal dopamine receptors in mice did not show a retardation of the decline previously seen in rats. As stated earlier, the effects of diet restriction on the endocrine system appear to be different in rats and mice. Severe diet restriction (50%) prevents the oestrous cycling of mice (Nelson *et al.*, 1985) whereas a similar regimen in rats does not alter the normal 5-day cycle (Merry *et al.*, 1985).

According to Totter (1985), it makes good evolutionary sense not to have babies when food is scarce and to divert reproductive energy to personal survival, thus allowing the individual to outlive the time of scarcity. It also makes sense, from an evolutionary point of view, that after a period of 'food restriction' there is a rejuvenating effect on the reproductive system when food becomes plentiful again. Quigley *et al.* (1987) found that, after a 10-wk period of severe (50%) dietary restriction, young rats ceased their oestrous cycling. Thereafter when they were once again fed *ad lib.*, they started to cycle again. It was also noted that older restricted-fed rats that had already ceased cycling also recommenced their oestrous cycles when re-fed *ad lib.*

The reproductive system clearly influences the immune system, by neuroendocrine feedback mechanisms (Grossman, 1985). One can thus make an intriguing case for an evolutionary interpretation of some of the effects of dietary restriction.

Some of the changes discussed above may not be wholly attributable just to daily calorie intake (Conybeare, 1988). How and when rats are fed may also be important determinants.

15. *Effects on urinalysis parameters*

Pickering and Pickering (1984b) fed weanling Wistar rats of both sexes either *ad lib.* or at several levels of dietary restriction for a 5-wk period and measured a wide variety of parameters including several involving urinalysis. Associated with the diet restriction were higher urinary volume, higher pH and lower specific gravity.

16. *Effects of diet on clinical observations*

The reason for the between-group differences in incidence of wet-tail and manifestations of radiculoneuropathy are discussed above.

17. *Effects on body weight, survival and tumour development*

Numerous studies have shown that dietary manipulations resulting in reduced body weight gain also result in reduced tumour incidence and/or prolonged survival. In line with this, it has often been observed that non-specific toxicity which leads to reduced body weight is associated with both reduced tumour incidence and prolonged survival. Sometimes such non-specific toxicity simply reflects reduced food consumption, because admixture of the test compound with the diet renders it unpalatable.

Using a self-selection feeding regimen, where animals could select from a range of different diets, Ross *et al.* (1982) showed that animals which chose the diets offering the higher calorie intake had the fastest post-weaning growth rates, the highest body weights and the highest incidence of tumours. Fast growth rate was also associated with reduced survival (Ross *et al.*, 1983a), but neither survival nor tumour incidence were associated with either protein intake or carbohydrate intake. On the other hand, there were strong correlations between the incremental weight gain early in life and both the amount of food consumed later in life and mature body weight (Ross *et al.*, 1983b).

Turnbull *et al.* (1985) reported that, even within animals fed the same diet, there is an inverse relationship between body weight gain and survival. Using data from 200 CFY strain and 240 CD strain rats which had acted as controls in a number of routine chronic toxicity studies, and which were fed *ad lib.*, these investigators reported a striking relationship between body weight early in life and probability of survival to the end of the study. Among 86 rats in the heaviest quintile of body weight at week 54 (for a given strain and sex) only 14 survived (16%). In contrast, 46 out of 82 (56%) in the lowest body weight quintile survived.

Rao *et al.* (1987) analysed data derived from control groups in 29 National Cancer Institute–National Toxicology Program rodent carcinogenicity studies conducted in the USA. Their analyses were complicated by the fact that in some studies test substances were administered by gavage using corn oil as the vehicle and in others by admixture with the diet. However, in female F344 rats they found a highly significant ($P < 0.005$) correlation between body weight and benign mammary tumour incidence. In male F344 rats corn oil gavage was associated with increased body weight gain, increased survival, increased incidence of pancreatic acinal cell adenomas but decreased incidence of leukaemia. By contrast, in females, corn oil was associated with both decreased body weight and decreased incidence of pituitary adenomas but no effect on survival. In so far as the 29 studies were conducted at a number of different contract research laboratories at different dates, the findings should only be accepted with caution.

Deerberg *et al.* (1990) reported that reduction of calorie intake by restricting the period of time each day during which male rats of the HAN:SPRD strain had access to food was associated with reduced body weight gain, prolonged survival, reduced incidences of chronic nephropathy and prostatitis and reduced age-standardized risk of development of neoplasia.

A particularly important finding in the present study was that despite the wide variations between different groups in the diets they were given and the forms of calorie restriction to which they were submitted, there were overall highly significant correlations between body weight early in life and the risks of premature death from any cause and of development of life-threatening malignant neoplasm (see Tables 30 and 31). The strengths of these correlations suggest a need for further analyses of data derived from other studies to see how useful body weight at 6 months would be as a predictor of premature death and subsequent tumour development.

McCay *et al.* (1935) found that, in growth-retarded animals, the power to grow in response to increased calorific intake persisted to an advanced age, particularly for males. On the other hand, maximum body weight cannot be achieved if growth is restricted early in life.

Early dietary manipulation had no effect on survival, supporting the previous findings of Nolen (1972). The tendency, although not statistically significant, was for early dietary restriction followed by *ad lib.* feeding to be marginally detrimental by leading to poorer survival. Turnbull *et al.* (1985) and the present study both show an inverse correlation between body weight and survival.

Conybeare (1988) reported that dietary restriction of suckling dams and pups before weaning produced offspring which reduced body weights that never managed to catch up even when they were later fed *ad lib.* The differences between these studies may be that restricting the food intake of animals during pregnancy and lactation prevents the formation of fat cells and hence reduces adult body weight, with no correlation between body weight and survival.

The time-restricted (interrupted) feeding regimen did not seem to control food consumption, but did seem to control body weight, so that survival rates correlated better to body weight (Turnbull *et al.*, 1985) than to calorific intake (Masoro, 1984). This discrepancy between food/calorie consumption and body weight may, in part, be because interrupted-fed animals have a different feeding pattern from *ad lib.*-fed animals. The larger meal of the interrupted-fed animal passing through the gut as a bolus, may result in reduced calorie absorption. Alternatively, the emptiness of the food hopper

during the majority of each day may affect hormonal status.

Studies in species other than rats and mice have provided evidence that dietary restriction slows fundamental ageing processes, for example in protozoa [Tokophyra] (Rudzinska, 1952), water fleas [Daphnia] (Ingle *et al.*, 1937) and guppies [Lebistes] (Comfort, 1963). Expectation of life in these species may be increased by as much as 80%, depending on the proportion of the lifespan during which food intake is restricted.

II. Present study

1. *Effects of calorie restriction by rationing to 80% ad lib. food intake throughout life (SBR/SMR v. SBA/SMA)*

Calorie restriction reduced water consumption to the same extent as food consumption and energy consumption (see Tables 5A, 5B, 7A and 7B). A combination of reduced water consumption and the effect of rationing on when rats ate and drank during the 24 hr may partly or wholly explain some of the apparent effects of SMR on various haematological and clinical chemistry parameters as measured in blood samples taken during the morning of blood sampling days (see Tables 9A, 9B, 10A and 10B). Some of the apparent effects on urinalysis findings (Tables 11A and 11B) may be explained on the same basis, and reduced water intake during the night, when ambient humidity fell, was probably largely responsible for the particularly high incidence of ringtail in SMR-fed rats (see Table 12).

As expected in the light of numerous other studies reported in the literature, the rationing of food intake was associated with highly significantly ($P < 0.001$ in both sexes) improved survival to 30 months (see Table 17B) and highly significantly ($P < 0.001$) lower body weight gain (see Table 18A). This reduction in body weight (about 11% in males and 17% in females at 33 months but approximately 30% in both sexes earlier in the study) was associated with significantly lower absolute weights of heart, liver, kidneys, adrenals and pituitary, but with no decrease in absolute weights of brain, testes, seminal vesicles or ovaries (see Table 18A). Liver, kidney, pituitary and adrenal weights relative to body weight were also significantly lower in SMR compared with SMA rats (see Table 18B).

The effect of dietary restriction on relative liver weight has previously (Conybeare, 1988) been shown to be associated with a longer phenobarbitone-induced sleeping time—a finding which is consistent with the restricted rats being in a lower state of *P*-450 enzyme induction. This particular finding is consistent with calorific restriction leading to a reduction in metabolic capacity and metabolic rate. In this regard it is noteworthy that evidence for a reduction in metabolic rate in response to decreased energy intake in man and other animals (Hervey and Tobin, 1982; Lusk, 1928), is in disagreement with the conclusions of Masoro *et al.* (1982) and McCarter *et al.* (1985) (see below).

The significant effect of calorie restriction on kidney weight may also reflect a lower rate of metabolism. However, the more obvious explanation of this finding is that it was due to the highly significant reduction in incidence and severity of manifestations of chronic progressive nephropathy.

Likewise, the effects of calorie restriction in reducing pituitary weight are explained by the dramatically lower incidences of focal hyperplasia and/or neoplasia of the anterior and intermediate lobes of the pituitary. The presumption is that most of the proliferative changes in the anterior pituitary involved prolactin-secreting cells. However, differential staining techniques were not applied to the pituitary lesions in the present study. Also, we do not know whether the lesions in the intermediate lobe in *ad lib.*-fed rats are functional and if so, what their function is. Since the reduction in incidence of mammary gland tumours in the SMR rats (see Table 26) is consistent with lower circulating prolactin levels, we were surprised to find no striking between-group differences in prolactin levels in blood samples taken from satellite animals at 6, 12 and 18 months between SBR/SMR (group 5) and SBA/SMA (group 1). Even in blood samples taken from animals killed at the termination of the study prolactin levels were only moderately less ($P < 0.05$) with SMR (groups 3 and 5) than with SMA (groups 1, 7 and 9).

By contrast with the weakness of the effect of calorie restriction on prolactin levels, there was a markedly higher ($P < 0.001$) level of 17β-oestradiol in SMR than in SMA terminally killed females. This difference possibly reflects the fact that calorie restriction postpones reproductive senescence and prolongs fecundity (see Holehan and Merry, 1985). However, the higher circulating oestradiol level in the SMR rats was not associated with any observed effect on the uterus or ovaries.

We observed, as have many others, significantly lower incidences of a number of ageing-related conditions in the SMR compared with the SMA rats. As shown in Table 26, the list of non-neoplastic conditions reduced in incidence/severity includes: haemorrhage/degeneration of the adrenal cortex, chronic myocarditis and myocardial fibrosis, chronic progressive nephropathy, periductule biliary fibrosis (males only), lymphoid aggregates around airways in the lungs, lymphoid hyperplasia and cyst formation in the cervical lymph node, mammary gland acinar hyperplasia, secretory activity and galactocoele formation, polyarteritis (females only), acute prostatitis, and radiculoneuropathy. For neoplastic lesions, SMR was associated specifically with significantly reduced incidences of pulmonary adenoma/adenocarcinoma, benign and malignant mammary gland tumours (females only), haemangioma/haemangiosarcoma of the mesenteric

lymph node (males only) and pituitary adenomas/carcinomas–epidermal tumours (sexes combined), subcutaneous tumours (males) and pancreatic exocrine and endocrine tumours (males). In fact there was no kind of neoplasm that occurred more frequently in SMR rats than SMA rats, and for benign or malignant neoplasms of all sites and types, rationing the food intake of rats to 80% of that consumed by *ad lib.*-fed animals reduced incidence highly significantly ($P < 0.001$) in both sexes (see Table 26). It is particularly important to note that this beneficial effect of calorie restriction affected the overall incidence of malignant tumours since this is the parameter which is most relevant for human epidemiological studies in which the endpoint is usually cancer mortality as distinct from tumour incidence.

2. *Effect of calorie restriction to 80% ad lib. starting 13 weeks post weaning compared with lifelong restriction (SBA/SMR v. SBR/SMR)*

The effects of these two regimens (groups 3 and 5) were broadly very similar. However, there were indications that restriction started at weaning was even more beneficial than restriction started 13 weeks post weaning. This was so for reduction in end-of-test prolactin in females, radiculoneuropathy (in males), islet-cell tumours of the pancreas, proliferative lesions of the anterior and intermediate lobes of the pituitary gland (in males), benign or malignant tumour at any site (in males). In no instance were the beneficial effects seen in response to SBA/SMR greater than those in response to SBR/SMR.

As pointed out elsewhere, dietary restriction (either SBR or SBI) before week 13 post weaning was associated with increased incidence of corticomedullary nephrocalcinosis in males and decreased incidence of the same lesion in females (see Table 21). Also (as shown in Table 23), SBR during the 13 weeks post weaning (with adjustment for subsequent diet) reduced the incidence/severity of chronic progressive nephropathy. In other words, the reduction in nephropathy associated with SMR after week 13 was enhanced by restriction (SBR) before week 13. SBR before week 13 also reduced the incidences of polyarteritis (the sexes combined $P < 0.05$) and mammary gland secretory activity in females ($P < 0.05$).

3. *Effects of calorie restriction to 80% ad lib. during 13 weeks post weaning without subsequent restriction (SBR/SMA v. SBA/SMA)*

Calorie restriction limited to the 13 weeks post weaning (group 7 *v.* group 1) had an enduring but only small effect on body weight gain in both sexes during the first 2 years of the study. These small effects were associated with significantly ($P < 0.05$) lower serum prolactin levels in females killed at the end of the study but with virtually no significant effects on end-of-test organ weights, survival or incidence of ageing-related diseases or neoplasia. Thus, in this study, simple moderate calorific restriction during the rapidly growing phase of life from the time of weaning had very little or no effect on longevity or disease incidence unless some form of restriction was continued into later life.

4. *Effects of limiting daily access to food to 6 hr throughout the study (SBI/SMI) or only from 13 weeks post weaning (SBA/SMI) compared with SBA/SMA*

Leveille (1972) reported reduced weight gain, better survival, and various liver enzyme changes in rats given access to food for only 2 hr per day. Later, we (Salmon *et al.*, 1990) reported that restricting the access of rats to food to 6.5 hr per day led to significantly reduced body weight gain (e.g. after 54 wk: males −21%; females −21%), significantly better survival to 2 years ($P < 0.01\%$), significantly reduced liver weight relative to body weight ($P < 0.001$ in both sexes), significantly reduced kidney weight relative to body weight in males ($P < 0.05$), and significantly reduced incidences of myocardial fibrosis, myocarditis, chronic progressive nephropathy, polyarteritis, testicular atrophy and benign and malignant tumours at any site. In the light of the results of that earlier experiment, conducted in the same strain of rats, we expected the rats in group 4 (SBA/SMI) and group 8 (SBI/SMI) in the present study to exhibit beneficial effects of calorie restriction similar to those in group 3 (SBA/SMR) and group 5 (SBR/SMR). However, this expectation was not fulfilled. During the first 13 weeks of the study SBI and SBR had similarly marked effects in reducing body weight gain in females, but SBI was less effective in doing so in males (see Figs 1A and 1B). During the study as a whole SBI/SMI was only about half as effective as SBR/SMR in reducing body weight gain (see Figs 4A and 4B). It seems that the rats, particularly the males, in the present study learned to compensate for the intermittency of the availability of food by taking a huge meal just before the food basket was blocked from their reach. In fact, throughout the second phase of the study, SMI males consumed significantly ($P < 0.001$) more food than SMA males and from about 70 weeks onwards SMI females consumed slightly ($P < 0.05$) more food than SMA females.

Total WBC were similarly reduced by SMI and SMR compared with SMA in both sexes, whereas effects on red cell and platelet parameters tended to be less marked in SMI than SMR rats (see Tables 9A and 9B). Effects on various clinical chemistry and urinalyses parameters were evident in SMI rats and these were generally similar in kind to, but usually less marked than, those seen in SMR rats (see Tables 10A, 10B, 11A and 11B). Unlike SMA rats, SMI rats did not show any increase in the incidence of low-humidity-induced ringtail (see Table 12) whereas the effect of SMR in reducing the age-standardized incidence of radiculoneuropathy ($P < 0.001$) in females was only weakly evident in

SMI females ($P < 0.05$) (see Table 13B). These findings are in conflict with those of Berg (1976) who reported that diet restriction had no beneficial effect on the incidence of this ageing-related disease. The reduced level of growth hormone in SMR males killed at the end of the study compared with SMA males was scarcely evident in SMI males and the elevated level of 17β-oestradiol seen in terminally killed SMR females ($P < 0.001$) was far less evident in SMI females ($P < 0.05$). Like SMR, SMI significantly ($P < 0.001$) improved survival in males but had no significantly beneficial effect in females (see Table 17B). Many of the effects of SMR on absolute and relative organ weights were also evident in SMI rats (see Tables 18A and 18B). Like SBR, SBI, during the first 13 weeks of the study, was associated with a significant increase in the incidence of corticomedullary mineralization in males (see Table 21). Like SMR, SMI reduced the incidences of several non-neoplastic and neoplastic lesions; however, the effects were much less marked and far fewer types of lesion were affected (see Table 26).

Our observations in the present experiment viewed in the context of those in the earlier experiment (Salmon *et al.*, 1990) lead us to the opinion that one cannot rely on restriction of time per day of access to food to bring about reduced food consumption in the same way as rationing.

5. *Effects of feeding the PR diet throughout the experiment instead of SBA/SMA*

As shown in Table 1B, the nutrient composition of the PR diet is essentially similar to that of the SB diet, although the ME density of PR diet was only 87.5% that of SB diet. Probably because of the difference in ME density, PRA-fed rats of both sexes consumed more food than SBA-fed rats during the first 13 weeks of the study (see Tables 4A and 4C), although clearly calorific adjustment was incomplete because the PRA-fed rats gained marginally less weight during this period (see Figs 1A and 1B). From week 13 onwards the 'control' (SBA/SMA) rats, and also rats in groups 7 and 9 were given the SM diet of which only 14.3% was protein, compared with the 19.8% protein in the PR diet. During this second phase of the study, group 12 rats (PRA/PRA) consistently ate more food than the rats fed the SMA diet (see Tables 4B and 4C). Despite this difference in food consumption, body weight gain was no greater in response to PRA/PRA than to SBA/SMA (see Figs 4A and 4B). Water consumption was higher throughout the study in PRA/PRA rats than in SBA/SMA rats (see Tables 5A and 5B). In terms of ME, PR and SM were similar, but the ME of PR was only 87.5% that of SB (Table 6A). However, taking both food consumption and ME into account, PRA rats of both sexes took in significantly ($P < 0.001$) more ME than SMA rats (Table 7B). PRA rats also took in significantly more ME expressed as energy consumption per 100 g body weight per day (see Table 8).

The only haematological values to differ significantly between PRA and SMA rats were higher platelet counts in male PRA rats in blood samples taken at 6, 12 and 18 months ($P < 0.001$) and marginally lower WBC in samples in females taken at 18, 24 and 30 months ($P < 0.05$) (see Tables 9A and 9B). Throughout the study PRA was associated with higher BUN levels in males and the same is true for the sample taken at 6 months in females. Other between-group differences shown in Tables 10A and 10B vary between the sexes and/or between sampling times and are accordingly difficult to interpret. Urine volume and protein content were consistently higher up to 18 months in PRA-fed males than SMA-fed males (see Table 11A). This difference may reflect the greater severity of chronic progressive nephropathy in the PRA-fed group although it is disputed whether the high protein intake causes the nephropathy or vice versa. Levels of circulating hormones in rats surviving until the end of the study showed only one significant difference, namely a higher ($P < 0.01$) level of circulating progesterone in PRA males (see Table 15).

PRA neither increased nor decreased survival compared with SMA (see Tables 17A and 17B). Relative to body weight, the weights of the liver and kidney were higher in PRA males than SMA males (liver, $P < 0.01$; kidney, $P < 0.001$) and that of liver in PRA females was higher than SMA females ($P < 0.05$) (see Table 18B). These changes may reflect higher metabolic activity in the liver, and more severe nephropathy of the kidney. In previous studies, one of us (GT) found that liver weight tends to reflect recent energy intake. In females the feeding of PRA diet throughout the study was associated with a highly significantly lower incidence of corticomedullary nephrocalcinosis than seen in rats fed SBA during the first 13 weeks of the study and diets other than PRA after 13 weeks (see Table 21). There is no clear explanation of the finding of a significantly higher incidence of pelvic mineralization in PRA rats (males, $P < 0.01$; females, $P < 0.001$) (see Table 24). One would need to conduct careful mineral balance studies in order to identify the cause of these differences. However, the striking between-group and between-sex differences in respect of the incidences of corticomedullary and pelvic nephrocalcinosis seen in this study, which involved no deliberate exposure to any xenobiotic chemical, indicate the need for caution in the interpretation of histopathological evidence of mineral desposition in the kidney in toxicological studies.

As shown in Table 23, compared with SBA, PRA during the first 13 weeks of the study was associated with more bile-duct hyperplasia ($P < 0.001$) and more periductular fibrosis ($P < 0.001$) in both sexes, and more testicular

atrophy ($P < 0.05$). Feeding PRA throughout the study, compared with SMA, was associated with a higher incidence of proliferative changes (hyperplasia, benign or malignant tumour) of the adrenal medulla in both sexes, more myocarditis and myocardial fibrosis in males ($P < 0.001$) more chronic progressive nephropathy (males, $P < 0.001$; females, $P < 0.05$), more acinal hyperplasia of the mammary gland in males ($P < 0.01$), more polyarteritis of the pancreatic and/or mesenteric artery (males, $P < 0.001$; females, $P < 0.1$), more generalized hyperplasia of the spleen ($P < 0.05$ in both sexes), more arteriolitis of the testis ($P < 0.01$), more marked distension of the uterine horns ($P < 0.01$) and more endometrial hyperplasia ($P < 0.05$) (see Table 24).

In relation to the higher incidence of proliferative lesions of the adrenal medulla seen with the PR diet, it is interesting to note that this effect has previously been reported to be associated with increased calcium absorption from the gut (as seen in animals on a high lactose diet) and with increased pelvic nephrocalcinosis as seen in both sexes with the PRA diet in the present study (Hodgkinson *et al.*, 1982; Roe and Baer, 1985).

Perhaps the most important aspects of the findings with PRA/PRA and SBA/SMA is that both these dietary regimens are the same or closely similar to those that are commonly used in long-term toxicity studies in many laboratories. Hence the big differences between the findings in the animals on these two dietary regimens illustrate the importance of taking the details of the composition of the diet into account. Also, the differences illustrate the fragility of historical control data in circumstances where there are, or may be, between-experiment differences in dietary composition and/or dietary regimen.

6. *The effects of substituting the standard breeding diet (SBA) by a high fibre diet (LBA) during the first 13 weeks post weaning*

The LB diet contained less protein and more fibre than the SB diet (see Table 1B) and animals fed LBA during the first 13 weeks of the study gained somewhat less weight than those fed SBA or PRA (see Figs 1A and 1B). In males the reduction in weight gain was less than that seen with SBR, but in females the reductions with LBA and SBR were similar. Animals of both sexes compensated for the lower ME value (see Table 6) of the LBA diet by eating more of it than animals given SBA (see Tables 4A and 4C). Nevertheless, in neither sex were LBA-fed animals able to consume as much ME per day as SBA-fed animals (see Table 7A). Water consumption was also higher in LBA-fed males than in SBA-fed males (see Table 5A). In females fed LBA during the first 13 weeks of the study, there was a significantly ($P < 0.001$) lower incidence of corticomedullary mineralization than in females fed SBA during the same period (see Table 21). Histopathologically, animals fed LBA during the first phase of the study exhibited more bile-duct hyperplasia ($P < 0.001$), less myocardial fibrosis ($P < 0.05$) less mammary gland secretory activity ($P < 0.01$) less polyarteritis ($P < 0.05$) but more chronic prostatitis ($P < 0.05$) than SBA-fed animals (see Table 23).

As far as we know, the effects of diets similar to LBA and SBA have not previously been compared. Most of the effects can be explained as simply effects of calorie restriction, but the increased incidence of bile-duct hyperplasia requires a different explanation for which further research would be needed.

7. *The effects of feeding, from 13 weeks post weaning, the high fibre low energy diet (LMA) instead of SMA*

In so far as a total of 200 males and 200 females (groups 2, 6, 10 and 11) were fed *ad lib.* the LM diet from week 13 onwards, and those in group 11 were fed it throughout life, the present study provides a good opportunity for comparing the effects of this low energy maintenance diet with those of the standard maintenance (SM) diet given *ad lib.* to 150 rats of each sex (groups 1, 7 and 9) from week 13 onwards. LMA-fed rats ate more food (males, +19%; females, +13%) than SMA-fed rats (see Table 5B). However, on a per animal basis, they obtained significantly less ME (males, −8%; females, −14%) (see Table 7B). On a per 100 g body weight basis, however, LMA-fed males compensated completely for the low ME value of the diet, whereas the LMA females did so at one time point (60 weeks) but not at another (28 weeks) (see Table 8). As shown in Tables 9A and 9B, LMA had many of the same effects on haematological values (e.g. reduced WBC in both sexes, increased PLT in males) as SMR. Also, like SMR, LMA was associated with reduced plasma protein levels in both sexes and higher BUN levels in females, whereas both SMR and LMA led to increased liver enzyme levels in both sexes (see Tables 10A and 10B).

Urinary pH was higher in LMA than SMA animals but this effect was not as great and not as persistent in old age as in SMR animals (see Tables 11A and 11B). Like SMR, LMA significantly reduced urinary protein levels compared with SMA. Haematuria was reduced in incidence in LMA females compared with SMA males and ketones were higher in LMA than SMA males. Both these differences match those seen with SMR (see Tables 11A and 11B). LMA females were less prone to develop radiculoneuropathy than SMA females (see Tables 13B and 25) and like SMR females, LMA females had lower ($P < 0.01$) prolactin levels at the end of the study (see Table 15).

Compared with SMA, life expectancy was increased significantly ($P < 0.001$) in LMA females but no such effect was seen in males (see Table 17B). Absolute body weight and the absolute median weights of the heart, liver, kidneys, adrenals and pituitary were highly significantly lower in LMA animals of both sexes than in SMA animals (Table 18A). These effects were closely similar to

those seen with SMR. Further, in males, LMA like SMR was associated with a higher end-of-test median testes weight ($P < 0.01$). By contrast, the reduction in liver and kidney weights relative to body weight seen with SMR were not seen with LMA (Table 18B). In fact the liver weight relative to body weight was actually higher than in SMA rats (males, $P < 0.05$; females, $P < 0.01$).

The main effects of LMA compared with SMA on histopathological findings are summarized in Table 25. They included reductions in myocarditis (females, $P < 0.001$); chronic progressive nephropathy (both sexes, $P < 0.001$); increased bile-duct hyperplasia (males $P < 0.001$); reduced lymphoid hyperplasia and cystic change in cortical lymph nodes (females, $P < 0.01$); decreased mammary gland acinal hyperplasia (females, $P > 0.001$); decreased mammary gland secretory activity (both sexes, $P < 0.001$); decreased galactocoele formation (females, $P < 0.01$); decreased mammary gland neoplasia (females, $P < 0.001$); decreased neoplasms of the anterior lobe of the pituitary gland (males, $P < 0.01$; females, $P < 0.001$); and decreased neoplasia of the intermediate lobe of the pituitary gland (both sexes, $P < 0.001$).

As discussed in relation to the genetic profile of the Wistar strain of rat used for the study (see above) LMA, compared with SMA, increased the incidence of haemangiomas and haemangiosarcomas of the mesenteric lymph node (males, $P < 0.001$) and of adenomatous tumours of the uterus ($P < 0.01$). Another uterine change seen in higher incidence in LMA rats was hypertrophy of the cervix wall ($P < 0.01$). Finally, males fed LMA had a higher incidence of Leydig-cell tumours of the testis ($P < 0.001$).

Overall, despite the adverse effects of LMA on the incidence of neoplasms of the mesenteric lymph node and of the uterus, the incidence of benign or malignant tumours of any site was significantly lower ($P < 0.001$) in female rats fed this diet than in female rats fed SMA. On the other hand, the incidence of tumours of all sites in males was no different in response to LMA than to SMA.

An important question in relation to our findings concerning the effects of the LMA diet is to what extent the benefits of rationing animals to 80% of *ad lib.* food intake (i.e. SMR) can be achieved by feeding a low ME (high fibre) diet *ad lib?* The first thing to say in this regard is that the LMA diet would be uneconomic and not suitable for routine use because of the 66% spillage rate, and the fact that the rats clearly did not like it. Otherwise there were considerable similarities between the effects of LMA and SMR in terms of survival, body weight gain, organ weights, effects on incidences of radiculoneuropathy, myocarditis, chronic progressive nephropathy and tumour incidence (other than of mesenteric lymph node and uterus in females). One important difference, however, is that whereas SMR was highly effective ($P < 0.001$) in reducing overall tumour incidence in males, LMA was without effect in this regard. It is hoped that further research will lead to the formulation of a low energy diet which rats find palatable and that can be fed to animals *ad lib.* with the same benefits on incidence of ageing-related degenerative diseases and neoplasia as rationing the food intake of animals fed a standard chow, such as SM or PR.

Turek and Desjardins (1979) studied the development of Leydig-cell hyperplasia and tumours of the testis in F344 rats. They invariably found evidence of involution of seminiferous tubules in association with Leydig-cell proliferation. They also noted that during periods in which such proliferative changes appear, circulating serum prolactin and oestradiol levels are rising and although testosterone levels remained normal until rats were 18 months of age they rose strikingly thereafter. Unfortunately, the distinction between causes and effects in relation to the associations they describe is not clear. In this regard, our finding that the LMA diet fed *ad lib.* resulted in a significantly increased incidence of Leydig-cell tumours but a reduced level of circulating prolactin in terminally killed animals suggests that increased prolactin levels are not implicated in causation of these tumours. On the other hand, the possibility that the significantly higher circulating progesterone levels observed in terminally killed LMA-fed males was causally implicated in their higher incidence of Leydig-cell tumours will now merit further study.

8. *Peculiarities of the Wistar rat strain used in respect of mesenteric lymph node tumours and endometrial carcinomas and the enhancing effect of LMA on the incidence of tumours of these two kinds*

Of the 600 males in the study, 131 (21.8%) developed benign and/or malignant haemangiomatous tumours of the mesenteric lymph node. The crude incidence of such lesions in the 600 females was 47 (7.8%). We considered these tumours to be haemangiomas or haemangiosarcomas. However, in some of them, connective tissue proliferation dominated the picture with spaces containing lymph or blood being only sparse. Some of these latter lesions might have been diagnosed more properly as lymphangiomas. Multiplicity of lesions was common. The crude lifetime incidence in the three groups fed the SMA diet from 13 weeks (groups 1, 7 and 9) was 18.7% in males and 6.7% in females. By contrast, despite far better survival, the crude lifetime incidences in animals with a calorie intake restricted to 80% *ad lib.* from 13 weeks (groups 3 and 5) were only 8% and 3% in males and females, respectively, whereas the crude lifetime incidences in animals fed the LMA regimen from 13 weeks (i.e. groups 2, 6, 10 and 11), were 38.5% in males and 15.5% in females. After appropriate standardization for differences in survival it was found that calorie restriction (SMR) significantly ($P < 0.01$ in the sexes combined) reduced incidence

compared with SMA (see Table 26) and LMA significantly ($P < 0.001$) increased it compared with SMA (see Table 25).

At first when we observed the higher incidence of lesions of the mesenteric lymph node in rats fed the LMA diet, we wondered whether the addition of lignosulfonates to this diet might be causally implicated since effects on bone marrow macrophages and Kupffer cells in the liver had been reported as toxic effects following oral exposure to chemicals belonging to this group (Luscombe and Nicholls, 1973; Suzuki *et al.*, 1989). However, we could not find any evidence of the haematological effects or of the effects on Kupffer cells reported by these investigators. Moreover, as shown in Tables 1E and 1F, the amounts of lignosulfonates added to the SMA diet were similar to those added to the LMA diet. We concluded, therefore, that the addition of lignosulfonates to the LMA diet was not responsible for the higher incidence of mesenteric lymph node lesions seen in the LMA-fed animals.

The incidence of mesenteric lymph node tumours seen in our study in rats fed the SMA diet from 13 weeks (males 18.7%, females 6.7%) or the PRA diet from 13 weeks (males 12%, females 0%) can be compared with that observed in control rats of the same strain in carcinogenicity studies in the same laboratory fed PRA diet (1975–79 males 6.9%, females 3.4%; 1981 males 6.1%, females 2.2%; 1982 males 5.2%, females 0.9%). The higher incidences in our study no doubt reflect its greater length (2.5 *v.* 2 years). Deerberg *et al.* (1982) report a striking rise in incidence of these tumours in relation to the age of the animals. The higher incidences in males than in females is not only consistent with the historical control data but also with the results of Deerberg *et al.* (1982).

Another kind of neoplasm which occurred in unusually high incidence in the study as a whole was uterine carcinoma—either adenocarcinoma or anaplastic carcinoma. No less than 54 of the 600 female rats in the study (9.0%) developed such tumours. Calorie restriction (SMR) did not significantly protect against the development of this tumour; however, as shown in Table 25, feeding the LMA diet from week 13 of the study significantly increased the age-standardized risk of developing it ($P < 0.05$) with 33/200 (16.5%) of animals in groups 2, 6, 10 and 11 developing such tumours. A comparison of the incidences of malignant and/or benign glandular neoplasms of the uterus in the LMA compared with the SMA groups (41/200 *v.* 9/150) was more strongly statistically significant ($P < 0.01$).

The incidence of uterine adenomas/adenocarcinomas seen in our study in rats fed the SMA diet from 13 weeks (6%) or the PRA diet from 13 weeks (0%) is not dissimilar from that observed in control rats of the same strain in carcinogenicity studies in the same laboratory fed PRA diet (1975–79, 5%; 1981, 1.9%; 1982, 3.1%). The greater length of our study (2.5 *v.* 2 years) to some extent invalidates the comparison, although the rise in incidence with age seems much less for uterine tumours than is the case for the mesenteric lymph node tumours (Deerberg *et al.*, 1982).

The high incidences of tumours of the mesenteric lymph node seen in the present study were almost certainly partly determined by the genetic characteristics of the strain of Wistar rats used for the study. In a study of up to 48 months' duration of virgin male and female Wistar rats of the outbred Han:Wistar strain, Deerberg *et al.* (1980 and 1982) observed incidences of 26.3 and 17.5% of what they diagnosed as lymphangiomas of the mesenteric lymph nodes in males and females, respectively, and an incidence of 30.3% adenocarcinomas of the uterus. They considered that genetic factors were responsible for over 70% of the mesenteric lymph node tumours in males and over 40% of them in females. The description of the mesenteric lymph node as 'essentially haemorrhagic' and the illustrations in the 1980 paper are consistent with the lesions seen by Deerberg *et al.* being the same as those diagnosed by us in the present study as haemangiomas or haemangiosarcomas. On cytological grounds, and because of evidence of local invasiveness, we considered many of the tumours to be malignant. However, distant metastases were not seen either by us or by Deerberg *et al.* (1980 and 1982). The beneficial effect of calorie restriction (SMR) and the adverse effect of the high fibre diet (LMA) on the occurrence of these mesenteric lymph node tumours are seemingly new findings.

Deerberg and Kaspareit (1987) suggested that female BD II/Han inbred rats, which also have a high incidence of uterine adenocarcinomas, provide a useful model of a spontaneous hormone-induced tumour. They found that the incidence of such tumours in a lifetime (up to 48 months) study was lower (60%) in germ-free rats and in retired breeders than in conventionally maintained virgins (90%). However, they saw no difference in incidence between rats fed a commercial cereal-based diet and rats fed a purified diet. Thus our finding that the feeding of a high fibre diet (LMA) led to an increased incidence of uterine carcinomas appears to be new. The possibility that this effect was due to a higher exposure of the LMA-fed rats to dietary phyto-oestrogens in some way associated with the fibre cannot be excluded. A simple shift from progesterone dominance to oestrogen dominance by which Richardson *et al.* (1984) explained the low pituitary tumour incidence and high uterine tumour incidence in rats exposed to bromocriptine cannot be the whole explanation in the present case since SMR, which greatly reduced pituitary tumour incidence, had no significant effect on the incidence of uterine carcinomas. No significant between-diet differences in the ratio of oestrogen to progesterone were seen in females in the present study.

9. *Indirect effects of dietary restriction regimens engendered by effects on diurnal eating and drinking patterns*

The findings in the present study illustrate the fact that many of the parameters measured in routine toxicity studies are capable of being influenced by the dietary regimen. In general, rats and mice drink when they eat. Hence the more they eat, the more water they drink and during periods when food is not available, they drink only little. The higher incidence of gangrene of the tail (so called 'wet-tail') in the SMR groups, when the ambient RH fell during cold winter spells, is explained by the fact that the animals concerned drank too little water during the night time (see above).

Findings in other studies (Conybeare, 1988) illustrate the need to relate blood-sampling times to feeding and drinking patterns since serum biochemistry and circulating hormone values are influenced by the interval between the previous meal and the time of blood sampling, irrespective of overall calorie intake or the composition of the diet.

In some laboratories this particular problem is tackled by subjecting all animals in all groups to overnight starvation before blood sampling. However, in our view, this creates as many problems as it solves, since the sudden imposition of starvation on an animal that is used to feeding intermittently throughout the night is a sure way of disturbing its overall diurnal rhythm, its behaviour and its physiological status.

10. *The effects of spikey chaff in the LBA and LMA diets*

The syndrome, consisting of gaseous intestinal distension and respiratory distress, secondary fistulation of the palate, and of inflammation, abscess formation and squamous carcinoma of the oral cavity, which was encountered most frequently in SMA-, LMA- and PRA-fed rats, has been described previously by Robinson (1985) and Madsen (1989). Earlier, Deerberg (1971) also drew attention to the effect of spikey chaff in causing gingivitis and rhinitis in rats. Clearly it is not desirable that animals in long-term toxicity/carcinogenicity tests should be put at risk unnecessarily of developing such lesions. Therefore, steps should be taken to avoid the inclusion in rodent diets of sharp fibrous splinters capable of piercing the oral epithelium. This happens if diets containing wheat or oat chaff are not sufficiently finely ground.

11. *Effects of different diets on the incidence of corticomedullary and pelvic mineral deposition in the kidney*

The data for incidence of corticomedullary mineralization in the kidney in relation to diet during the first 13 weeks of the study (see Table 21) lack any simple explanation. In females, as compared with SBA, all the other diets/dietary regimens significantly reduced the incidence of this change. In males, however, restricted intake of SB [either by rationing (SBR) or reduced time of access (SBI)] was strongly associated with the development of this form of nephrocalcinosis. Reduced water consumption in these diet-restricted groups could have been responsible for this effect in males but, if this is the explanation, it is difficult to understand why females were not similarly affected. Unfortunately, the urinalysis data collected do not include data for electrolytes and no such data were collected during the study.

The effect of feeding the PR diet throughout the study in increasing the incidence of pelvic mineralization in both sexes (Table 24) may have been associated with the overall higher intake of absorbable carbohydrates by animals on this diet. Calcium is absorbed from the small intestine along with glucose and other monosaccharides (Bergeim, 1926; Casey *et al.*, 1978; Greenwald & Gross, 1929; Hodgkinson *et al.*, 1982). Calcium, in excess of that required for bone growth etc., is for the main part excreted in the urine and, if this is paralleled by high phosphate excretion, precipitation of calcium phosphate is apt to occur in the kidney in the calyx region between the base of the papilla and the lining of the medulla. Such precipitation constitutes pelvic nephrocalcinosis.

Numerous dietary factors have been studied in relation to the incidence of various forms of mineralization of the kidney in laboratory rodents, but the literature is confused because of the failure of many of the investigators when studying just one variable to control for other important variables and/or to distinguish between the several different forms of nephrocalcinosis and/or analyse mineral deposits chemically. Among the list of factors known to be important are: calcium intake; phosphate intake; magnesium intake; calcium:phosphorus ratio in the diet; intake of other electrolytes; urinary pH; dietary composition in terms of proportion of protein, fat and carbohydrate, intake of carbohydrates; type of dietary carbohydrate; vitamin D intake; oxalate intake; and citrate intake (Coburn and Packett, 1962; DuBruyn, 1972; Eklund *et al.*, 1973; Forbes, 1963; Gershoff and Andrus, 1961; Györy *et al.*, 1970; Hitchman *et al.*, 1979; Hodgkinson *et al.*, 1982; Jacob and Forbes, 1970; Magnusson and Ramsay, 1971; Pansu *et al.*, 1971; Phillips *et al.*, 1986; Roe, 1993b; Sager and Spargo, 1955; Theophilus and Barnes, 1974; Van Reen, 1962; Van Reen *et al.*, 1959 and 1964; Vaughan and Filer, 1960). For the present study we simply do not have enough relevant data relating to the variables listed above to explain the findings.

Of further interest in relation to the higher incidence of pelvic nephrocalcinosis in the PRA-fed animals is the fact that adrenal medullary focal hyperplasia and/or neoplasia was seen in significantly higher incidence in rats fed PRA than in rats fed SMA from week 13 (see Table 24), the difference

being evident in both sexes. As reported by us (Roe and Baer, 1985) dietary factors, such as lactose, which increase calcium absorption, also increase adrenal medullary proliferative changes in rats.

12. *Effects of calorie intake on organ weight relative to body weight and the implications of the findings in relation to toxicity testing*

It is important to stress that none of the forms of dietary restriction studied in the present experiment was sufficient to stunt growth as determined by bone length or absolute brain weight. The main effects of dietary restriction were on the principal organs involved in metabolism, namely the liver and the kidney, and on those endocrine organs that are particularly prone to the development of hyperplastic and neoplastic changes in *ad lib.*-fed animals.

The lack of any effect of calorie restriction on brain weight is consistent with the observation of Goodrick (1984) that lifelong-restricted feeding is associated with better complex maze performance than is *ad lib.* feeding.

Several factors influence absolute organ weights and organ weights relative to body weight. If exsanguination at autopsy is incomplete, organs such as the liver, lungs and spleen are heavier because of their higher content of blood. The data for animals killed at the end of the present study are not subject to variation for this reason because of the care taken by the prosectors concerned.

The fact that calorie-restricted animals remain sexually active for many months longer than do *ad lib.*-fed animals probably accounts for the fact that absolute testis, prostate and ovary weights were not reduced in the SMR rats in the present study (see Table 18A). The other forms of dietary restriction studied (SMI and LMA) exhibited a similar pattern of effects to SMR.

Another factor which influences organ weights is disease. Reduced kidney weight relative to body weight in SMR males and females is explained by the lower incidence/severity of chronic progressive nephropathy in these animals than in the SMA rats. In males the high protein diet fed *ad lib.* (PRA) significantly increased kidney weights over and above those of SMA-fed rats and PRA-fed females had significantly higher liver weights than SMA-fed rats. Likewise the lower pituitary weights relative to body weights seen in SMR and LMA animals of both sexes (see Table 18B) is explained by the lower incidence/severity of focal hyperplasia and neoplasia in the anterior and intermediate lobes.

From a toxicological viewpoint, however, it is the highly significantly lower liver weight relative to body weight in SMR and SMI rats of both sexes which is probably of greatest importance. The fact that this reduction has been found to be associated in female rats with a prolongation of phenobarbitone sleeping time is consistent with *ad lib.*-fed animals being in a higher state of *P*-450 drug metabolism enzyme activity (Conybeare, 1988). Whether the same explanation is true for males is uncertain. By contrast, the significantly higher liver weights in LMA and PRA rats of both sexes compared with SMA rats suggests that these diets led to the liver being involved in a higher level of metabolic activity.

Since liver weight, particularly liver weight relative to body weight, is commonly used as an index of toxicity and since any associated difference in liver enzyme activity may be relevant to how animals metabolize test substances, it is clearly important to take both food consumption and water consumption into account in the interpretation of differences of liver parameters in toxicity studies.

Since liver enzyme levels and liver weights fluctuate throughout the day and are influenced by when food is eaten, and we only had liver weight data for animals killed at the end of the study, we did not attempt to relate the liver enzyme data that we collected during the study to liver weight as determined at autopsy.

13. *Effects of different diets on circulating hormone levels*

In the present study the rise in serum prolactin levels with age was, as expected, much lower in diet-restricted rats than in *ad lib.*-fed rats. Apart from this, diet restriction was associated, particularly in males, with lower circulating growth hormone levels. Also, in agreement with Merry *et al.* (1985), 17β-oestradiol levels were found to be higher in SMR- and SMI-fed groups than in SMA-fed groups. Another finding was of higher progesterone levels in low energy-fed (LMA) males and it is possible that this relates to the higher incidence of Leydig-cell tumours seen in LMA-fed rats. Prolactin is essential for the maintenance of LH receptors in Leydig cells, and a fall in the level of circulating prolactin leads, as a result of a feedback control mechanism, to increased LH production. Theoretically this may help to explain why the feeding of the LMA diet predisposed to Leydig-cell tumour development, even though no elevation in LH was observed in rats killed at the end of the study, nor in satellite animals.

An interesting parallel to some of the above findings is that chronic treatment with the dopamine agonist, bromocriptine, appears to correct the ageing-related endocrine dysfunction that occurs in female rats in much the same way as dietary restriction (Richardson *et al.*, 1984). Thus both bromocriptine and diet-restriction:

(i) reduce the age-related rise in serum prolactin;
(ii) reduce the age-related decline in pituitary dopamine; and
(iii) increase the circulating level of 17β-oestradiol.

Although dietary restriction delays the start of regular oestrous cycling, its effect in delaying the age-related decline in fecundity is much more impressive (Conybeare *et al.*, 1986; Holehan and Merry,

1986; Merry and Holehan, 1979). These effects, like those on circulating hormones, indicate that dietary restriction affects hypothalamic function. However, the precise mechanisms involved remain to be elucidated. It is possible, for instance, that regulatory peptides such as vasoactive intestinal polypeptide (VIP) are involved. This particular hypothalamic peptide stimulates prolactin secretion and is affected by dopaminergic tone and oestrogen levels. The observed increase in VIP levels with age may be a factor in the development of prolactinomas, since it has been shown to be a growth factor for lactotrophs (Prysor-Jones *et al.*, 1987a and 1987b; Samson *et al.*, 1979). Further, it needs to be borne in mind that the anterior pituitary cells synthesize and secrete a number of autocrine and other growth factors, which may be mediated by feeding regimens.

14. *Effects of different diets on urinalysis findings*

Some of the between-group differences seen in the present study were probably related more to the time when urine samples were collected in relation to when animals previously ate food and drank water than to the composition of the diet or to the amount of food consumed each 24 hours. The two main exceptions to this were, first, the increased urinary protein levels seen in rats fed the high protein (PR) diet and the high urinary ketone levels seen in rats fed the high fibre (LM) diet. Ketosis occurs when acetoacetyl coenzyme A, derived from oxidation of fatty acids, is hydrolysed in the liver to form acetoacetic acid which is then released into the blood stream. Why the acetoacetyl coenzyme A takes this route rather than being oxidized to oxaloacetate which enters into the citric acid cycle to release energy or hydrogenated as a precursor for fat synthesis is not always clear.

Ketosis can be brought about in several ways, but is typically found when less than 15% of the energy utilized comes directly from carbohydrate and the Respiratory Quotient is less than 0.75. Such conditions are likely to occur for example during fasting when body fat may be metabolized to provide energy or when the diet contains a low level of available carbohydrate and a high fat content. It is also likely to occur in meal-eating animals when the large short-lived influx of carbohydrate saturates the animal's ability to store it as glycogen in the liver, and the excess is converted into fat; later the animal will metabolize the fat to provide energy during the period of 'fasting'.

For high fibre diets, not only may there be a shortage of available carbohydrate, but there may be fermentation of fibre in the large intestine to produce volatile fatty acids (VFA) such as acetic acid, butyric acid and propionic acid which may be absorbed. In the case of acetic and butyric acid this may add to the ketone load. There is also evidence that both adrenocortical and pituitary hormones may regulate the shift towards ketone body production.

Although none of the animals in this study had very high levels of ketone bodies in the urine (i.e. ketosis), there were elevated levels in the SMR-, SMI- and LMA-fed groups. The increases were more noticeable in the LMA-fed animals than in the SMR-fed animals, despite the fact that both groups had low plasma glucose levels, which may have been the trigger for ketogenesis. It is not clear why the levels of ketones were higher in the LMA-fed rats, but the possibility of absorption of VFA derived from fermentation in the large intestine that give rise to ketones, or some hormonal influence exists.

As the study progressed, between-group differences in urinalysis parameters were influenced increasingly by the differences in the incidence and severity of chronic progressive nephropathy which are curtailed by dietary restriction. Lower urinary levels of protein and blood in the SMR-, SMI- and LMA-fed groups are largely explained in this way.

The urine samples derived from SMR- and SMI-fed males were on all occasions alkaline while those from SMA- and PRA-fed males were on all occasions acidic. The same is true for SMR females while the urine from SMI females hovered around neutral. The pH of urine from LMA-fed animals was significantly less acidic than that of the SMA-fed animals at 6 and 12 months in both sexes and in males the same was true at 18 and 24 months. It happens that these effects of the different diets on urinary pH paralleled those on longevity, with the result that urinary pH at, say, 6 months appeared to be correlated significantly with survival time (see above). However, since there is no obviously plausible direct mechanism to explain the association, we presume that it is non-causal in nature.

15. *Implications of findings in relation to the design and interpretation of carcinogenicity tests in laboratory rodents*

The results of the experiment reported in this paper illustrate the fact that factors unassociated with exposure to genotoxic carcinogens can markedly influence the risk of development of a wide range of neoplasms in laboratory rats. The most striking difference seen was between rats fed *ad lib.* the SM diet and those fed only 80% *ad lib.* the same diet from 13 weeks (groups 3 and 5). It is inconceivable that the huge effects of such slight calorie restriction could have been due to a 1:0.8 difference in intake of traces of genotoxic carcinogens in the SM or SB diets. The amount that individual animals eat may be influenced by several factors, including genetic constitution, events during early life, palatability of diet, toxicity of test substances, disease status and endocrine status. Despite the multiplicity of these factors, body weight gain is widely used as a marker of general toxicity, with reduced weight gain being taken as evidence of adverse effect. However, the results of the present study show quite conclusively that in animals not deliberately exposed to any toxin, reduced weight

gain is associated with highly statistically significant beneficial effects on health in terms of increased longevity, delayed onset of ageing-related diseases and reduced tumour incidence. There must be an argument, therefore, for standardizing calorie intake in toxicity tests, so that any effects of exposure to a test substance can be compared in groups of animals that do not differ in life expectancy and/or age-standardized risk of development of neoplasia because of differences in the amount of food eaten. Food conversion efficiency, which is certainly relevant to toxicity, can be measured just as easily in calorie-restricted animals as in *ad lib.*-fed animals.

As has been argued elsewhere (Abelson, 1992; Roe, 1981, 1988a and 1993a; Roe and Lee, 1991) *ad lib.*-fed rats, with their high incidences of endocrine disturbances and endocrine and non-endocrine tumours, cannot be regarded as appropriate models for detecting carcinogenicity relevant to humans, particularly when it is clear that differences in calorie intake are strongly associated with differences in tumour incidence.

In 1973, we (Roe and Tucker, 1974) drew attention to the fact that in an outbred colony of mice, despite constancy of dietary formula over a 10-year period, there were in untreated control animals dramatic increases in the mean adult body weight and overall tumour incidence and, at the same time, a decreased life expectancy. More recently, similar drifts have been observed in rodent strains (e.g. B6C3F$_1$ hybrid mice, F344 rats, Sprague–Dawley rats) commonly used for carcinogenicity testing in the USA and elsewhere (Nohynek *et al.*, 1993; Rao and Haseman, 1993). Genetic drift (because of selection by animal breeders for large litters and rapid early body growth) is blamed. Certainly there are now very substantial differences in the growth rate and survival rate of the 'same' strain of rat, the Sprague–Dawley, from different suppliers (Harlan Sprague Dawley Inc., personal communication). Nevertheless, in the short term at least, part of the answer to the problem must be to reduce the calorie intake of the animals used for carcinogenicity tests (Abelson, 1992).

16. *The relationships between body weight during the first year of the study (irrespective of dietary regimen) and (a) longevity and (b) incidence of ageing-related diseases and neoplasia*

In the present study the effects of a variety of different diets and dietary regimens were compared. We were interested to see whether, notwithstanding these differences, there was any relationship between body weight at different times during the observation period and (a) longevity and (b) the development of ageing-related diseases and neoplasms. The results of analysis of our findings in this regard have been published elsewhere (Roe *et al.*, 1991). Ignoring diet and dietary regimen we divided the 600 males and 600 females in the study into quintiles of approximately 120 animals each according to their body weight 26 weeks post weaning. We found highly significant correlations between body weight quintile and premature death ($P < 0.001$ in both males and females) development of benign and/or malignant neoplasms at any site ($P < 0.001$ in males and $P < 0.01$ in females) and the development of malignant neoplasms at any site ($P < 0.001$ for the sexes combined). Numerous kinds of neoplasm contributed to these overall correlations. The most significant were pituitary tumour ($P < 0.001$), mammary tumour ($P < 0.001$), squamous or anaplastic carcinoma of the jaw ($P < 0.001$) and subcutaneous tumours of mesodermal origin ($P < 0.05$). The 20% rats that were heaviest were more than twice as likely to die before the age of 33 months than the 20% that were lightest (2.55 times, males; 2.12 times, females) and almost twice as likely to develop a malignant tumour (1.89 times for the sexes combined). These findings are summarized in Tables 30, 31 and 32 whereas Table 29 illustrates the relationship between mean body weight and incidence and severity of chronic progressive nephropathy and body weight 12 months post weaning.

As shown in Table 31, the picture for malignant tumours in females was complicated by the fact that LMA was associated both with a reduction in body weight gain and an increased incidence of adenocarcinomas of the uterus (see above). The picture is also complicated by the fact that the LMA diet led to an increased incidence of haemangiomas and haemangiosarcomas of the mesenteric lymph node. Despite these adverse effects of the body weight-reducing diet, the overall correlation between weight gain during the first 6 months of the study and eventual cancer incidence remained highly significant.

Similar relationships between body weight early in life, lifespan and cancer incidence were reported by Turnbull *et al.* (1985). More generally, numerous investigators (e.g. Rao *et al.*, 1987) have reported that diet restriction leads both to reduced body weight gain and reduced cancer incidence, but the relationship between body weight early in life and longevity and cancer incidence in an experiment involving different diets and dietary regimens has not previously been analysed statistically. It is already well known that in chronic toxicity/carcinogenicity tests reduced weight gain in high exposure groups tends to be associated with reduced chronic renal disease in rats and reduced incidences of certain kinds of tumour in both rats and mice. The question now is whether this effect should be taken into account in the statistical evaluation of studies by adjusting downwards the expected incidence of tumours in high dose groups to take into account the body weight effect.

During recent years there has been concern in relation to carcinogenicity testing in many laboratories, including those involved in the National Toxicology Program in the USA (Rao and Haseman,

1993) because the survival of animals for the full 2 years that regulatory authorities require has been increasingly difficult to achieve. This is because the tumour incidence in untreated control groups has been rising despite standardization of diets and animal husbandry procedures. For various reasons, the most plausible explanation is that, despite close genetic control, there has been genetic drift because of selection for large litters and rapid growth in animal breeding establishments. Whether or not this is the only explanation, there can be little doubt that calorie restriction of all animals in carcinogenicity tests would improve survival and cut down background tumour incidence. Nevertheless, one should not expect a calorie-restricted genetically large animal to be biologically the same as an *ad lib.*-fed genetically smaller animal. A paper by Ross *et al.* (1983a) is relevant in this regard. These investigators found that the mature body weight of male rats of an outbred strain permitted to select their own diets throughout life correlated linearly with the incidence of tumours that they developed. Most of the between-animal variance in mature body weight could be explained by a model that took into account food intake relative to body weight, the proportion of protein and carbohydrate in the diet, the intake of each of these constituents and the efficiency with which the diet consumed was used for growth during early post-weaning life. By comparison with this list of variables, the actual level of food or calorie intake proved less informative. Presumably, genetic differences between individual rats determined which of the available diets they selected.

III. Theoretical mechanisms by which calorie restriction affects incidences of ageing-related diseases, including neoplasia

For many years investigators working in the field of gerontology have been drawing attention to relationships both between age and degenerative disease and between age and the development of neoplasia (Weindruch *et al.*, 1986; Weindruch and Walford, 1982). However, the possibility that increased risk of development of tumours might actually be a part of the ageing process and that similar mechanisms may be implicated in the causation of both phenomena has, until recently, been largely ignored by oncologists involved in carcinogenesis research. To such scientists the concept that cancers are caused by mutations due to exposure to environmental mutagens has been more attractive. Increasing evidence that calorific restriction simultaneously increases lifespan, and reduces both the age-standardized risks of developing many different ageing-related diseases and those of developing many different types of cancer is, nowadays, demanding that the relationships between ageing and neoplasia be taken seriously.

Masoro (1988) provided a thoughtful review of the mechanisms that might theoretically underlie the relationships between ageing-related diseases and neoplasia. In relation to the effects of calorie restriction on lifespan, he pointed out that one needs first to distinguish between prevention of premature death and retardation of the ageing process. A difficulty here is that the true maximum lifespan of no species is known for certain. Despite this difficulty, after weighing up the evidence, Sacher (1977) concluded that calorie restriction actively retards the ageing process. Next, a distinction needs to be made between the effects of calorie restriction on age-related changes in physiological processes [examples being: crystallization of the lens of the eye in mice (Leveille *et al.*, 1984); loss of immune functions in mice (Fernandes, 1984); effects on the ability of adipocytes to respond to the lipolytic effects of hormones (Bertrand *et al.*, 1987); delay in reproductive senescence (Holehan and Merry, 1985); and loss of learning ability (Ingram *et al.*, 1987)] and slowing or preventing the onset of age-associated degenerative diseases.

McCay *et al.* (1935) suggested that calorie restriction increases lifespan simply by slowing growth and development. If this were an important mechanism then one would not expect that dietary restriction begun in adult life (i.e. after growth and physical development are complete) would prolong life or reduce the incidence of degenerative diseases. However, several investigators (Cheney *et al.*, 1983; Goodrick *et al.*, 1983; Stuchlíková *et al.*, 1975; Weindruch and Walford, 1982) have reported significant benefits of dietary restriction started late in life.

Another theory that has, according to Masoro (1988), been ruled out is that proposed by Berg and Simms (1960) to the effect that calorie restriction prevents ageing by reducing body fat. In reality, however, this is not a 'mechanism-based' theory. It is only a 'description' of a negative association between obesity and longevity. In this connection, a paper by Holliday *et al.* (1967) is interesting. It seems that there tends to be an inverse relationship between body weight and basal metabolic rate, not only between species but also within species. Thus the basal metabolic rate per kilogram (basic metabolic rate/kg) of a human infant is about twice that of a normal adult human, and the basic metabolic rate/kg of an obese adult may only be two-thirds that of a lean adult. This subject has been expertly reviewed by Blaxter (1989). Within the body the most important component to contribute to basal or resting metabolism is the lean body mass; fat tissue is energetically relatively inert. Thus one would expect that metabolic rate is similar for two individuals or groups of similar age whose difference in weight can be attributed to fat tissue, and this is confirmed in practice. Adjusting metabolic rate for body weight results in a reduced rate for the obese individuals. Therefore it seems unlikely that the higher cancer incidence seen in heavy rats compared with light rats or in obese humans as compared with lean humans is caused by

a higher basal or resting metabolic rate per kg of body weight in the heavier, fatter individuals.

Sacher and Duffy (1979) reported a negative correlation between metabolic rate (resting or average) and longevity among the male progeny from 21 out of 25 possible matings of five inbred mouse strains. However, according to Masoro (1988), the plausibility of the theory that calorie restriction leads to a reduction in the production of mutagenic and tissue-damaging oxygen free radicals during the conversion of food into energy (Harman, 1981) because it leads to a reduction in the metabolic rate has been disproved. In the light of their own research, Masoro *et al.* (1982) and McCarter *et al.* (1985) concluded that calorie restriction is not associated with any decrease in metabolic rate per unit of lean body mass and that the energy consumption per unit of lean body mass is the same in calorie-restricted and *ad lib.*-fed animals. We, however, question this conclusion in the light of our finding that both absolute liver weight and liver weight relative to body weight in SMR groups were lower than that in SMA groups. Further, it is well accepted that during food restriction resting metabolic rate decreases more than can be accounted for by loss of lean tissue [see Blaxter (1989) for a review].

Masoro (1988) suggested that calorie restriction brought about retarded ageing by effects on endocrine and neuroendocrine regulatory systems. In so far as *ad lib.* feeding (which some investigators, including ourselves, regard as 'overfeeding') is associated in rats with abundant evidence of endocrine disturbance and of increased incidences of endocrine tumours, this hypothesis seems not implausible. Also, support for this theory was provided by the finding of Ross *et al.* (1983c) that, in rats, a high growth rate and a high rate of conversion of food into body mass early in life are correlated with high incidence of anterior pituitary tumours. However, in mice, ageing is not closely associated with manifestations of endocrine disturbances and the main effects of calorie restriction in relation to neoplasia are on the incidences of tumours of the lung, liver and lymphoreticular system, none of which are to be regarded primarily as endocrine tissues.

Yu (1989) found that the longevity of rats was increased by 10–15% by reducing protein intake without reducing calorific intake, whereas reduced protein intake in conjunction with calorific restriction increased longevity by closer to 50%. The beneficial effect of protein restriction in the absence of overall calorific restriction was attributed to a beneficial effect on the incidence/severity of nephropathy. By contrast, an important consequence of calorific restriction is a slowing down of the age-related decline in microsomal cytochrome *P*-450 activity and a consequential preservation of the detoxifying activity of this group of enzymes. It is well known that while one can vary the intake of protein over a wide range, the rate of net accretion of protein in the body varies very little. While the increase in availability of protein does stimulate protein synthesis, it is balanced by an increase in protein catabolism. Thus there is an increase in protein turnover—including presumably of DNA. This may also explain the findings of Yu (1989).

Chipalkatti *et al.* (1983) suggested that calorie restriction may retard ageing by decreasing oxygen free radical mediated cell damage. Later, Koizumi *et al.* (1987) confirmed tht calorie restriction reduces lipid peroxidation and promotes hepatic catalase activity (which protects against oxidative damage) in mice.

Cotton and Rogers (1993) discussed the possible importance of age-dependent changes in mitochondrial DNA in relation to ageing and cancer. They suggested that life on earth originated at a time when there was no free oxygen in the atmosphere. However, as photosynthetic activity increased, there arose a need for living cells to develop a way of dealing with toxicity from oxygen and from oxygen-derived free radicals and mitochondria evolved as a consequence of this need. Wild-type mitochondrial DNA which is circular in type (i.e. like bacterial DNA) has a different genetic code from nuclear DNA. However, like nuclear DNA, it is subject to mutation, and certain genetic disorders are associated with mutations of mitochondrial DNA. Cotton and Rogers (1993) suggest that mutations in mitochondrial DNA may reduce the protection of cells from oxidative damage and thereby predispose to premature ageing and cancer. This intriguing theory awaits substantiation.

In 1990, there appeared a spate of papers expounding a new theory, according to which increased cell turnover rates predispose to increased risk of mutation. Cohen and Ellwein (1990) pointed out that a common characteristic of non-genotoxic agents which predispose to cancer of the urinary bladder is that they increase cell proliferation in the bladder epithelium. Heller *et al.* (1990) found that food restriction reduces cell proliferation in the small intestine of the rat. Lok *et al.* (1990) reported similar findings in several tissues in mice. Citing these and many other papers, Ames and Gold (1990) boldly advanced the theory that "mitogenesis increases mutagenesis". Basic to this theory is the fact that during mitosis there is a brief phase in which the two strands of DNA are separated. DNA damaged by oxygen free radicals during this phase is far less amenable to repair than during other phases of mitosis, with the result that mutations are 'fixed' and passed on to daughter cells. A variety of defence mechanisms other than DNA repair, ensure that almost all mutant cells produced in this way do not become clones of deviant cells. Most such cells will not be stem cells anyway, and will die out by natural apoptosis, others will not be fully viable because of the DNA damage, and others may become the victims of immune surveillance. Notwithstanding these protective mechanisms, cell division is associated with a finite, although

doubtless small, risk of persistent mutations and, as the available lifespan passes, such mutations which gradually accumulate give rise to both manifestations of age-related changes and increased cancer incidence.

Underlying the concept that mitogenesis increases mutagenesis is a recognition of the fact that potentially mutagenic chemicals are produced within cells (i.e. endogenously) during the ordinary processes involved in the conversion of food into energy (Ames, 1989a,b; Totter, 1980a,b) and that DNA damage and repair are processes that are proceeding at high rates in all body cells all the time. Ames (1989b) estimated that the DNA of the average cell in the rat is damaged by endogenous oxidants approximately 10^5 times each day and that the comparable figures for man is 10^4 times per cell per day. Apart from oxidant change, DNA may suffer methylation, deamination and depurination as a consequence of normal intracellular metabolic processes (Ames, 1983; Totter, 1980a,b). Evidence of oxidative damage to DNA—which is probably the most important form of DNA damage—can be found in the urine in the forms of thymol glycol and thymidine glycol which are produced during the repair of oxidized DNA (Ames *et al.*, 1984; Cathcart *et al.*, 1984). The higher the basal metabolic rate, the higher the rates of DNA damage and repair and the higher the rates of cell turnover. Thus it is not surprising that there is a relationship between all these variables, lifespan and age-standardized cancer risk.

All in all, the concept outlined above offers plausible answers to many questions and at the same time takes into account the potentially important protective anticarcinogenic role of exogenous antioxidants (e.g. β-carotene) and endogenous antioxidants (e.g. uric acid). However, Weinstein (1991) has seen fit to warn against over-glib acceptance of the overall concept. First, he stresses that carcinogenesis is a multistage process, involving not only progressive genetic changes, but also non-genetic changes in the course of tumour promotion, clonal expansion and progression from benign to increasing malignancy. Secondly, he questions whether DNA damage caused by xenobiotic exogenous agents is comparable with endogenous DNA damage in the sense that the latter is likely to be more amenable to repair by mechanisms that have developed during the course of evolution. While heeding Weinstein's caution, we do not consider that his arguments falsify the concept, particularly as it relates to the effects of calorie restriction on longevity, ageing-related diseases and risk of developing cancer. In other words, it is our opinion that calorie restriction is associated with decreased cell turnover rates, and therefore, with reduced risk that unrepaired DNA damage caused by either exogenous or endogenous mutagen will be passed on to viable daughter cells. Further, we question whether the stages of carcinogenicity, particularly the stages of tumour initiation and tumour promotion that have been amply demonstrated in highly controlled animal tests, are commonly involved as distinct stages in human carcinogenesis (Roe, 1988a). Such a sequence has never seemed likely for hormonal carcinogenesis (Roe, 1988b). Finally, we are not impressed by Weinstein's argument that cancers do not arise more commonly in tissues with high cell turnover rates than in tissues with lower cell turnover rates because he fails to consider the distinction between stem cells and non-stem cells. Clearly, mutations in the former are far more likely to be relevant to cancer risk than mutations in the latter.

Despite the important developments of the last few years, there remains much scope for further research into how calorific restriction reduces the risk of cancer development. Licastro *et al.* (1986) reported that dietary restriction retards the age-related decline in the DNA repair capacity of mouse splenocytes. Tice and Setlow (1985) also found that diet restriction slowed the loss of capacity for DNA repair with age in addition to reducing the endogenous production of oxidants. Weindruch (1992) did not rule out any of the following possible mechanisms: " ... less cellular oxidative damage, retarded immunologic ageing, hormonal changes, less energy available for cell proliferation, reduced exposure to dietary carcinogens and promoters, enhanced DNA repair and less carcinogen activation".

There is published evidence that ageing is not associated with increased susceptibility to the effects of genotoxic carcinogens. Several years ago, in collaboration with Richard Peto (Peto *et al.*, 1975), we compared the responses of female mice to repeated once-weekly doses of the carcinogen, benzo[*a*]pyrene, applied to the dorsal skin. Separate groups received their first dose at 10, 25, 40 and 55 weeks of age. Thereafter, dosing was continued until the development of a malignant skin tumour or death (or euthanasia on humane grounds) from other causes. The object of the study was to see whether response to the same carcinogenic treatment remains constant throughout life or becomes more or less marked with age. The result of the experiment was unequivocal. The incidence of malignant epithelial tumours among survivors in each group increased steeply with time and the increase was associated directly with duration of exposure. Given duration, the incidence was independent of age at the start of exposure. Further, the growth rates of tumours after they appeared was similar in all four groups. Thus, in this study, no intrinsic effects of ageing (such as failing immunological surveillance or age-related hormonal changes) on carcinogenic response, were evident. How do the results of this earlier experiment fit the conclusions that we are drawing, albeit tentatively, from the present study? We suggest the following answer. Benzo[*a*]pyrene is a fairly potent genotoxic carcinogen for mouse skin. The cumulative doses applied were sufficient to give rise to skin tumours in virtually all mice which survived for

100 weeks from the start of exposure. The mouse study was not concerned, as is the present rat study, with the incidence of neoplasia at all sites in animals that are not deliberately exposed to any genotoxic agent. For the mouse study, virtually no skin tumours would have arisen in the absence of exposure to benzo[*a*]pyrene. The only relevance of the results of the mouse study to the interpretation of the rat study is that the former provides no evidence that the incidence of tumours in the rats in the present study was influenced by an age-related increase in sensitivity to a genotoxic stimulus. If this is so, then one may deduce that there are no grounds for suspecting that calorific restriction reduced tumour incidence by reducing the sensitivity of animals to environmental genotoxic agents such as might have been present inadvertently in the food fed to them, etc. In a recent review Clayson *et al.* (1994) consider the ways in which oxidative DNA damage may be implicated in carcinogenesis either by genotoxic agents or non-genotoxic agents. Their conclusions are consistent with our own.

In summary, it is our view that the most likely explanation of the various beneficial effects of dietary restriction is that oxygen free radicals, which have been shown to be capable of damaging either irreversibly or reversibly, not only nucleic acids, but also cell proteins, free amino acids, lipids, lipoproteins, carbohydrates and connective tissue macromolecules (Cross, 1987) are involved in both ageing processes and carcinogenesis, and that a combination of these activities shortens life. Further, it is likely that cell damage, particularly if it results in premature cell death followed by regenerative hyperplasia, predisposes to cancer by increasing the risk that DNA damage is not repaired before cells divide into daughter cells. By cutting down the frequency of mitosis, calorie restriction reduces this latter risk.

IV. Implications of findings in relation to ageing-related diseases and cancer mortality in man

In theory, humans in affluent Western countries are similar to *ad lib.*-fed laboratory rodents. However, in practice there are important differences. Humans are not kept in cages with nothing to do except eat, but have duties to perform and problems to face. Further, they are free to take physical exercise. By contrast, laboratory rats and mice live in an artificial environment which bears little relationship to that of their wild forebears. They are protected from debilitating infections, parasitic diseases and predators, deprived of sexual fulfilment, and do not have to forage for food or go without it if they cannot find any. It is likely, therefore, that the sluggish, grossly obese *ad lib.*-fed laboratory rodent with its lacklustre coat and unhealthy skin is far from representative of rodents in the wild. In other words, it is a laboratory artefact.

Be that as it may, an important aspect of the observed benefits of calorie restriction is to consider whether similar benefits might accrue from calorie restriction in humans.

Epidemiologists aiming to answer this question are faced with formidable difficulties. Comparisons of life expectancy and cancer mortality in different countries reveal large differences which might be due to either genetic or environmental factors or a combination of the two. Studies on migrants, for instance, from Japan to the USA, point to environmental factors being more important than genetic ones and to dietary differences being of paramount importance. In particular, a change from a low-fat oriental diet to a high-fat occidental diet seem to be of crucial importance (Haenszel, 1970). However, attempts to reduce the incidence of typical ageing-related diseases (e.g. coronary heart disease) or cancer in Westernized populations by dietary manipulation have given essentially equivocal results (e.g. Pearce and Dayton, 1971). One difficulty is that such studies tend to be started when people are too old to show benefits; years of eating too much, particularly, perhaps, too much fat, have already set in train the sequence of changes leading up to premature senescence and elevated risk of developing cancers. Although experiments in rodents have shown that calorie restriction started in middle life has statistically significant benefits (Cheyney *et al.*, 1983; Goodrick *et al.*, 1983; Weindruch and Walford, 1982), the extent of such benefits is less than that of calorie restriction begun earlier in life. Indeed, the overall picture is of an association between duration of restriction and benefit.

Another problem is that virtually all the dietary intervention studies on humans have involved attempts to restrict fat consumption without controlling the overall calorie consumption. In this connection, it is important to stress that the diets used in the present study all had a similarly low (about 3%) fat content. In other words, we controlled calorie intake without changing dietary composition in respect of fat content, whereas human intervention studies have attempted to control the proportion of daily calories ingested as fat without controlling calorie intake.

Nor is the case:control comparison approach of any value for studying the effects of calorie restriction in humans. People generally are notoriously unreliable in relation to providing information on what and how much they eat (Wynder and Shigematsu, 1967). They often, for instance, cannot recall what and how much they ate during the week before being questioned, let alone during the previous 20–40 years.

Lew and Garfinkel (1979), in a huge American prospective study, investigated how cancer mortality, adjusted for age, sex and tobacco use, varied according to body weight. Subjects were categorized according to actual weight divided by the average weight for people of similar height and sex. People who were sick, or had a history of cancer, heart disease or stroke, or who had lost 10 pounds or more in weight

over the preceding year were omitted from the analysis in order to avoid bias due to the effects of pre-existing cancer on initial weight. Compared with men of average weight (risk = 100%), obese men (i.e. at least 40% heavier than average) were at significantly higher risk of dying from cancer (colon/rectum, 173%; prostate, 129%; pancreas, 162%; stomach, 188%; all cancers, 133%), while men less than 85% average weight had significantly lower risks of dying from the four types of cancer specifically listed above. Even so, the risk that less than average weight men would die from *any* form of cancer was seemingly not less than that of men of average weight. The situation in women was more consistent, with those who were 40% overweight being at much higher risk of dying from a long list of cancers (endometrial, 542%; uterine cervix, 239%; gall bladder, 358%; kidney, 203%; colon/rectum, 122%; breast, 153%; any cancer, 155%) and those less than 85% average weight being at less (77–95%) risk of the same cancers (overall risk = 92%). One of the difficulties with the interpretation of these findings is that the populations studied were heterogenous. Consequently it is not possible to be sure to what extent the body weight of individuals reflects genotype and to what extent it reflects over-indulgence in food. More generally, it is a matter of common observation that body weight does not correlate very well with food consumption in heterogenous populations.

Seemingly, the best evidence of a beneficial effect of low calorie intake in humans comes from a comparison of food intake and death rates from heart disease, cerebrovascular disease and cancers in Okinawan Japanese and Japanese in general, between whom genetic differences are small (Kagawa, 1978). Food consumption data are obtained annually from randomly chosen households in hundreds of different districts in Japan. This collection of data involves detailed personal interviews and the direct weighing of the foods eaten during a period of 3 consecutive days. Okinawans eat fewer calories per day throughout life than mainland Japanese (children, 62%; adults, 80%). Deaths per 100,000 in the 60–64-year age group in Okinawa and deaths from heart disease or cerebrovascular disease are only 59% of those in mainland Japan, with deaths from cancers being only 69%. Not surprisingly, Okinawans are shorter and lighter than other Japanese. This reflects the fact that calorie restriction starts during childhood before mature body size is obtained.

Preston-Martin *et al.* (1990) considered that an increased rate of cell division may well play a causative role in the genesis of cancers in man and related this theory to examples of increased cancer risk from hormones, drug, infectious agents, chemicals, physical or mechanical trauma and chronic irritation generally. They pointed out that a series of distinct genetic alterations takes place in a cell before it becomes malignant and that several of the changes involved (e.g. those resulting in the activation of oncogenes or the suppression of tumour suppressor genes) can only occur during cell division. This concept, they say, fits what is known of the epidemiology of human cancer. They point out, for instance, that the risk of induction of breast cancer by X-rays is particularly high if exposure takes place when mammary cell proliferation rates are high (e.g. at puberty or during pregnancy).

Doll and Peto (1981) estimated that diet is responsible for some 35% of all cancer deaths. Since cancer deaths typically form some 20–25% of all deaths in many populations, this implies dietary related cancer is responsible for approximately 80,000 cancer deaths per million. Using the carcinogenic potency database established by Gold *et al.* (1984), Lutz and Schlatter (1992) calculated that, apart from alcohol, known dietary carcinogens could not account for more than a few hundred of these. They then explored the possibility that the discrepancy might be accounted for by over-nutrition. Using data from the present (Biosure) study, and taking the cancer incidence in the SMR groups as the control value, and the higher tumour yields in the SMA groups as a carcinogenic response, they calculated that the TD_{50} (= the dose rate in milligrams per kilogram body weight per day which would halve the probability of an animal remaining tumour-free by the end of the standard lifespan for the species) for calories in excess of those ingested by the SMR groups is 16 g/kg/day. In another calculation they estimated that over-nutrition in humans living in Switzerland averages 5.5 kcal/kg/day (= 1.9 g excess food/kg/day). On this basis they concluded that over-nutrition could be accounting, by its effects on cancer alone, for 60,000 out of every million deaths.

V. Comments on the present study in the light of the findings in a large contemporary study

During the period when the Biosure Study was in progress, a study with a rather similar purpose was being undertaken in the Merck Research Laboratories (MRL) with Dr Kevin Keenan as the principal investigator. It seemed, therefore, appropriate to ask Dr Keenan to comment on the findings in the Biosure Study in the light of those of the MRL study. His comments are set out in Appendix 2.

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Availability of data—The full data from the Biosure Study and the results of further analyses not presented in this report may be obtained from Peter Lee at a nominal cost. Mr Lee would also be prepared to undertake additional analyses on request.

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APPENDIX 1

THE BIOSURE STUDY: PRELIMINARY NUTRITIONAL EXPERIMENTS

Before the main Biosure Study, two preliminary studies were carried out to determine the adequacy of the experimental diets with respect to protein content and composition, and their metabolizable energy (ME) density. In addition to achieving the primary objectives of the studies, valuable information was gained on the influence of the diets on body weight gain and body composition. These two studies are reported below.

A. Protein quality study

Introduction

The purpose of this study was to determine the adequacy of the protein in the experimental diets to be used in the main Biosure Study. We were concerned to ensure that growth restriction through any inadequacy of protein content or composition did not confound the primary objective of the Biosure Study, namely to assess the impact of energy restriction on body weight gain and subsequent pathology and survival.

Methods

Diets. Four experimental diets were specially formulated and used in the study. These were: Standard Breeder diet (SB); Standard Maintenance diet (SM); Low Nutrient Breeder diet (LB); Low Nutrient Maintenance diet (LM).

The fifth diet was the commercially available Porton Rat diet (PR). This diet had been used for many years by Smith Kline and French Research Ltd (SKF), and thus provided a control diet with substantial background data.

All the diets were formulated and manufactured by Biosure (Lavender Mill, Manea, Cambs, UK). Except for PR, the diets used the same raw materials as the sources of the main nutrient constituents. The two 'Standard' diets (SB, SM) were formulated to provide nutrient levels that were typical of current breeding and maintenance diets in use at that time. The 'Low Nutrient' diets were intended to be similar to the standard diets except that the density of the available energy was decreased by the addition of fibre sources, such as wheatfeed and oatfeed, at the expense of the cereals, maize and wheat. The protein content of the diets was varied by the manipulation of two high protein sources, white fish meal and Pruteen (a single cell protein produced on a methanol feed stock by Imperial Chemical Industries).

The rate of inclusion of the mineral and vitamin premixes was adjusted to compensate in part for the expected adjustment in food intake that was likely to occur as the animals attempted to regulate their ME intake of diets that differ substantially in ME density.

The details of the diet formulations are given in Table 1A of the main body of the paper. The calculated analyses of the diets, based on analysis of the ingredients, is given below in Table A1.

Animals. Wistar albino rats from the SKF colony, and aged 21 days on arrival, were used.

Housing. On arrival, each animal was ear numbered. Six rats were then placed in a mesh-bottomed plastic-sided cage and fed PR diet *ad lib.* Water was freely available. The sexes were housed separately in two banks of cages. After grouping (see below), the rats were housed two per cage. Beneath each cage was a plastic tray to collect faeces and any spilled food.

Environment. The experiment was performed in a controlled-environment room, maintained at 21°C (range ± 0.5°C) and at 50% humidity (range ± 5%). Lighting was on from 06.00 to 18.00 hr.

Grouping. The experiment used groups of two rats, selected to be as uniform as possible for mean body weight and rate of weight gain. The 75 rats of each sex were weighed daily for 7 days before grouping. A computer program, developed by Professor G. R. Hervey of the Department of Physiology at the University of Leeds, was used to regress each rat's weight against time to obtain rate of weight gain and 'smoothed' weight on the day of grouping. Body weight and rate of weight gain were given equal weighting in the selection of animals, and on this basis the rats were then placed in ranking order. The most deviant animals were then discarded, leaving the 60 rats required for the experiment.

The ranked series was then divided into two 'rows' (the number of rats per group). The rats in each row were assigned by random permutation to the 30 groups; for each permutation the group means and their standard deviations about the global mean were calculated. This was repeated, and the grouping that gave the lowest standard deviation for the combined attributes of rate of gain and smoothed body weight was recorded. The process stopped when there had been no improvement after 50 repetitions.

The whole procedure was carried out separately for the two sexes.

The close matching of the treatment groups can be seen from the means and standard errors (SE) of body weights on the day of grouping and the rate of weight gain over the previous week presented in Table A2.

Protocol. After grouping, 25 pairs of female rats were allocated to an equal number of cages in a 5 × 5

Table A1. Calculated composition of the Biosure diets (values adjusted to 12.5% moisture)

	Diet				
	SB	SM	LB	LM	PR
Dry matter (g/kg)	875	875	875	875	875
Crude protein (g/kg)	202	143	167	136	198
Crude oil (g/kg)	31	31	34	30	30
Crude fibre (g/kg)	25	63	78	106	54
Dietary fibre (g/kg)	103	194	264	317	146
Ash (g/kg)	49	52	60	67	62
Gross energy kJ/g	16.6	16.4	16.4	16.7	16.7
Digestible energy kJ/g	12.8	11.6	11.0	10.3	12.6

Table A2. Grouping data (values are the means of five pairs of rats; the values are expressed per rat ± SE)

	Diet				
	SB	SM	LB	LM	PR
Males					
Body weight	102.0 ± 0.9	101.1 ± 1.2	102.9 ± 1.3	101.3 ± 1.3	102.7 ± 2.1
Rate of gain (g/day)	6.32 ± 0.02	6.14 ± 0.11	6.30 ± 0.15	6.36 ± 0.13	6.29 ± 0.19
Females					
Body weight	91.7 ± 0.6	93.2 ± 1.9	93.4 ± 0.3	93.4 ± 1.1	93.5 ± 1.0
Rate of gain (g/day)	5.65 ± 0.16	5.56 ± 0.10	5.61 ± 0.15	5.53 ± 0.10	5.56 ± 0.17

array. Diets were allocated to the cages in a Latin Square design, that is each diet appeared in each row and column once only. The procedure was repeated with a separate bank of cages for the male rats.

The remaining five matched pairs of each sex were killed humanely for carcass analysis and were used to predict the starting body compositions of the animals to which the diets were to be fed.

The animals were weighed daily during the 21-day experimental period. The amount of food removed from the food hopper and the amount of spillage collected in the plastic trays below the cage were also

Table A3. Food intake, spillage, weight gain, dry matter digestibility and parameters of protein quality of the five diets fed to male rats

	Diet				
	SB	SM	LB	LM	PR
Food intake g/21 days per rat	493	512	547	526	498
Spillage g/21 days per rat	61	162	187	351	47
Weight gain g/21 days per rat	146	120	123	100	132
Dry matter digestibility	0.856	0.754	0.672	0.552	0.747
Nitrogen digestibility	0.826	0.729	0.674	0.608	0.732
Proportion of N intake retained	0.295	0.305	0.262	0.252	0.262
Proportion of N absorbed retained	0.357	0.419	0.388	0.415	0.357
	Statistical significance tests				
	F	E.M.S.	L.S.D. 0.05	L.S.D. 0.01	
Food intake g/21 days per rat	8.97***	545.9	22.8	31.9	
Spillage g/21 days per rat	136.26***	1098.4	32.3	45.3	
Weight gain g/21 days per rat	25.55***	110.2	10.2	14.3	
Dry matter digestibility	1378.02***	0.00005	0.0093	0.0131	
Nitrogen digestibility	151.27***	0.00022	0.0202	0.0284	
Proportion of N intake retained	18.22***	0.00015	0.0169	0.0237	
Proportion of N absorbed retained	17.38***	0.00026	0.0222	0.0311	

***Significant treatment effect at $P < 0.005$. If the variance ratio (F) is significant then the treatment means are significantly different if the difference between them is greater than the least significant difference (L.S.D.).
E.M.S. = Error mean square.

Table A4. Food intake, spillage, weight gain, dry matter digestibility and parameters of protein quality of the five diets fed to female rats

	Diet				
	SB	SM	LB	LM	PR
Food intake g/21 days per rat	347	411	424	445	385
Spillage g/21 days per rat	50	131	171	291	38
Weight gain g/21 days per rat	80	73	70	64	76
Dry matter digestibility	0.858	0.760	0.680	0.559	0.752
Nitrogen digestibility	0.833	0.754	0.703	0.595	0.751
Proportion of N intake retained	0.240	0.263	0.233	0.210	0.214
Proportion of N absorbed retained	0.288	0.349	0.332	0.354	0.285
	Statistical significance tests				
	F	E.M.S.	L.S.D. 0.05	L.S.D. 0.01	
Food intake g/21 days per rat	24.63***	334.5	17.8	25.0	
Spillage g/21 days per rat	73.76***	1439	37.0	51.8	
Weight gain g/21 days per rat	12.21***	26.9	5.1	7.1	
Dry matter digestibility	1243.48***	0.00005	0.0097	0.0136	
Nitrogen digestibility	156.39***	0.00024	0.0215	0.0301	
Proportion of N intake retained	24.08***	0.00009	0.0134	0.0188	
Proportion of N absorbed retained	16.06***	0.00034	0.0253	0.0355	

***Significant treatment effect at $P < 0.005$. If the variance ratio (F) is significant then the treatment means are significantly different if the difference between them is greater than the least significant difference (L.S.D.).
E.M.S. = Error mean square.

Table A5. Estimates of metabolizable energy (ME) intake of the five experimental groups compared with weight gain (the ME density was obtained in the second of the preliminary studies)

	Diet				
	SB	SM	LB	LM	PR
Males					
ME intake kJ/day	323	288	279	225	285
Weight gain g/day	6.95	5.71	5.86	4.76	6.28
Females					
ME intake kJ/day	246	239	215	193	222
Weight gain g/day	3.81	3.48	3.33	3.05	3.62

determined daily and daily food intakes calculated. Faeces from the plastic trays were also collected daily and stored at −20°C for later analysis.

At the end of the 21-day period, all the animals were killed humanely and subjected to carcass analysis. The faeces collected from each group were individually dried at 105°C to constant weight, and then weighed.

Carcass composition. The rats were finally weighed (ante-mortem weight), killed humanely with CO_2 and reweighed (post-mortem weight). Any faeces produced at this stage were recovered, weighed and the weight added to that of the gastro-intestinal contents.

Carcasses were analysed by the method of Hervey and Hervey (1967); the method allows the analysis of the entire body and therefore does not introduce sampling errors. The contents of the gastro-intestinal tract—food, partly digested food and faeces—were weighed and discarded. Body water content was measured by drying the chopped whole carcasses at 105°C. Body fat content was measured by transferring the dried carcass quantitatively to 600-ml Soxhlet extraction thimbles, and then extracting them for approximately 18 hr with 40–60°C b.p. petroleum ether. The fat extracted and the fat-free residue were both weighed. The dry fat-free solids were ground to a fine powder and sampled for nitrogen analysis.

The body composition of the groups fed the experimental diets for 21 days was predicted at the start of the experimental period, when the change of diet occurred, by regressing the amount of each carcass component against live body weight. Thus for animals of known weight, their composition could be predicted with known accuracy (Armitage *et al.*, 1983).

Nitrogen analysis. Samples of finely ground diet, dried faeces and fat-free solids were weighed with an accuracy of 0.1 mg. Sample weights were typically about 1.5 g for diet and faeces and 0.45 g for the fat-free solids. The samples were digested in a Technicon digestion block for 1 hr at 200°C with 10 ml conc. H_2SO_4 and 5 g K_2SO_4 containing selenium and copper as catalysts. The digested samples were distilled in a Buchi distillation unit, and the distillate collected in an accurately known volume of 0.05 M H_2SO_4 containing methyl red as an indicator. The acid remaining after collection of the ammonia distilled off was back-titrated with 0.05 M NaOH. From this, the nitrogen content of the sample was determined. Samples were analysed in duplicate and the analyses were all found to agree to within 2% of the mean.

Statistical analyses. Where appropriate the data were subjected to analysis of variance from a 5 × 5

Table A6. Body composition of male rats at the end of the experiment (values are means of individual rats ± SE)

	Diet				
	SB	SM	LB	LM	PR
Body weight (g)	248.00 ± 1.79	220.87 ± 3.36	226.24 ± 4.66	201.30 ± 2.67	234.54 ± 4.66
Gut contents (g)	17.44 ± 0.45	16.89 ± 0.35	24.06 ± 1.42	27.33 ± 1.07	20.76 ± 0.23
Carcass weight (g)	230.56 ± 3.50	203.99 ± 3.53	202.19 ± 3.99	173.97 ± 1.62	213.79 ± 4.51
Water (g)	154.20 ± 2.77	139.06 ± 2.48	139.03 ± 2.58	124.23 ± 1.19	146.34 ± 3.33
Fat-free solids (g)	53.05 ± 0.84	46.69 ± 0.96	47.45 ± 0.90	40.98 ± 0.46	50.31 ± 1.19
Fat (g)	22.33 ± 1.07	18.29 ± 0.64	15.72 ± 0.87	8.76 ± 0.10	17.41 ± 0.50
Proportion of water in lean tissue	0.744 ± 0.001	0.748 ± 0.001	0.746 ± 0.001	0.752 ± 0.001	0.744 ± 0.001

Table A7. Body composition of female rats at the end of the experiment (values are means of individual rats ± SE)

	Diet				
	SB	SM	LB	LM	PR
Body weight (g)	171.40 ± 0.91	165.83 ± 2.17	163.65 ± 2.63	157.57 ± 1.58	169.03 ± 2.00
Gut contents (g)	11.23 ± 0.20	13.27 ± 0.20	16.30 ± 0.92	20.68 ± 0.72	15.11 ± 0.18
Carcass weight (g)	160.18 ± 0.97	152.56 ± 2.14	147.35 ± 1.94	136.89 ± 0.96	153.93 ± 1.98
Water (g)	104.80 ± 0.62	101.46 ± 1.45	100.55 ± 1.29	95.72 ± 0.68	102.38 ± 1.42
Fat-free solids (g)	38.45 ± 0.24	36.59 ± 0.48	36.28 ± 0.46	33.30 ± 0.13	37.74 ± 0.54
Fat (g)	16.93 ± 0.33	14.52 ± 0.54	10.53 ± 0.27	7.88 ± 0.40	13.81 ± 0.75
Proportion of water in lean tissue	0.732 ± 0.001	0.735 ± 0.001	0.735 ± 0.001	0.742 ± 0.001	0.731 ± 0.001

Table A8. Protein content measured in the diet and the fat-free fraction of the carcass of rats fed one of the five diets (values are means ± SE of five determinations)

	Diet				
	SB	SM	LB	LM	PR
Protein in diet as fed (g/kg diet)	197.8 ± 1.4	150.7 ± 0.8	166.0 ± 0.9	140.5 ± 0.6	203.1 ± 1.2
Protein in fat-free solids (g/kg)					
Males	825.2 ± 1.3	821.4 ± 7.5	819.7 ± 9.0	816.7 ± 1.4	826.8 ± 7.3
Females	798.8 ± 6.0	808.1 ± 17.2	819.8 ± 11.8	794.9 ± 6.4	800.2 ± 12.5

Latin square design. The tables include the mean treatment values, the variance ratio (F), the error mean square (E.M.S.) and the least significance differences at the 0.05 and 0.01 probability (P) levels.

Results and Discussion

General. A summary of the results of the study are given in Tables A3 (male rats) and A4 (female rats). Although the experimental unit is composed of two rats, the data are expressed on a 'per rat' basis.

Food intake. Male and female rats both had the lowest food intakes on the SB diet; intake of the PR diet was similar, though slightly higher. The next highest food intake was of SM diet. The highest intakes were on the LM and LB diets. The pattern of food intake corresponded with estimates of the dietary fibre (indigestible carbohydrate) and the measurements of ME density reported in the second study, and were consistent with the suggestions that animals will adjust their food intake to regulate ME intake until the physical bulk of the diet prevents continued adjustment (Hervey and Tobin, 1983).

Spillage. Typically spillage amounted to about 10% of food eaten, and this was the figure seen with PR diet; spillage with SB diet was similar at about 12–13% of food eaten. With the other diets spillage was much higher: SM, 32%; LB, 37%; and LM, 66%. The level of spillage corresponded with the fibre level of the diets. It was not possible to determine definitively whether the extent of the spillage was associated with the physical nature of the diet *per se* (i.e. the ease with which it might crumble) or with palatability. However there was no obvious crumbling of the diets, whereas spillage certainly increases independently of the physical nature of the diet for quinine-adulterated diets. Whatever the cause, such severe spillage on high fibre diets places severe constraints on the practicality of their use in toxicology.

Weight gain. The weight gain over the 21-day experimental period can be seen in Table A3 for males and Table A4 for females.

The mean body weight of the animals that subsequently provided the five groups were similar over the first 7 days when they were all fed PR diet. The rate of body weight gain of the five groups diverged once they were given access to the different experimental diets. The rate of gain was generally consistent with the amount of ME ingested (Table A5), being highest in animals fed SB and PR diets; and lowest in animals fed LM diet. The weight gain data should be treated with some caution as a measure of energy and protein gain since the contribution of the weight of gut contents to body weight varied substantially with treatment (see Tables A6 and A7).

Body composition. A summary of the final body compositions of the male and female rats fed the five diets is given in Tables A6 and A7 respectively. Since the comparable starting body compositions of the animals was similar because of the grouping procedure, the final body compositions reflects the effects of the different diets.

The body weights are distorted by the variation in the amount of the gut contents which reflected the amount of indigestible matter in the diet. The true difference in tissue deposition, and thus true body size, between animals fed SB and LM diets is much greater than live body weight measurements alone would suggest. The low nutrient diets consistently produced rats that were smaller and much leaner than the rats fed standard nutrient diets. The amount of fat in the body was closely associated with ME intake (r^2 0.99 for males, 0.94 for females). It is interesting to note that the proportion of water in lean tissue was very similar for the five diets.

Dry matter digestibility. The dry matter (DM) digestibility (Tables A3 and A4) is the proportion of the dry matter intake which was apparently absorbed by the animal (the term apparently absorbed is used because some secretions into the gut are eliminated and compensate for some of the dry matter that is absorbed). The low nutrient diets had very low DM digestibilities because of their high fibre content. The SB diet was remarkable for the small amount of faeces produced by animals fed it, and this gave a high DM digestibility figure.

Digestibility was always somewhat higher in female rats and this possibly reflects a longer transit time associated with their lower food intake and lesser gut distension.

Nitrogen digestibility. Nitrogen digestibility (the proportion of nitrogen ingested that was apparently absorbed) reflected the overall DM digestibility.

Protein deposition. The amount of protein deposited in the body has been calculated both as a proportion of the protein eaten and as a proportion of the protein apparently absorbed. In calculating protein deposition, the amount of body protein has been determined by multiplying the weight of the fat-free solids by its protein content. The main variation in the protein content of the fat-free solids of rats given the same treatment is caused by sampling errors (the fat-free solids are not strictly homogenous being composed of muscle, bone and hair, and even

Table A9. Metabolizable energy (ME) density of the five Biosure experimental diets

	Diet				
	SB	SM	LB	LM	PR
Gross energy (kJ/g diet as fed)	16.64	16.36	16.45	16.67	16.74
Dry matter content (g/kg diet as fed)	886.8	890.8	899.0	883.0	904.1
ME density (kJ/g diet as fed)					
Males	13.75	11.83	10.72	8.98	12.01
Females	13.80	12.22	10.64	9.11	12.11

	Statistical significance test			
	F	E.M.S.	L.S.D. 0.05	L.S.D. 0.01
ME density (kJ/g diet as fed)	1877.05***	0.001	0.17	0.23

***Significant treatment effect at $P < 0.005$. If the variance ratio (F) is significant then the treatment means are significantly different if the difference between them is greater than the least significance difference (L.S.D.).
E.M.S. = Error mean square.

after grinding it is possible to distinguish the different components). Consequently the individual weights of fat-free solids of animals on each diet have been multiplied by the mean protein content of the fat-free solids of the animals fed that diet—this procedure can be justified by the uniformity of the composition of fat-free solids as judged by the precision of measurements of proportion of water in the lean tissue. The main protein content was based on five estimates, one from each pair of animals on the diet, each in duplicate (Table A8).

The protein deposited is calculated by subtracting the prediction of the initial body protein content from the final body protein content for each animal.

The influence of digestibility on protein retention and apparent protein quality can be seen by comparing nitrogen (protein = N × 6.25) retention as a proportion of nitrogen intake and of apparent nitrogen absorption in rats fed the LM diet. The proportion of nitrogen retained from that ingested is lowest in animals fed LM diet, but expressed as a proportion of that absorbed it is the highest of all the groups.

The high nitrogen retention of that absorbed seen with animals fed the SM and LM diets may reflect a slightly higher protein quality but may also be a function of the total amount of protein available; as increasingly more protein is supplied than required, a smaller proportion will be retained and vice versa.

Female rats had consistently lower nitrogen retention values than male rats, reflecting their inherently lower rate of nitrogen (protein) deposition.

There appears to be little evidence of a primary limitation on growth that can be attributed to poor protein quality or insufficient protein.

B. Metabolizable energy density study

Introduction

The purpose of the study was to determine the metabolizable energy (ME) density of the diets that were to be used in the study.

Methods

Diets. Details of the diets that were studied are described in Section A above.

Animals. Wistar rats were used from the SKF colony. Male rats weighed 200–220 g and female rats 140–155 g on arrival.

Housing. On arrival the rats were grouped in single-sex pairs so that the body weight of the pairs were as similar as possible within each sex. Each pair was placed in a separate mesh-bottomed plastic-sided cage. Beneath each cage was a plastic tray to collect faeces and any spilled food. The sexes were housed separately in two racks of cages.

Environment. The experiment was performed in a controlled-environment room, maintained at 21°C (range ± 0.5°C) and at 50% humidity (range ± 5%). Lighting was on from 06.00 to 18.00 hr.

Protocol. After grouping the rats into pairs, the five diets were randomly allocated to three pairs of rats of each sex. The rats were allowed 14 days to adapt to the diet before the experiment began. The experiment was carried out over a 5-day period during which the amount of food removed from each food hopper was measured. Excreta and food spillage were collected in plastic trays beneath each cage. A small amount of 5% sulfuric acid was added to the trays to prevent bacterial contamination of the excreta and loss of energy.

In some cases, where spillage was high, it was removed daily, dried and weighed. At the end of the 5-day period, spillage was separated from the trays and also dried and weighed. The remaining excreta was transferred to metal containers, dried and weighed.

The amount of food eaten was calculated by subtracting the weight of dried spilled food from the weight of dry matter removed from the food hopper. The gross energy content of diets and dried excreta were analysed by a Gallenkamp adiabatic bomb calorimeter; a calibration chart had been prepared previously by combusting numerous samples of the thermochemical standard, benzoic acid (McLean and

Tobin, 1987). Each sample was measured in duplicate, and the duplicates were only accepted if they agreed to within 1%.

ME density was calculated as follows:

$$\text{ME density} = \frac{\text{gross energy intake} - \text{gross energy of excreta}}{\text{weight of food eaten}}$$

Statistical analysis. The data have been analysed using analysis of variance for a randomized factorial experiment. The table of results includes the ME values for all five diets, for both males and females, the variance ratio, the error mean square and the least significant differences at the 0.05 and 0.01 probability levels.

Results and Discussion

A summary of the results is given in Table A9. As expected the low nutrient diets have a considerably lower ME density than the standard diets. The value measured for PR diet was similar to that previously measured in this laboratory. The values for females are usually higher than those for males. This may reflect an increased transit time and an increased efficiency of absorption in females.

A close relationship was observed between the calculated dietary fibre content of the diet and ME density; the relationship could be expressed as:

$$\text{ME density (kJ/g)} = 15.7 - (0.20 \times \text{dietary fibre})$$

The coefficient of determination (r^2) was 0.94. Significantly the intercept at zero dietary fibre lies close to the theoretical ME density (16 kJ/g) of a diet made up primarily (*c.* 700 g/kg diet) of available carbohydrate (16 kJ ME/g), with about 200 g/kg protein (17 kJ ME/g) and a small amount of fat (37 kJ ME/g).

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APPENDIX 2

COMMENTS ON FINDINGS IN THE BIOSURE STUDY BY KEVIN P. KEENAN DVM, PhD*

It is timely that the massive database of this well-controlled study is being published in such a complete and accessible format. As cited by the authors, this work was first presented at the 1990 International Life Sciences Institute (ILSI) Conference on the "Biological Effects of Dietary Restriction" held in Washington, DC. Since that time a second ILSI conference has been held in 1994 on the implications of using dietary restriction in toxicity and carcinogenicity bioassays; the United States Food and Drug Administration is presently drafting a "Points to Consider" document on this subject and many academic, regulatory and industrial institutes are considering the importance of studies like this in future bioassay design and interpretation of existing and ongoing studies.

In the extensive literature in this field it is rare to have access to such a complete dataset as offered to the scientific community for further utilization, evaluation and interpretation. Data from this study have already been utilized in risk assessment models by Lutz and Schlatter (1992) in determining the effect of over-nutrition, by its effects on cancer alone, of possibly accounting for 60,000 out of every million human deaths. This study demonstrates that the unlimited access to food (so-called '*ad lib.*' feeding) is clearly over-nutrition with many adverse outcomes including premature death from the early onset of degenerative diseases and cancer.

The husbandry utilized in this carefully controlled study was typical of many European rodent carcinogenicity studies that group-housed five rats per cage. The authors point this out and properly report nutritional and energy intakes as per cage averages. The potential problems of conducting dietary restriction studies with group-housed animals have been reviewed with many other practical considerations by Masoro (1991). It has been recently observed that wide differences in food consumption and subsequent body weight and survival can be seen in group-housed rats with restrictive feeders that limit access to feed or feeders that permit unlimited food intake (Dr Patricia L. Lane, personal communication). This potential difficulty appears to have been carefully controlled in this study, but must be considered when comparing results from different studies utilizing different husbandry practices and feeds.

A typical situation in pharmaceutical bioassays in the USA and Canada is for rats to be singly housed and to be provided with unlimited (*ad lib.*, AL) access to food. Many consider this an appropriately controlled method. However, AL feeding results in massive over-nutrition with many physiological and pathological consequences leading to decreased longevity. Researchers at the Merck Research Laboratories (MRL) have observed a steady decline in 2-year survival in rat carcinogenicity studies undertaken or sponsored by the pharmaceutical and chemical industries (Gumprecht *et al.*, 1993; Keenan *et al.*, 1992 and 1994a,b; Keenan and Soper, 1995). This decline has been observed in rats of all stocks and strains, including the relatively long-lived Fischer strain. The Sprague–Dawley (SD) stock is the principal stock used in the USA. In a retrospective study of 58 control groups of Charles River SD rats from 2-year carcinogenicity studies, singly housed and AL fed Purina 5002 Rodent Chow, a significant negative correlation has been noted between food consumption and survival (Keenan *et al.*, 1994a). The average survival for males in these studies varied from 7 to 73% at the end of 2 years. Their average daily food consumption (g/day) or energy intake (kcal/day) ranged from 21.7 to 33.2 g/day or 66.6 to 102.2 kcal/day. This wide variability in food consumption and, as a consequence, in average body weights and final survival, appeared to be due to differences in access to feed and the result of husbandry and feeder configuration. All these data are from carcinogenicity studies that have been submitted to regulatory agencies. They demonstrated a close correlation between food consumption and survival with considerable variability noted from laboratory to laboratory. It should be noted that only rodents (rats and mice) are maintained by unlimited access to feed. Other laboratory animals (i.e. dogs and primates) are typically provided measured amounts of feed and it is considered poor scientific practice to do otherwise.

The MRL workers designed a prospective study to compare the effects of AL overfeeding and moderate dietary restriction (DR) of two different diets on the 2-year survival of 700 SD rats and the development of spontaneous degenerative and neoplastic disease. The level of DR used in this study provided food amounts within the lower range of the 58 AL studies described above (Keenan *et al.*, 1994a). The Charles River SD rats were fed Purina 5002 Rodent Chow or a modified chow, 5002-9, that had lowered protein, fat and metabolizable energy content and increased fibre content similar to the dietary manipulations described in the Biosure Study. These diets were fed AL or an approximate 35% DR by measurement or by time (6.5 hr of daylight feeding). By 106 weeks more than 50% of the SD rats fed AL had died. Only moderate DR by daily measurement consistently resulted in lower mortality for both sexes fed either diet (Gumprecht *et al.*, 1993; Keenan *et al.*, 1992 and 1994a,b; Keenan and Soper, 1995).

At a 52-week interim autopsy and 106-week final autopsy the SD rats fed either diet AL had similar brain weights as DR-fed rats but the AL animals had greater body weights, body fat content, and higher

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heart, lung, kidney, liver, adrenal, thyroid and pituitary weights as well as an increased incidence and severity of degenerative disease and proliferative lesions in these organs. For example, moderate DR by measurement of either diet delayed the progression of chronic nephropathy by preventing the early development of glomerular hypertrophy seen at 52 weeks, which constitutes the initial change in the pathogenesis of glomerular sclerosis and nephron loss in an accelerated fashion in the AL-overfed animals (Gumprecht *et al.*, 1993). Moderate DR of either diet lowered the incidence, severity and progression of cardiomyopathy and other degenerative age-related lesions and appeared to delay the development of reproductive senescence in the SD females as determined by oestrous cycle analysis.

The most common cause of early mortality in SD rats of both sexes fed either diet AL was pituitary tumours followed by mammary gland tumours in females and renal and cardiovascular degenerative disease in males. The overall tumour incidence was remarkably similar between the AL and DR groups and did not fall outside the lower or upper ranges of historical controls at MRL or the published incidence rates of tumours for this SD stock in other laboratories (Keenan *et al.*, 1994b; Keenan and Soper, 1995). However, compared with the AL group, an age-adjusted Peto analysis demonstrated a decreased incidence of pituitary adenoma in the male DR groups. This decrease was seen in the female DR by measurement groups but not in the time restricted females. Compared with the AL group, DR males had a decrease in the age-adjusted incidence of pancreatic islet-cell carcinoma. Compared with the AL group, DR females had a decrease in age-adjusted incidence of mammary gland tumours, although this incidence was not outside the range of historical controls for SD rats. Additional analysis of mammary gland tumour growth showed that growth time (time from initial palpation until death), tumour doubling time and tumour volume were generally not significantly different between AL and DR groups, although AL females could sustain larger tumour volumes (Keenan *et al.*, 1994b; Keenan and Soper, 1995). There were no other significant differences in the age-adjusted incidence of tumours at any site in the SD rats given either diet or subjected to moderate DR (Keenan *et al.*, 1992 and 1994b; Keenan and Soper, 1995). The conclusion drawn from this 2-year prospective study was that this moderate level of dietary restriction delayed death due to fatal cardiovascular or renal disease and spontaneous tumours, particularly those of the pituitary and the mammary glands. However, this moderate level of DR appears only to delay the time of onset but not the progression of these spontaneous tumours whether measured by age-adjusted incidence, growth time, tumour doubling time or time between initial palpation and death (Keenan *et al.*, 1994b; Keenan and Soper, 1995).

The MRL workers have also conducted 14-week studies that compare the effects of AL overfeeding and moderate DR on the toxicological response of SD rats to five different pharmaceutical agents administered at or near the maximum tolerated dose. The compounds tested included phenobarbital, clofibrate, an HMG-CoA reductase inhibitor, cyclosporine A, and a dopamine agonist, all given by oral gavage. While quantitative differences were seen, the qualitative appearance of all significant clinical, haematological, biochemical and pathological changes were detected both in the AL and moderate DR groups given these compounds. Liver samples indicated no significant difference in microsomal cytochrome *P*-450 contents and drug metabolizing enzyme activities. While quantitative differences were seen, the qualitative changes in individual biochemistry parameters, organ weights and pathological lesions were similar for each compound under both feeding regimens (Keenan *et al.*, 1994b; Keenan and Soper, 1995). Considering the adverse effects of AL overfeeding and the beneficial effects of moderate DR (that does not fall out of the range of AL food consumption seen in other laboratories), it is clear that chronic toxicity induced by these classes of compounds would be readily detected in animals maintained under this moderate DR. Moreover the beneficial effects of moderate DR in preventing the long-term spontaneous degenerative diseases and delaying the onset of spontaneous diet-related endocrine tumours would result in the rats being exposed to test compounds for a considerably longer period of time (3–5 months) in the 2-year bioassay and thus allow for a better assessment of the test compound's chronic toxicity and potential carcinogenicity (Gumprecht *et al.*, 1993; Keenan *et al.*, 1992 and 1994a,b; Keenan and Soper, 1995).

While both genetic and environmental factors are involved, it is clear that poor survival in laboratory rats closely correlates with AL overfeeding and can be prevented by simple moderate dietary (calorific) restriction as practised in the MRL studies and the Biosure Study. MRL has undertaken additional studies at even a more moderate level of DR (approximately 25% DR of MRL's high AL levels) with 1400 SD rats to demonstrate that this moderate level of restriction will also improve survival through the benefits of reducing the onset and severity of spontaneous disease and tumours associated with overfeeding.

The data from the 1200 rat Biosure Study and the studies from Merck Research Laboratories are consistent with the observations of others over the past four decades that reducing energy intake by moderate caloric restriction will increase lifespan, retard senescence and delay or prevent the appearance of age-related diseases and spontaneous tumours. Although the multiple mechanisms underlying these effects are not completely understood, data from these studies are consistent with the most widely held scientific

hypotheses supporting the observation that caloric restriction acts by modulating the characteristics but not the rate of fuel use in such a fashion as to prevent long-term damage of such fuel use from oxidative damage or glycation while preserving protection mechanisms against such damage (Gumprecht *et al.*, 1993; Keenan *et al.*, 1992 and 1994a,b; Keenan and Soper, 1995). In contrast, present methods of overfeeding rodents AL clearly accelerated these ageing processes and resulted in lower survival and the early onset and increased severity of age-related degenerative disease and diet-related endocrine tumours. These events confound the interpretation of long-term carcinogenicity bioassays. The effect of moderate DR results in the animals being exposed to test compounds for a significantly longer period of time while reducing background degenerative disease and tumours that may interfere with interpretation of compound-related effects. Therefore, this moderate method of dietary control will improve the sensitivity of the bioassay to detect compound-specific toxicity and carcinogenicity. The results presented in the Biosure Study are consistent with those of MRL and many other laboratories throughout the world and point out the uncontrolled nature of AL overfeeding and demonstrate clearly that moderate DR does not adversely affect the rat's health and thus improves the carcinogenicity bioassay as a model for the evaluation of human safety.

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