The Problem of Pseudocarcinogenicity in Rodent Bioassays

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OVERVIEW

Pseudocarcinogenicity is defined as the enhancement of tumor risk by a nongenotoxic mechanism in physiologically abnormal animals. In the case of rats, severe endocrine disturbance secondary to overfeeding is the commonest cause of physiological abnormality. Pseudoanticarcinogenicity is the counterpart of pseudocarcinogenicity. The literature abounds with, and is seriously confused by, examples of these phenomena, which could be largely avoided by sensible choice of animal strain and intelligent animal husbandry. The fact that pseudocarcinogenic effects are reported alongside true carcinogenic effects while pseudoanticarcinogenic effects are usually ignored not only adds to the confusion, but also introduces serious bias into the overall picture.

INTRODUCTION

It is now reasonable to subdivide carcinogens into two categories: genotoxic and nongenotoxic. However, we need a term to describe apparent effects on tumor incidence that are neither examples of genotoxic carcinogenicity nor examples of nongenotoxic carcinogenicity but which are, in effect, laboratory artifacts. To fill this need, I have proposed the term pseudocarcinogenicity (Roe 1983), which applies to the enhancement of tumor risk by a nongenotoxic mechanism in physiologically abnormal animals but not in physiologically normal animals.

It is not intended that pseudocarcinogenicity should apply to apparent effects which, in reality, are due to the statistical mismanagement of data, e.g., failure to age-standardize data or failure to allow for the fact that the expectation of encountering statistically significant differences apparently attributable to treatment increases with the number of comparisons made. Nor is it intended that the term should apply to a situation in which animals are inadvertently exposed to carcinogens.

The commonest source of both pseudocarcinogenic and pseudoanticarcinogenic phenomena is overfeeding. In rats, overfeeding profoundly influences hormonal status and increases the incidence of hormonally mediated neoplasia. Effects of overfeeding on other forms of neoplasia are less evident in the rat. In mice, overfeeding increases the incidences of lung, liver, and lymphoreticular neoplasia by mechanisms about which we can at present only speculate.

Banbury Report 25: Nongenotoxic Mechanisms in Carcinogenesis

ANIMAL MODEL

A rat, or mouse, is a highly complex, intricate, and integrated living system that has taken millions of years to evolve. It is not simply a list of protocol tissues to be checked by a Quality Assurance officer. Animals are subject to a myriad of variables, both genetic and environmental. The experimentalist must strive to avoid interference by these variables and must not imagine that good experimental design is proof against possible bias from such interference. This is particularly true if animals are obtained from a breeder according to requirements specified only in terms of strain, sex, and weight. Littermates vary in birth weight and in growth rate before weaning, and birth weight and growth before weaning vary with litter size. Thus, a batch of young animals of similar body weight may be of different ages and have different expectations of maximum achievable weight. It is normal for an experienced breeder to cull runts and to discard young animals with obvious abnormalities, such as hydrocephalus or other birth defects, that have not already been rejected by the dam. However, animals with inobvious abnormalities find their way into long-term studies. Bias from undetected variation between animals can be largely obviated by a proper system of randomization of animals between groups. But let no one imagine that there is no inhomogeneity between animals of the same sex and weight even if they are derived from the same inbred strain.

EFFECTS OF OVERFEEDING IN RATS

Historically, the current practice of supplying animals with overnutritious food ad libitum for 24 hours each day has several roots. First, nutritionists have led experimentalists to believe that maximum growth is a hallmark of optimal nutrition. Arguably, this may be true for those responsible for fattening turkeys for Thanksgiving Day, but as pointed out by Berg and Simms (1960), it certainly is not true for rats and mice in untreated control groups in long-term toxicity/carcinogenicity tests. Second, in the bad old days when animal houses were left unattended on weekends and public holidays, it was obviously convenient to supply animals with an excess of food. Third, in the eyes of animal lovers, to deprive animals of food savors of cruelty and is therefore something to be avoided. Today, no animal house is left unattended for as long as 24 hours, so there is no need to supply excessive amounts of food. Furthermore, it is now clear that it is more inhumane to allow animals to become obscenely obese, and to predispose them to renal disease, endocrine disturbances, cancer, and an early grave, than to control what, when, and how much they eat. Three aspects of the effects of overfeeding merit separate attention: (1) effects on survival, progressive nephropathy, and other nonneoplastic diseases in rats, (2) effects on risk of tumor development, and (3) effects on hormonal status.

OVERFEEDING AS A CAUSE OF NEPHROPATHY AND OTHER NONNEOPLASTIC DISEASES IN THE RAT

Berg (1960) and Berg and Simms (1960) were among the first to demonstrate the role of caloric intake in the etiology of three particular nonneoplastic life-shortening diseases in the rat: chronic progressive glomerulonephritis, polyarteritis, and myocardial degeneration. They found that diet restriction from the time of weaning to 67% or 54% of the food intake of rats fed ad libitum greatly reduced the age-standardized incidence of these three increasingly debilitating and fatal diseases (see Table 1). A quarter of a century later, in the course of a routine carcinogenicity study involving Sprague-Dawley rats fed ad libitum on a standard laboratory chow, I observed in the male control group a 67% incidence of severe glomerulonephritis and an 83% incidence of chronic degenerative myocarditis. The animals had simply been fed ad libitum on a standard diet. Parathyroid hyperplasia secondary to nephropathy was evident in two thirds of the animals and, in half of these, metastatic calcification of the aorta and other tissues had occurred.

Bras and Ross (1964) concluded the following from a study involving 1000 male Sprague-Dawley rats: "A remarkable reduction in the prevalence of progressive glomerulonephrosis (PGN) was found in those experimental groups which were restricted in their intakes of protein, of carbohydrates, or of calories. The most beneficial effects were obtained in those groups whose carbohydrate intake and concomitant calorie intake was reduced regardless of the level of protein intake. The

Table 1
Effect of Food Intake on Survival and Incidence of Glomerulonephritis, Polyarteritis, and Myocardial Degeneration

	Days	Ad libitum %	67% of Ad libitum	54% of Ad libitum
Males				
deaths	0–800	52	13	19
G	0–800	97	0	7
	800+	100	36	0
P	0–800	83	0	0
	800+	63	17	3
MD	0–800	69	17	0
	800+	96	29	3
Females				
deaths	0–800	6	4	5
G	0–800	69	0	0
	800+	57	0	0

Data from Berg and Simms (1960).
Abbreviations: (G) glomerulonephritis; (P) polyarteritis; (MD) myocardial degeneration.

Table 2
Effect of Overnutrition on Survival and Renal Disease in Rats

	Sex	24 hr access/day	6.5 hr access/day
Survival to 2 years	male	8/20	18/20
	female	14/20	16/20
Incidence of moderate or severe nephropathy	male	13/20	1/20
	female	12/20	0/20

Data from Harleman et al. (1984).

greatest prevalence as well as the earliest appearance of PGN was found in those rats fed a commercial diet (Purina Chow) ad libitum.''

In the literature there are many examples of treatment-related and dose-related reductions in incidence/severity of chronic progressive nephropathy in rats. Indeed, one should expect to see such reductions wherever reduced body weight gain is a feature of response. It is somewhat ironic that by poisoning animals with high doses of test substances, we end up rendering them healthier than untreated controls!

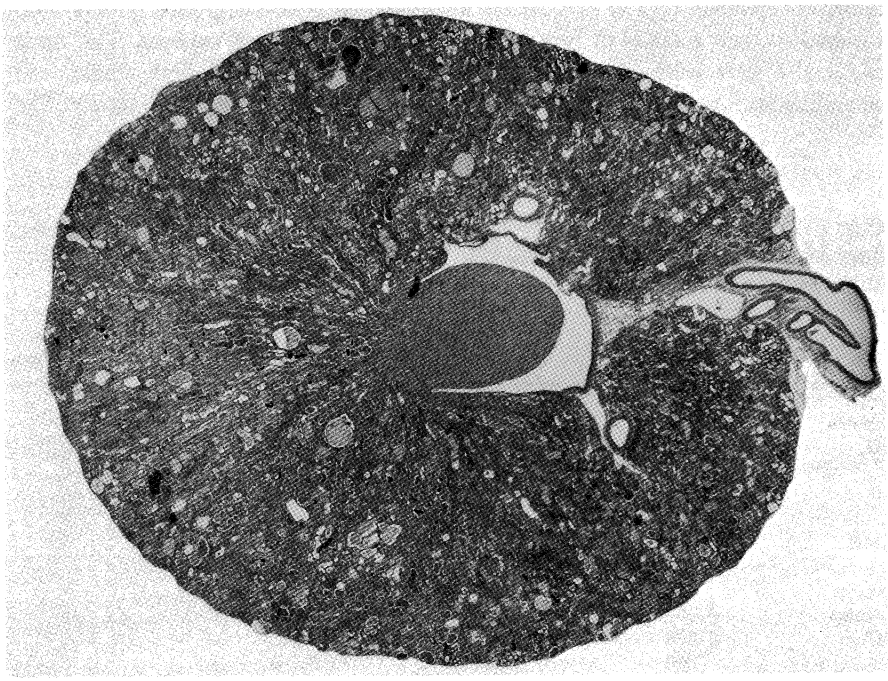

Figure 1
Kidney of a 25-month-old male Wistar rat given free access to a standard laboratory diet for 24 hr/day. There is gross enlargement because of advanced chronic glomerulonephritis.

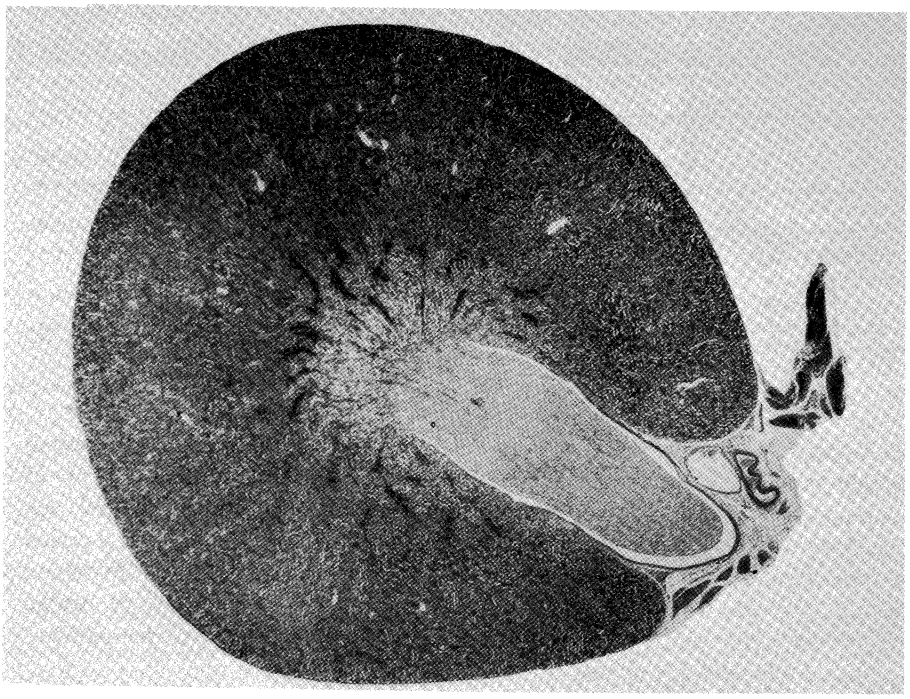

Figure 2
Kidney of a 25-month-old male Wistar rat given free access to the same standard diet (see Fig. 1) but for only 6.5 hr/day. It is histologically normal.

Recently, we (Harleman et al. 1984) reported that the survival of rats can be improved and the incidence of chronic renal disease dramatically reduced by the simple stratagem of reducing the number of hours per day during which they have free access to food from 24 to 6.5 (see Table 2). Figures 1 and 2 illustrate the appearance of the kidneys from rats fed 24 hours/day and 6.5 hour/day, respectively. In the same small study, we witnessed a significantly lower incidence of testicular arteriolitis in the 6.5 hour/day group.

OVERFEEDING IN RELATION TO NEOPLASIA

Many investigators have reported a direct association between food intake and tumor risk in laboratory rodents. In the rat study of Berg and Simms (1960) referred to above, there was a marked reduction in the incidence of benign tumors, particularly in females, but no effect on the incidence of malignant tumors (see Table 3). Tucker (1979) reported an even more striking effect of 20% diet restriction on tumor incidence in rats despite improved survival (see Table 4). In a survey of

Table 3
Effect of Food Intake on Tumor Incidence in Rats Surviving for 25 Months or More

	Ad libitum (%)	67% of Ad libitum	54% of Ad libitum	Trend
Males				
benign/malignant tumor	58	36	26	$p < 0.01$
malignant tumor	0	2	5	n.s.
Females				
benign/malignant tumor	43	14	12	$p < 0.1$
malignant tumor	2	5	0	n.s.

Data from Berg and Simms (1960). n.s. indicates not significant.

25 2-year cancer bioassay studies in F344 rats, Haseman (1983) found that reduced weight gain during the studies was associated with reduced incidences not only of pituitary and mammary tumors, but also of adrenal medullary, thyroid C-cell, and pancreatic islet-cell tumors (see Table 5). Ross and Bras (1971) found that a period of restriction in food intake early in life permanently influenced the growth pattern of rats and was associated with a decreased risk of development of benign connective tissue tumors and tumors of epithelial tissue, particularly pituitary adenomas and islet-cell tumors of the pancreas.

Clearly, the predominant pattern in rats is that overfeeding predisposes them to

Table 4
Effect of 20% Food Restriction on Percentage of Survival to 2 Years and Percentage of Tumor Incidence in Rats

	Ad libitum (%)	20% Restricted
Males		
survival to 2 years	72	88
one or more tumors	66	24[a]
>1 tumor	22	2[b]
pituitary	32	0[a]
dermis	12	2[c]
Females		
survival to 2 years	68	90
one or more tumors	82	57[b]
>1 tumor	26	10[c]
pituitary	66	39[b]
mammary benign or malignant	34	6[a]
malignant	12	2[c]

Data from Tucker (1979).
[a] $p < 0.001$.
[b] $p < 0.01$.
[c] $p < 0.05$.

Table 5
Relation between Low Weight Gain and Tumor Incidence in 25 Carcinogenicity Tests on F344 Rats

Site	Incidence
Males	
pituitary	down
thyroid (C-cell)	down
adrenal medulla	down
pancreas (islet-cell)	down
monocytic leukemia	up
Females	
pituitary	down
mammary (fibroadenoma)	down
monocytic leukemia	up

Data from Haseman (1983).

endocrine and mammary tumors. In contrast, overfeeding predisposes mice toincreased incidence of a wide variety of tumors, but mainly of a nonendocrine origin. Both Conybeare (1980) and Tucker (1979) found the biggest effects to be on tumors of the lung, liver, and lymphoreticular system (Table 6). An important feature of Conybeare's study was the significant difference he saw between ad-libitum-fed and diet-restricted mice of both sexes in the overall incidence of malignant tumors.

Table 6
Effect of Overfeeding on Percentage of Tumor Incidence in a Mouse Study of 18 Months Duration

Tumor site	Ad libitum	75% of Ad libitum
Males (160 per group)		
any tumor at any site	44	22.5[a]
any malignant tumor	11	4[b]
lung	19	12[c]
liver	29	7.5[a]
lymphoreticular	2.5	0.6
other	5	2.5
Females (160 per group)		
any tumor at any site	31	11[c]
any malignant tumor	14	4[c]
lung	15	5[c]
liver	4	0.6[b]
lymphoreticular	7	2.5[b]
other	7.5	2.5[b]

Data from Conybeare (1980).
[a] $p < 0.001$.
[b] $p < 0.05$.
[c] $p < 0.01$.

EFFECT OF OVERNUTRITION ON HORMONAL STATUS

The pattern of enhancement of endocrine and mammary tumor incidence in overfed rats is clearly associated with disturbance of hormonal status. Most of the pituitary tumors that arise in rats fed ad libitum are prolactinomas, and the high circulating levels of prolactin to which these tumors give rise increases the risk of development of mammary tumors in rats of both sexes.

A recent study by G. Conybeare (pers. comm.) has provided important information about the influence of overfeeding on the sex hormone status of female rats. He found that female rats given free access to food for 24 hours each day reached sexual maturity a week or so earlier than females given free access to food for only 6 hours each day. However, by 1 year of age, most of the animals fed ad libitum were cycling irregularly and subfertile while most of the restricted animals were still normal in these respects (see Table 7). In the normally cycling young female rat, serum prolactin levels range from 20 to 80 ng/ml, according to the stage of the cycle. In the irregularly cycling 1-year-old ad-libitum-fed females, Conybeare found higher serum prolactin levels ranging over 310 ng/ml. But even this latter level is modest compared to the levels of over 1000 ng/ml that may occur in older ad-libitum-fed females with hyperplasia or neoplasia of prolactin-producing cells in the pituitary.

The influence of food intake on other hormones awaits further study. Disturbances of growth hormone, insulin, calcitonin, and the hormones produced by the adrenal medulla almost certainly occur in association with the increased incidences of tumors at sites where these hormones are produced in overfed rats. However, published data are generally lacking.

Table 7
Effects of Overfeeding on Onset of Sexual Maturity, Regularity of Estrus Cycling, and Reproductive Performance in Female Wistar Rats (Ten per Group)

	Ad libitum	80% of Ad libitum
Opening of vagina		4 days later than in ad libitum
Success of mating at 8 weeks of age (no. of litters)	10	7
Success of mating at 10 weeks of age (no. of litters)	—	10
Cycling irregularly at 12 months of age (no. of litters)	8	2
Success of mating at 12 months of age (no. of litters)	2	6
Mean litter size of successfully mated females	2	5
Total offspring from mating at 1 year	4	30

Data from G. Conybeare (pers. comm.)

DO HIGH LEVELS OF CIRCULATING PROLACTIN PREDISPOSE TO NEPHROPATHY IN RATS?

The results of a 2-year feeding study on bromocriptine, which blocks prolactin release, revealed a number of dose-related beneficial effects not only on the incidences of prolactin-associated pituitary and mammary tumors, but also on the incidences of nephropathy and polyarteritis nodosa (see Table 8) (Richardson et al. 1983). It was suggested (Richardson and Luginbuehl 1976) that excessively high levels of circulating prolactin adversely affect the rat kidney directly. It was also suggested that the reduction in prolactin levels caused by bromocriptine permit estrogen dominance and that this explains the treatment-related increase in inflammatory, hyperplastic, metaplastic, and neoplastic uterine changes that they saw. Richardson et al. (1983) further suggested that the reduction in adrenocortical tumors in males is a consequence of prolactin suppression. In support of this, they point out that prolactin has been found to support the growth of experimentally induced tumors of this kind (Thomson et al. 1973).

INCREASED CALCIUM ABSORPTION AS A RISK FACTOR FOR PHEOCHROMOCYTOMA IN THE RAT

In a recent review of this topic, we (Roe and Bär 1985) sought to bring together scattered data relating increased calcium absorption with increased incidence of

Table 8
Effects of Bromocriptine in Rats (50/sex/group)

	% Incidence per dose (mg/kg/day)			
	0	1.8	9.9	44.5
Males				
survival to 2 years	28	44	58[a]	62[a]
moderate/severe nephropathy	84	62[b]	62[b]	44[c]
polyarteritis nodosa	50	16[c]	32[b]	24[a]
tumor-bearing rats	56	60	56	38
adrenal cortical tumors	38	24	28	6[a]
Females				
survival to 2 years	36	50	54	36
moderate/severe nephropathy	16	8	0[a]	0[a]
polyarteritis nodosa	18	4[b]	6	0[a]
tumor-bearing rats	88	58[a]	52[a]	40[a]
mammary tumors	80	30[a]	20[a]	16[a]
estrogen-associated endometrial changes	10	70[c]	82[c]	84[c]
uterine tumors	0	4	8[a]	9[a]

Data from Richardson et al. (1983).
[a] $p < 0.01$.
[b] $p < 0.05$.
[c] $p < 0.001$.

adrenal medullary hyperplasia and neoplasia in the rat. This association is seen when rats, but not mice, are fed on diets containing high levels (e.g., 10% or more) of lactose or various polyols, including sorbitol, mannitol, lactitol, and xylitol. With these substances, there is evidence of both increased calcium absorption from the gut and increased urinary output of calcium. Secondary to the latter, one observes pelvic, and sometimes other forms of, nephrocalcinosis. Brion and Dupuis (1980) reported that the adrenal medullary hypofunctioning secondary to hypocalcemia associated with vitamin D deficiency can be corrected by the administration of lactose in the diet. Even in rats deficient in vitamin D, dietary lactose can increase calcium absorption sufficiently to bring serum calcium levels into the physiological range, which is necessary for the normal functioning of the adrenal medulla. We suggest that excessive calcium absorption can give rise in the rat to hypercalcemia and that this predisposes them to adrenal medullary hyperfunctioning as evidenced histologically by hyperplasia and neoplasia.

An important cause of hypercalcemia in the rat is parathyroid hyperplasia secondary to severe nephropathy. Thus, we were not surprised to find (see Fig. 3) a highly significant ($p < 0.01$) correlation between metastatic calcification (secondary to hypercalcemia) and adrenal medullary hyperplasia/neoplasia in the carcinogenicity study referred to earlier.

Gilbert et al. (1958) dramatically brought down the incidence of adrenal medullary tumors in ad-libitum-fed rats of both sexes by reducing the level of carbohydrate in the diet (Table 9). This effect was probably mediated by reduced calcium absorption, since calcium is absorbed from the gut lumen along with monosaccharides.

		Adrenal medullary hyperplasia/neoplasia	
		-	+
Metastatic calcification (Aorta, Kidney, lung, etc.)	-	55	26
	+	14	24

Significance of positive association

$P = 0.01$

Figure 3

Correlation between hypercalcemia, as indicated by histologically evident metastatic calcification, and incidence of adrenal medullary hyperplasia and/or neoplasia among 119 male Wistar rats that survived for 64 weeks or longer in a routine oral carcinogenicity test on a nongenotoxic agent.

Table 9
Effect on Dietary Composition on Incidence of Pheochromocytoma in Rats Fed Ad Libitum

Diet (%)			Lifetime incidence of pheochromocytomas	
carbohydrate	protein	fat	male	female
64.4	15.2	11.3	63	47
3.6	81.6	10.2	13	15

Data from Gilbert et al. (1958).

In a survey of 25 cancer bioassay feeding studies in F344 rats, Haseman (1983) found that the incidence of tumors of the adrenal medulla in males was lower in animals that put on less weight than in those that put on more weight. Also, Berg and Simms (1960) reported that diet restriction reduced the incidence of pheochromocytomas in rats. It is not at present clear whether overfeeding predisposes directly to adrenal medullary proliferation disease in rats or whether the effect is mediated by high calcium absorption under conditions of overfeeding. I suspect that both factors are implicated.

In my opinion, most laboratory rat diets contain too much phosphate and too much calcium. In addition, some are deficient in magnesium. These faults render the laboratory rat prone to develop corticomedullary and pelvic nephrocalcinosis. They also stretch to the limit the ability of rats to maintain mineral homeostasis. For this reason, these rats are easily pushed into states of pathological nephrocalcinosis and adrenal proliferative disease by agents that increase calcium absorption.

CONCLUSIONS

At present, the literature is full of examples of what are almost certainly pseudocarcinogenic and pseudoanticarcinogenic phenomena that have been observed in studies in which control animals exhibit evidence of markedly abnormal physiological status. However, proof that these are not examples of true nongenotoxic carcinogenicity is usually lacking, since there are no parallel data derived from tests involving physiologically normal animals. Pending such proof, some may feel that pseudocarcinogenicity is simply a concept and not an actuality. If so, then the sooner we upgrade it from the status of concept to accepted fact the better.

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COMMENTS

TENNANT: In terms of the implication of the observations, would it not tend to mitigate against the classification of the substance X or Y as a carcinogen where the background frequency is increased? Just on a statistical basis the higher the spontaneous frequency of tumors in the control population, the

lower the probability that the chemical would be identified as a carcinogen simply because of the magnitude of effect that one has to see in order to statistically resolve between those. So, overall, one group of people would argue that in using the current dietary regimen we would be underidentifying substances as carcinogens because of the background statistical problem associated with it.

ROE: I expect you are right, but we haven't got enough data to support or refute that argument. However, that isn't really what I am worried about. Irrespective of the effect of background tumor incidence on the sensitivity of animal tests, surely it is just plain stupid to use as a model animals that are endocrinologically completely messed up. Methods are becoming available whereby it is possible to measure hormone levels repeatedly in individual living rats. Using such a technique for prolactin, we know that, from the age of 6 months onward, prolactin levels begin to rise far above the normal range. The main difficulty at present is that there are not enough comparative data for known carcinogens tested in diet-restricted and nonrestricted animals. The few data we have suggest that true carcinogenic effects are not missed if diet restriction is practiced. Diet restriction may reduce the age-standardized risk of tumor development, but the significance of the difference between the responses of treated and control animals may nevertheless have been increased because of the lower intensity of background noise.

TENNANT: I agree. What I was really speaking to was in terms of the overall issue of hazard identification. Of the last 75 substances that have been tested under the aegis of the National Toxicology Program, there has been, I believe, only one that has been classified as carcinogenic on the basis of endocrine tumors alone. I agree with you. On an individual chemical basis, you are absolutely right, in particular if you want to study the effects of that chemical. But, in terms of the overall use of these animals in their current state. . . .

ROE: The regulatory viewpoint is a very narrow one. Our main concern should be with the impact pseudocarcinogenic data have when they find their way to scientific data bases such as the data base provided by the IARC monographs. The consequences of this are not only serious with respect to the substance tested but also because chemically analogous substances fall under the same cloud.

TENNANT: That is the point I was speaking to. I can't answer for the IARC data base. The summary of the National Toxicology Program results released (related to a spontaneous high level of endocrine tumors in the rodent) have, with one exception, not contaminated the literature by identifying substances as carcinogenic.

ROE: Oh, yes they have. They have done it for a different reason.

NEWBERNE: Perhaps it is fortuitous that the National Toxicology Program uses the Fischer rat, which doesn't really get very fat. If you look at other strains, they all get obese if you hold them for 2 years.

ROE: I understand they get a very high incidence of renal disease, though.

NEWBERNE: Yes, they do, but I'm not sure we can correlate that directly with overfeeding in the Fischer rat. That doesn't eliminate the other causes.

ANDERSON: Masoro showed that very clearly. He restricted diet in male F344 rats and went from 80% end-stage kidney to zero, plus a great lengthening of life span.

HECK: Could you clarify one thing for me? In your slides you show the percentage ad libitum for a number of studies, but it is not clear to me what amount of food intake that represented. Does that represent any decrease in food intake?

ROE: In the studies which showed percentage of ad libitum food intake, the animals were rationed. The amount of food which the controls ate was measured. On each day during the following week, restricted animals were offered only 75% or 80% of the food consumed by the ad-libitum-fed animals during the previous week. Rats given access to food for only 6.5 hours per day ate only about 80% of the food consumed by rats fed 24 hours per day.