UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

MAY 5 1986

MEMORANDUM

OFFICE OF
PESTICIDES AND TOXIC SUBSTANCES

DATE:

SUBJECT: Review of Published Literature on Aliette (Fosteyl-Al)

TO: Henry Jacoby
PM 21
Registration Division (TS-767)

FROM: Margaret L. Jones M.L. Jones 4/29/86
Review Section III
Toxicology Branch (TS-769)

THRU: William Burnam
Acting Head
Review Section III
Toxicology Branch (TS-769)

W.L.Burnam 4.28.86

and

Theodore M. Farber, Ph.D., D.A.B.T.
Chief
Toxicology Branch (TS-769)

Chemical: Fosetyl-Al (Aliette)

Caswell No.: 12B

Data: Published article: "Enzootic and Epizootic Adrenal Medullary Proliferative Disease of Rats; Influence of Dietary Factors Which Affect Calcium Absorption" by F.J.C. Roe and A. Bar

Company: Rhone-Poulenc, Inc.

Action Requested: Review published article on Aliette which concerns the oncogenicity issue.

Conclusions: Aliette is at present being considered by the Toxicology Branch Peer Review Committee. The subject article provides interesting information about one possible mechanism of action of Aliette in tumorigenesis and this article will be considered as part of the "weight of evidence" decision. The article does not present any hard evidence to support the theory it proposes or to displace the existing body of evidence supporting the oncogenic potential of Fosetyl-Al.

Human Toxicol. (1985), 4, 27–52

Enzootic and Epizootic Adrenal Medullary Proliferative Disease of Rats: Influence of Dietary Factors which Affect Calcium Absorption

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Adrenal medullary hyperplasia and neoplasia occur both enzootically and epizootically in untreated laboratory rats. The lesions are typically chromaffin-negative and are found incidentally in animals that have died from unrelated causes or have been killed at the end of long-term toxicity/carcinogenicity tests. Urinary excretion of catecholamines is not usually increased. Environmental, particularly dietary, factors are seemingly much more important than genetic ones as determinants of the incidence of proliferative lesions. Recent observations of enhancement of adrenal medullary proliferative disease in rats by the feeding, in high dietary concentration, of certain polyols (sorbitol, mannitol, xylitol, lactitol), or of lactose, suggested that increased absorption of calcium from the gastrointestinal tract may be a risk factor. This evidence is reported and discussed in the light of other evidence linking disturbed calcium homeostasis with adrenal medullary function in the rat.

In man, adrenal medullary proliferative disease is relatively rare and there is no evidence of any relation between the hypercalcaemia associated with hyperparathyroidism and increased risk of phaeochromocytoma. Adrenal medullary proliferative disease in rats is usually seen against a background of multiple endocrine neoplasia, with the pituitary gland, the pancreatic islets and the thyroid C-cells being most commonly affected in addition to the adrenal medulla. A parallel between this situation and Sipple's disease in humans has previously been suggested.

We now stress the possible importance of three factors as determinants of enzootic and epizootic adrenal medullary proliferative disease in rats: excessive food intake, excessive dietary levels of calcium and phosphate and excessive intake of other food components, such as vitamin D and poorly absorbable carbohydrates, which predispose to increased calcium absorption.

Introduction

The purposes of this short review are, first, to draw attention to a common disease of laboratory rats which is presently posing problems in the interpretation of long-term toxicity studies on various foodstuffs, food additives and drugs and, secondly, to discuss the nature, significance and pathogenesis of several different endocrine disturbances which are seen very frequently in untreated laboratory rats. In

particular, there is an urgent need for basic research to investigate the mechanisms involved in the pathogenesis of enzootic adrenal medullary hyperplasia and neoplasia in untreated laboratory rats and the reasons why this enzootic disease, under some laboratory conditions, assumes epizootic proportions.

The suggestion is made that enhanced absorption of calcium is causally associated with increased risk of developing proliferative lesions of the adrenal medulla. If so, this would not be an isolated example of influence of inorganic minerals on endocrine tissues. Thus raised dietary levels of inorganic phosphate have been shown to predispose to parathyroid hyperplasia and neoplasia (Haase, 1978) and insufficient iodine is known to predispose to follicular tumours of the thyroid gland (Isler *et al.*, 1958)

Anatomy of the adrenal medulla

Anatomically, the adrenal medulla consists mostly of groups of anastomosing cords of ovoid cells which contain fine granules which become brown when oxidized by potassium bichromate. Because of this reaction, they are referred to us as 'chromaffin cells' or 'phaechrome cells'. The granules also stain green with ferric chloride, brown with osmic acid and yellow with iodate. Different cells contain different amounts of stainable granules, some staining deeply, some lightly and some not at all. The substances in the granules which stain are the catecholamines, adrenaline and noradrenaline. In addition to the chromaffin cells, there are a few ganglion cells. The fact that these are present in only small numbers results in their not being observed in most routine sections of the medulla.

Ultrastructural studies have shown that there are two main types of chromaffin cell which can be distinguished by the size and electron density of their content of membrane-bound cytoplasmic granules. The larger granules are thought to contain noradrenaline and the smaller ones adrenaline (Eranko, 1960). Proliferative lesions may consist of either type of cell according to DeLellis *et al.* (1973).

Nature of proliferative lesions of the adrenal medulla in man

In humans tumours of the so-called chromaffin cells of the adrenal medulla are rare. According to Symington (1969) the incidence is of the order of 0.005%, whereas Manger & Gifford (1977) refer to an incidence of 0.09% among nearly 16 000 consecutive autopsies carried out at the Mayo Clinic between 1928 and 1951. Those affected are mostly aged between 20 and 50 years, although young children are not exempt. The disease presents as vascular hypertension, typically paroxysmal in nature, and the diagnosis is usually established by the detection in the urine of the chief metabolite of both adrenaline and noradrenaline, namely, 3-methoxy-4-hydroxymandelic acid (vanillylmandelic acid, VMA). Calkins & Howard (1947) pointed out that less than a third of 176 cases of phaeochromocytoma in humans had been proved to be endocrinologically active, by the criterion of cessation of symptoms after surgical removal, and Walton's (1950) citing of their data might be taken to imply that non-functional adrenal

medullary tumours are more common than functional ones in man. This, however, does not seem to be true. According to Robinson (1980), phaeochromocytomas that are apparently asymptomatic may nevertheless be releasing excessive amounts of catecholamines as indicated by measurements of urinary excretion of metabolites. The same investigator writes 'It seems intrinsically unlikely that a phaeochromocytoma can be truly non-functional. If they exist, then they raise the question of whether such tumours have evolved from functional tumours that have in some way become spent or 'burnt'. Be that as it may, the position is that unsuspected adrenal medullary adenomas are occasionally found in the course of routine necropsy examinations, but these lesions almost always exhibit chromaffinity. Furthermore, in general, phaeochromocytomas in humans contain much higher quantities of noradrenaline, and sometimes also of adrenaline, than normal adrenal medullary cells.

Nature of proliferative lesions of the adrenal medulla in the rat

Nodular or diffuse hyperplasia precedes, and is closely associated with, increased tumour incidence and the incidence of both these changes increases steeply with age in both sexes, incidences usually being higher in males than in females.

From the limited number of published data, it appears that both enzootically occurring adrenal medullary hyperplasia and neoplasia consist usually of cells which show little or no chromaffinity. Whether there are between-strain differences in this respect is uncertain because the only sections prepared of most of these lesions are stained with haematoxylin and eosin, and this procedure makes for no clear distinction between chromaffin-positive and chromaffin-negative cells. Despite this, most pathologists use the term 'phaeochromocytoma' to describe the common adrenal medullary neoplasm of rats. In strict terms, this would seem to be a misnomer, unless the chomaffin-negative cells of which they are composed are known to possess the capacity to synthesize catecholamines (Thompson *et al.*, 1981).

In relation to functionality, Gillman *et al.* (1953), in their classic paper, describe the early hyperplastic lesion of the adrenal medulla of the rat as being essentially free of chromaffin particles. However, tumours, when they develop, may or may not contain chromaffin granules, and may or may not be associated with hypertensive disorders. This variation in appearance and behaviour, they suggest, is true for both man and rat. In qualitative terms this is undoubtedly true, but in quantitative terms it would seem to be misleading in so far as, in circumstances where adrenal medullary tumours are occurring in high incidence in rats, it is rare to find any that exhibit marked chromaffinity.

In keeping with the fact that the adrenal medullary tumours of rats are often non-functional and chromaffin-negative, it is noteworthy that in a Wistar-derived strain their presence was not accompanied by evidence of hypertension or increased urinary excretion of 3-methoxy-4-hydroxyphenylglycol (MHPG), the main metabolite of catecholamines in this species. Functional chromaffin-positive lesions were only occasionally seen (Bosland & Baer, 1984).

Distinction between hyperplasia and neoplasia of the adrenal medulla of rats

As pointed out by Strandberg (1983), there are no generally agreed criteria for distinguishing between hyperplastic foci and neoplasms. He writes 'Hyperplastic nodules may be distinguished by their diameter and by the fact that they do not compress or invade adjacent medullary or cortical tissue'. However, many pathologists would have regarded a lesion which (in his Fig. 20) Strandberg describes as a 'phaeochromocytoma' as nothing more than a large hyperplastic focus. Undoubtedly, the distinction is difficult, but we require there to be a discrete lesion with a definite edge and some evidence of compression of surrounding tissues before applying the diagnosis of neoplasia. Hollander & Snell (1976) solved the problem as far as they were concerned by simply dispensing with the term 'hyperplasia'. They wrote 'Because we have been unable to distinguish between hyperplasia, as described by Yeakel (1947) and tumour growth, we prefer to call all proliferative lesions of the chromaffin cells of the adrenal medulla phaeochromocytomas'. In our view it is unreasonable to regard small clusters of slightly basophilic cells as being equivalent to expansive lesions that compress surrounding medulla and/or cortex. Instead, we prefer to grade hyperplasia on a five-point scale which takes into account the size(s) and multiplicity of lesions, knowing that the distinction between grade 5 hyperplasia and benign neoplasia is uncertain. In experimental pathology the main objective is to distinguish between appearances in control animals and those in animals treated in different ways and not to provide an absolute diagnosis of each lesion. To achieve this objective, the disciplined application of arbitrary multigrade systems is of more value than a simple all-or-none categorization which combines together a wide range of lesions of different sizes and appearances.

In humans, most phaeochromocytomas are considered to be benign, although examples of local invasion and of distant metastasis are well documented (Manger & Gifford, 1977). The position in this respect is similar in the rat (Gillman *et al.*, 1953).

It is obvious to the practising pathologist that there is in the rat a continuous spectrum of changes from focal hyperplasia to malignancy. But this fact is unpalatable to the statistically minded regulator. What he wants the pathologist to tell him, in black and white terms, is how many rats of those at risk had hyperplastic lesions, how many had benign tumours and how many had malignant tumours. This, after all, is the philosophy that underlies the Delaney Clause. Most pathologists use their best endeavours to provide information in this form, however, they do so knowing that the distinctions are to a considerable extent arbitrary.

Characteristically, well-developed hyperplasia is manifest in the form of multiple clusters of smallish basophilic cells (Figures 1–4). Early cases may show only one or two such foci which are most often located in the juxtacortical region of the medulla. The cells are small because they have less cytoplasm than normal. The nuclei are approximately of normal size. Mitotic figures are occasionally in evidence, but not a prominent feature. Where hyperplasia is extensive, it may involve virtually the whole medulla. Where this pertains, it can be particularly

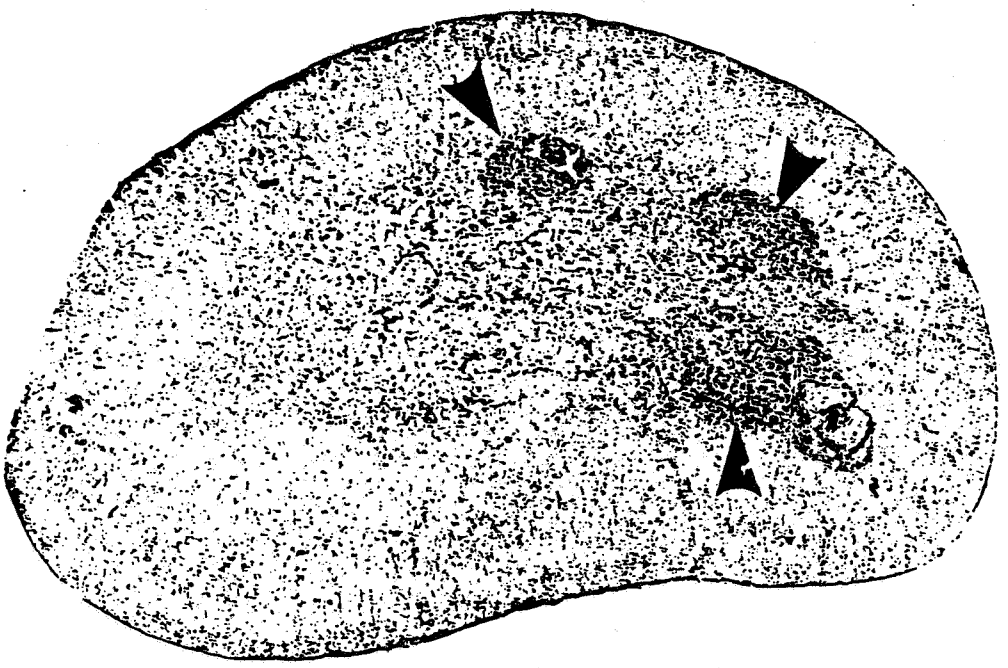

Figure 1 Typical appearance of marked adrenal medullary hyperplasia. Multiple areas of hyperplasia (arrows) situated on the periphery of the medulla (H & E × 22)

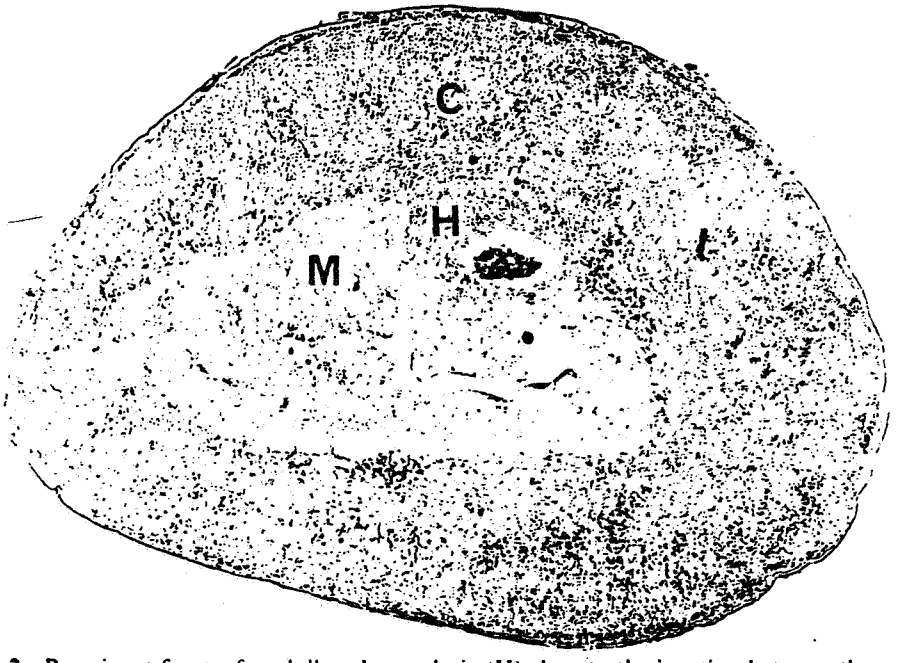

Figure 2 Prominent focus of medullary hyperplasia (H) close to the junction between the medulla (M) and the cortex (C) (H & E × 23)

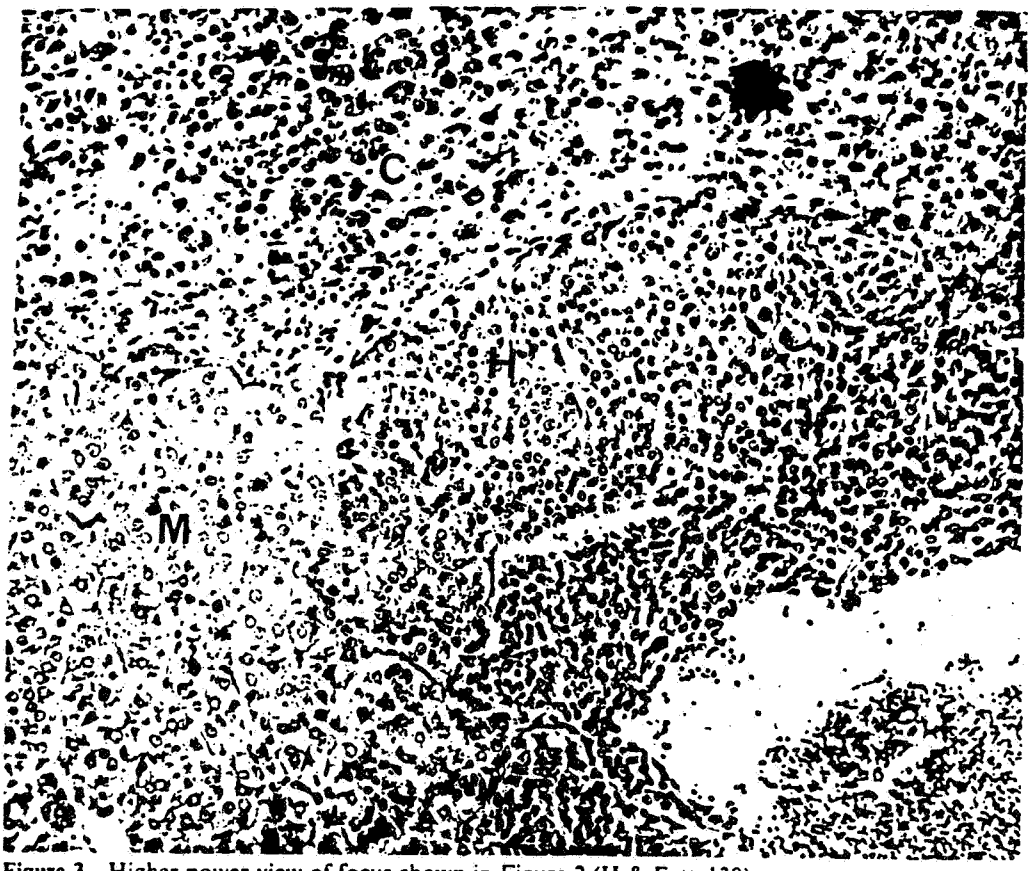

Figure 3 Higher power view of focus shown in Figure 2 (H & E × 130)

difficult to distinguish between hyperplasia and neoplasia.

Apart from the compression of surrounding structures that distinguishes benign neoplasms from hyperplastic lesions, there are often also cellular differences. For instance, the cells of adenomas tend to be larger and more basophilic than the cells of hyperplastic lesions. Also mitotic figures are sometimes more in evidence (Figures 5 & 6). Nuclear pleomorphism which is often a prominent feature of human phaeochromocytomas is not frequently encountered in the adrenal medullary tumours of rats. For a fuller description of the range of appearances of adrenal medullary neoplasms in the rat, the reader should consult Gillman *et al.* (1953). Extension by invasion through the cortex to the capsule or into blood vessels (Figures 7 & 8) is indicative of malignancy. Distant metastasis is rare.

Genetic constitution as a determinant of the incidence of proliferative lesions of the adrenal medulla in rats

Strandberg (1983) lists incidences of 'phaeochromocytoma' as reported in untreated rats of various strains and various ages. Percentages of rats affected range from nil to 85.5 in males of a Wistar strain (Gillman *et al.*, 1953) and from nil to 78.1

Figure 4 An adrenal exhibiting both a well-circumscribed benign medullary tumour (T) and irregular areas of medullary hyperplasia (H). The latter areas and the tumour are situated in the periphery of the medulla (H & E × 23)

in Copenhagen rats (Gilbert *et al.*, 1958). These values need to be regarded with caution in so far as the pathologists who reported them used different diagnostic criteria and most of them did not also report the incidence of medullary hyperplasia. Nevertheless, it is clear that the same, or ostensibly the same, strain of rat, whether inbred or outbred, may at different times and/or under different conditions exhibit very different risks of developing proliferative changes in the adrenal medulla (Table I). There is no doubt that genetic factors can play an important role. However, the variation illustrated in Table I suggests that environmental factors are likely to be even more important.

Environmental factors known to influence the incidence of proliferative lesions of the adrenal medulla in rats

Ionizing radiation and drugs

Until recently, literature concerning agents which cause adrenal medullary proliferation in rats was sparse.

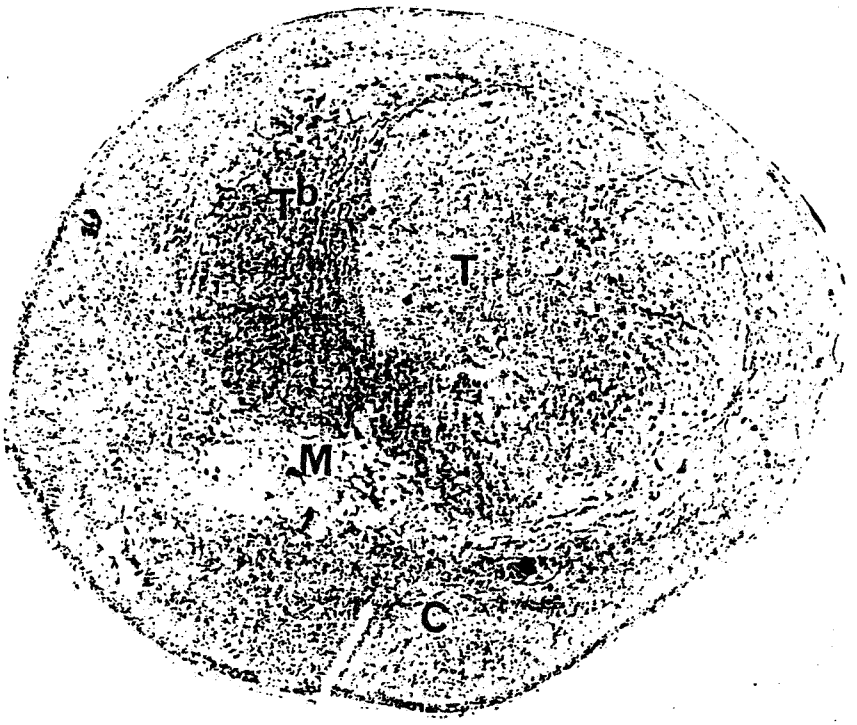

Figure 5 A well-circumscribed benign medullary tumour is displacing the remains of the normal medulla (M) to one side and bulging into the cortex (C). Part of the tumour is more basophilic (T^b) than the rest (T) (H & E × 23)

Warren *et al.* (1966) reported the induction of adrenal medullary neoplasms by ionizing radiation. One tumour so-induced was successfully transplanted serially and the recipients of the transplants developed hypertension, alteration in periadrenal brown fat, and renal and cardiac lesions secondary to the hypertension. These hormonally active transplants exhibited chromaffinity histologically. These findings of Warren *et al.* (1966), that are widely quoted, have tended to exaggerate the effect of ionizing radiation on the adrenal medulla. An article by Castanera *et al.* (1968) suggests that the tumorigenic effect of ionizing radiation on the adrenal medulla is, in reality, rather equivocal.

Nicotine, which produces its pharmacological effects by stimulating the release of catecholamines, gave rise to tumours of the adrenal medulla in rats, according to Staemmler (1935). The tumours showed less chromaffinity than normal medullary cells. More recently, Mohr *et al.* (1969) reported an increased incidence of adrenal medullary tumours in male BR (Wistar) rats in response to the subcutaneous injections of condensate.

In a small experiment Moon *et al.* (1950) investigated the effects of repeated injections of growth hormone in rats of the Long-Evans strain. This treatment was associated with the development of hyperplastic and neoplastic lesions of the

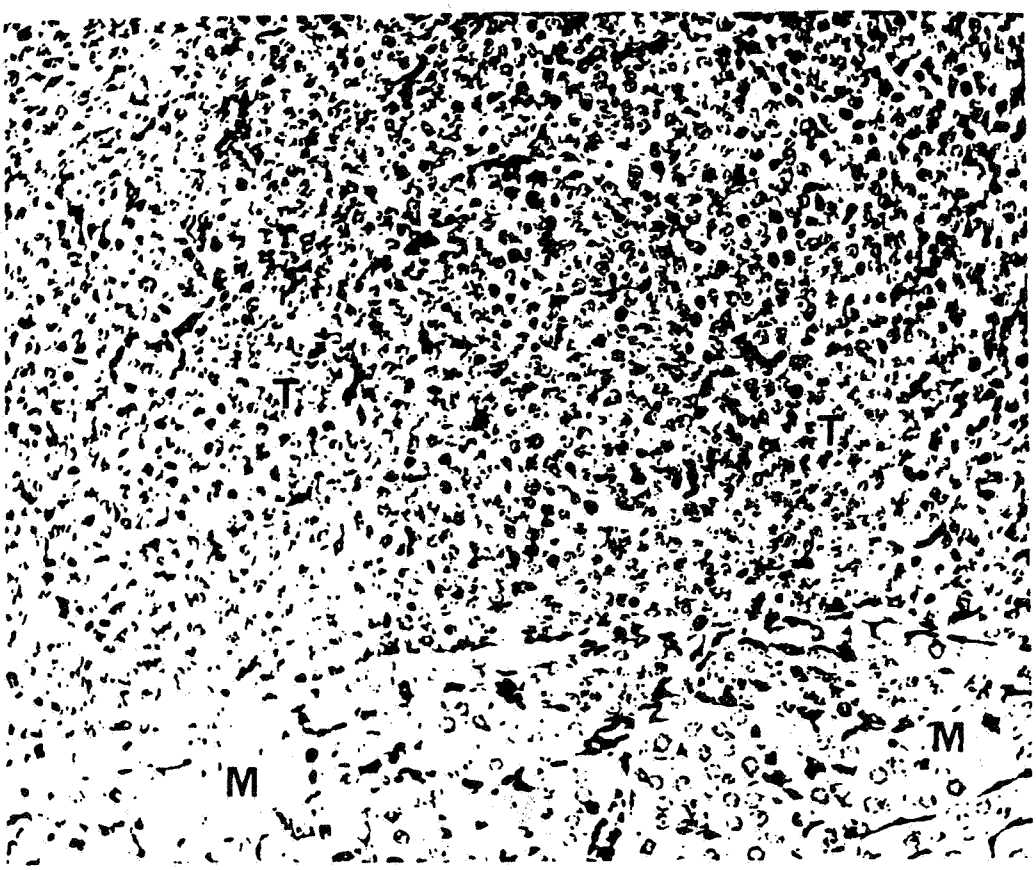

Figure 6 A higher power view of the tumour (T) shown in Figure 5. The cells are small and basophilic and well differentiated. There is no invasion of the surrounding medulla (M) (H & E × 240)

Table 1 Examples of high and low incidences of adrenal medullary tumours in untreated rats of various strains

Strain	*Age (months)*	*Males*	*Females*	*Reference*
Sprague–Dawley	1–18	0.1	0.2	Schardein *et al.*, 1968
	18–31	16	4	Thompson & Hunt, 1963
	0–26	51	8	Kociba *et al.*, 1979
Wistar-derived	11–43 (av. 33)	0	2	Boorman & Hollander, 1972
	Life-span	1	8	Burek, 1978
	13–18	82	50	Gillman *et al.*, 1953
	25–30	86	76	Gillman *et al.*, 1953
F344 (inbred)	Life-span	4	0.5	Sass *et al.*, 1975
	24–26	11	4	Goodman *et al.*, 1979
	18	45[a]	5[a]	Hollander & Snell, 1976
	13–30	37	12	Jacobs & Huseby, 1967

[a] All proliferative lesions regarded as neoplasma (see the text, p. 30)

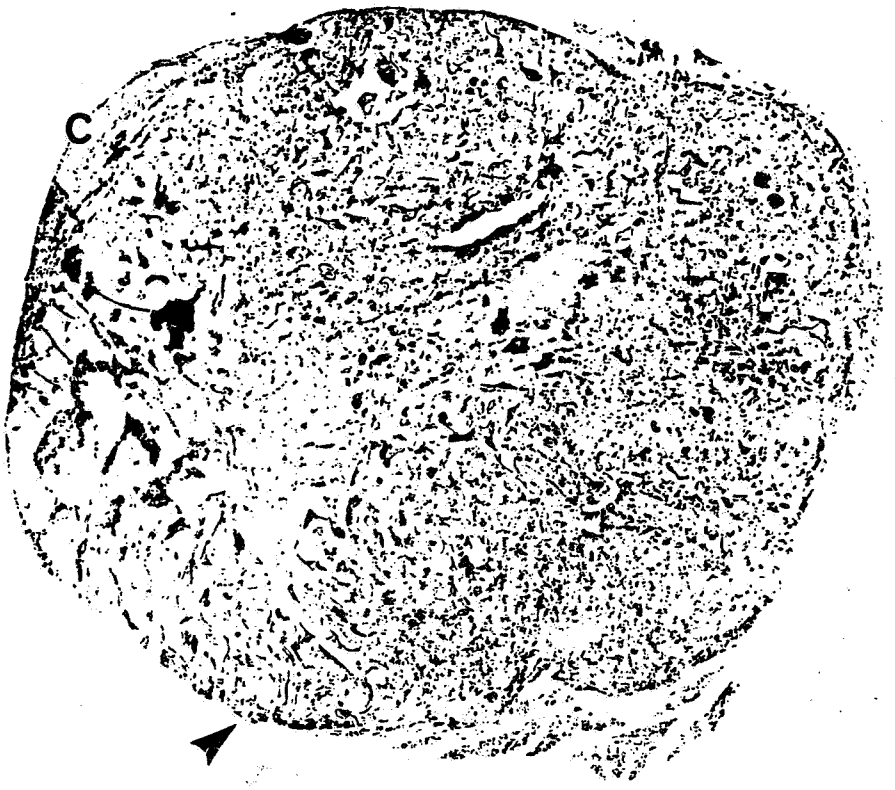

Figure 7 Virtually the whole of the greatly enlarged adrenal is replaced by a malignant medullary tumour which is extending through to the capsule (arrow). At one point (C) there is a rim of surviving cortex (H & E × 12)

adrenal medulla, but no increase in blood pressure compared with control rats. Similar results were reported by Russfield (1967).

In an early study on the response of rats to chronic exposure to thiouracil (Marine & Baumann, 1945) treatment was found to be associated with adrenal medullary hypertrophy and hyperplasia and a twofold increase in the adrenaline content of the gland. Subsequent studies in rats have neither confirmed this observation nor revealed any effect of treatment on the incidence of adrenal medullary neoplasia (IARC, 1974).

Grasso (1963) reported that short-term exposure of rats to alloxan caused hypertrophy of the adrenal medulla with the cells showing strong chromaffinity. Longer-term exposure led to adrenal medullary hyperplasia. These observations seemingly remain unconfirmed.

More recently, one of us (F.J.C.R.) has seen both hyperplasia and neoplasia of adrenal medulla of rats in response to a wide range of drugs, including various neuroleptics and a vitamin A analogue. In all these cases both kinds of lesion have been chromaffin-negative and the lesions have arisen against a background of high incidences of tumours of endocrine origin at other sites both in treated

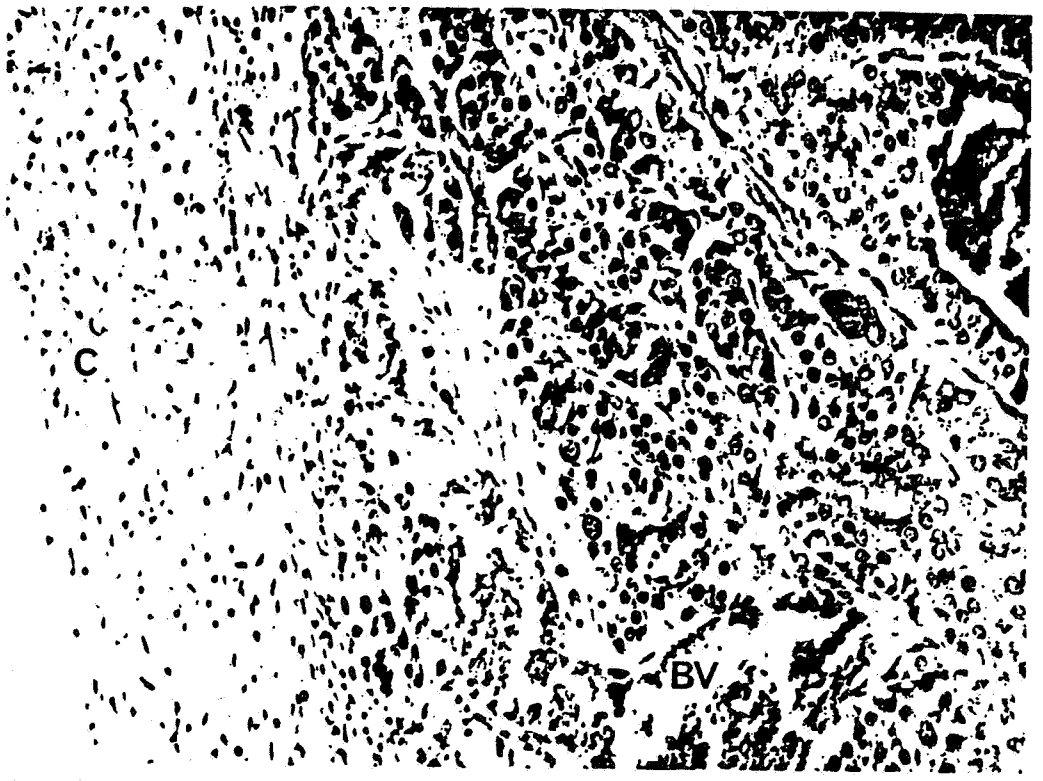

Figure 8 Higher power view of edge of lesion shown in Figure 7. There appears to be active invasion of the cortex (C) and of a blood vessel (BV) (H & E × 180)

and control animals. In some cases, as in the NCI study on reserpine (NIH, 1979), the effect of treatment on one kind of endocrine tumour (e.g. the pituitary) has been in a negative direction, whereas that on the incidence of tumours of the adrenal medulla has been one of enhancement (see Table 2).

Diet

Gilbert *et al.* (1958) were the first to note the effect of diet on the incidence of adrenal medullary neoplasia. They found, in a Wistar strain of rat, that they could reduce the incidence of adrenal medullary tumours by drastically lowering the carbohydrate content of the diet (see diet no. 4 in Table 3). Such treatment also reduced the incidence of pituitary, pancreatic islet-cell and gonadal tumours. By contrast, rationing the amount of basal diet (diet no. 1R in Table 3), although it reduced the overall risk of tumour development at all sites, had no effect on the incidence of tumours of the adrenal medulla. Unfortunately, the total number of animals on this restricted regimen was too small for any firm conclusions.

Goodman *et al.* (1980) compared the incidences of various kinds of neoplasm among Osborn–Mendel rats that had constituted the control groups in a number of carcinogenicity studies of 2 years' duration undertaken under the auspices of

Table 2 NCI study on reserpine in groups of 50 F344 rats (NIH, 1979)

	Males			*Females*		
Disease	*Control*	*Low dose*	*High dose*	*Control*	*Low dose*	*High dose*
Phaeochromocytoma	3	18**	24**	1	3	4
Pituitary adenoma	17	13	6*	21	27	28
Mammary fibroadenoma				14	18	14

Significance of difference from controls: $*P < 0.01$; $**P < 0.001$

Table 3 Effect of composition of diet on incidence of adrenal medullary neoplasm in rats of a Wistar-derived strain (from Gilbert *et al.*, 1958)

	Diet				*Life-time incidence of adrenal medullary tumours (%)*	
No.	*% carbohydrate*	*% protein*	*% fat*	*Availability*	*Male*	*Females*
1	60.4	15.2	11.3	Ad libitum	63	47
4	3.6	81.6	10.2	Ad libitum	13	15
2	78.0	12.3	8.1	Ad libitum	47	29
3	65.9	14.8	8.6	Ad libitum	41	37
1R	60.4	15.2	11.3	Rationed	46	57

the US National Cancer Institute's Carcinogenesis Testing Program. In some of these studies corn oil was used as a vehicle for the compound under test, so that the authors could compare tumour incidences in three sets of control animals: (i) completely untreated, (ii) rats given 2% corn oil mixed in with the feed and (iii) rats given corn oil by gavage (dose not stated). In males exposure to corn oil, especially if admixed with the food, was associated with a significant ($P < 0.01$) decrease in the incidence of adrenal medullary tumors (Table 4). In females corn oil, either in the diet or by gavage, was without significant effect on adrenal medullary tumour incidence. Presumably, the administration of corn oil either in the diet or by gavage results in an overall reduction in the intake of carbohydrate. If so, then these observations are consistent with those of Gilbert *et al.* (1958). However, since the data reviewed by Goodman *et al.* (1980) were not age-standardized, it is not certain that survival difference was not partly responsible for apparent differences in tumour incidence.

Berg & Simms (1960) reported that general diet restriction reduced the incidence of several kinds of neoplasm in rats, including phaeochromocytoma, but, unfortunately, these authors did not give values for individual tumour types. Others who have studied the effects of diet restriction on tumour incidence in rats (e.g. Tucker, 1979) were not in a position to see any reduction in the incidence of tumours of the adrenal medulla because the incidence of such tumours in control groups fed *ad libitum* was already very low.

In his survey of the results of 25 two-year cancer bioassay feeding studies conducted in Fischer 344 rats, Haseman (1983) found that incidences of tumours

Table 4 Influence of corn oil admixed with food or administered by gavage on incidence of 'phaeochromocytomata' in male Osborne–Mendel rats (from Goodman *et al.*, 1980)

	No. of rats	*No. with phaeochromocytoma*	*%*	*Significance of difference from control diet (P)*	
Control diet	245	12	4.9	—	
Corn oil added to diet	530	6	1.1	<0.01	<0.01
Corn oil administered by gavage	200	3	1.5	<0.1	

of the adrenal medulla and the thyroid (C-cell) in males as well as pituitary tumours in both sexes were lower in animals that put on less weight during the studies than in animals that put on more weight.

Lactose and various polyols

In various studies four different sugar alcohols, mannitol, sorbitol, xylitol and lactitol, and one sugar, lactose, have when fed in excessive doses to rats increased the incidence of adrenal medullary proliferative disease.

In a long-term (94 weeks) chronic toxicity/carcinogenicity study in Wistar rats, no effect of 1, 5 or 10% dietary mannitol was seen on the adrenal gland. However, there was a suggestive dose-related trend for benign tumours of the thymus in female rats (Saatman, 1978). Because of this finding, a second study was undertaken in which female rats of three different strains were exposed to 0, 1, 5 or 10% mannitol in the diet (Gongwer, 1978). No treatment-related excess of thymic tumours was seen in any strain. However, in one of three strains (the Fischer 344 strain) there was a significant ($P < 0.05$) excess of proliferative lesions of adrenal medulla (hyperplasia and/or neoplasia of one or both adrenals). For neither kind of lesion alone did the difference from controls achieve significance. No difference between treated and control animals was seen in rats of either the Wistar or Sprague–Dawley strains. Subsequently, mannitol was subjected to carcinogenicity testing in Fischer 344 rats and B6C3F$_1$ hybrid mice as a part of the US National Cancer Institute's National Toxicology Program (NCI, 1982). In these tests D-mannitol was given at levels of 0, 2.5 and 5.0% to animals of the two species. The results of both tests were completely negative.

Much more clear-cut relations between exposure to polyols in the diet and proliferative changes in the adrenal medulla of rats showed up in a 2-year carcinogenicity study on xylitol in which a group of rats exposed to sorbitol was included for control purposes. This study (Hunter *et al.*, 1978a) entailed the exposure of groups of 75 male and 75 female Sprague–Dawley rats to 0, 2.5, 5.0 or 20% xylitol in the diet or to 20% sorbitol or 20% sucrose, all these additions to the diets being made at the expense of starch. Because, after the first reading of the slides, there was an apparent excess of proliferative lesions of the adrenal medulla in the sorbitol and some of the xylitol groups, the adrenal gland sections were reviewed by Dr Agnes B. Russfield [in 'Two-year feeding study of xylitol,

sorbitol and sucrose in Charles Rivers (UK) rats: Adrenal Medulla', unpublished work, April 1981] who asked for some further sections to be cut. As described in her report, the incidences of medullary hyperplasia and neoplasia in the study were as shown in Table 5. For both 20% xylitol and 20% sorbitol there was, in comparison with the control and the 20% sucrose group, an excess of both medullary hyperplasia and tumours. However, the only differences which by themselves reached statistical significance were medullary hyperplasia in males given 20% sorbitol and medullary hyperplasia in females given 20% xylitol.

One of us (F.J.C.R.) has personally reviewed the adrenal sections derived from male animals in a life-span oral toxicity/carcinogenicity study in rats on lactitol. The full data from this study, which was supported by the EEC-Commission (under EEC-contracts 723/78, B35, 2935/79–56 and 271/82–45) and conducted at TNO/CIVO (Zeist, Netherlands), will be the subject of a future publication. In the meantime, we are grateful for permission to describe the findings for the adrenal medulla. It is particularly important for us to be able to do so since a group of rats exposed to 20% lactose was incorporated into the study design; it is the findings in these that serve, more than any others, to elucidate the problem of enzootic adrenal medullary disease in rats.

As far as lactitol is concerned, groups of 50 male and 50 female Wistar-derived rats were exposed *in utero*, during lactation, and subsequently up to the age of $2\frac{1}{2}$ years to diets containing 0, 2, 5 or 10% lactitol. Proliferative lesions (focal hyperplasia consisting of small basophil cells and/or of neoplasia) were present in

Table 5 Incidences of adrenal medullary hyperplasia or neoplasia in rats exposed to xylitol, sorbitol or sucrose for 79 or more weeks [Review by A. B. Russfield (unpublished work) of data reported by Hunter *et al.*, 1978 a]

% xylitol (X), sorbitol (So) or sucrose (Su) in diet	*0*	*2 (X)*	*5 (X)*	*10 (X)*	*20 (X)*	*20 (So)*	*20 (Su)*
Males							
No. of rats dying or killed at or after 79 weeks for which adequate sections were available	30	32	34	36	35	40	33
% with hyperplasia of one or both adrenal medullae	10	22	24	25	29	38**	6
% with neoplasia of one or both adrenal medullae	17	6	9	8	31	13	6
Females							
No. of rats dying or killed at or after 79 weeks for which adequate sections were available	42	38	37	41	44	46	39
% with hyperplasia of one or both adrenal medullae	5	5	24*	29**	25*	17	3
% with neoplasia of one or both adrenal medullae	2	0	5	5	14*	11	8

Significance of difference from controls: * $P < 0.05$, ** $P < 0.01$
Trend statistics for xylitol: hyperplasia, males not significant and females*; neoplasia, males* and females**

one or both adrenal medullae of 102 of the 200 males and of 44 of the 200 females. In females, there was no obvious difference between the lactitol-exposed and the control groups either in incidence of basophil foci (control = 7/50, 2% lactitol = 9/50, 5% lactitol = 8/50 and 10% lactitol = 11/50) or in incidence of medullary tumours (control = 1/50, 2% lactitol = 3/50, 5% lactitol = 2/50 and 10% lactitol = 3/50). In males, however, after age-standardization of the data by the method for incidental lesions described in the Annex to IARC Monographs Supplement 2 (IARC, 1980), there was some suggestion of enhancement by lactitol of proliferative changes in the adrenal medulla, although not of any statistically significant dose-related trend (see Table 6).

The age-standardized incidences of adrenal medullary hyperplasia and neoplasia for male rats in the parallel study on the effects of 20% lactose in the diet are shown in Table 7. Statistically significant effects ($P < 0.05$) are evident. As with lactitol, there was, in females, no obvious effect of lactose on the incidence of either of hyperplasia (reported as foci of basophil cells) (controls = 7/50, 20% lactose = 11/50) or of tumours of the adrenal medulla (controls = 1/50, 20% lactose = 2/50).

In another study (Hodgkinson *et al.*, 1982), an effect of feeding 30% lactose on the incidence of adrenal medullary hyperplasia was seen in female rats. The main aim of this study was to compare the effects of two chemically modified starches on the absorption and urinary excretion of calcium and on other aspects of mineral

Table 6 Incidence of proliferative changes in the adrenal medulla of male Wistar rats exposed to 0, 2, 5 or 10% lactitol in the diet

% lactitol in diet		0	2	5	10	Trend
No. of rats		50	50	50	50	
Hyperplasia and/or neoplasia in either medulla	O	18	30	23	31	
	E_1	23.98	26.13	25.22	26.67	
	E_2		24.78	20.97	26.00	
	P		*	NS	*	NS
Primary benign or malignant phaeochromocytoma of either medulla	O	10	18	8	18	
	E_1	12.09	13.82	13.24	14.85	
	E_2		15.06	9.02	15.17	
	P		NS	NS	NS	NS
Primary malignant phaeochromocytoma of either medulla	O	3	4	3	2	
	E_1	2.58	3.04	3.20	3.18	
	E_2		3.73	3.30	2.68	
	P		NS	NS	NS	NS
Bilateral hyperplasia and/or neoplasia of medullae	O	8	13	7	16	
	E_1	9.42	11.90	11.72	10.95	
	E_2		11.77	8.28	12.97	
	P		NS	NS	NS	NS

O = no. of rats observed with relevant condition
E_1 = no. of rats expected to have relevant condition assuming even distribution between all four groups
E_2 = no. of rats expected to have relevant condition in direct comparison with control group
P = probability based on Chi-squared: * = < 0.05; NS = not significant

metabolism. In this study four groups of 25 female Sprague–Dawley rats aged 3–4 weeks and four similarly sized groups aged 9 months were exposed to a control diet (a), diets in which 30% of the starch content (pregelatinized unmodified waxy maize starch) was replaced by chemically modified starches (b & c), or a diet in which 30% of the starch content was replaced by lactose. As expected, the lactose group showed caecal enlargement, increased urinary calcium and pelvic nephrocalcinosis and increased renal calcium as measured by chemical analysis. Although not examined at the time the study was reported, the adrenal glands of most of the rats in the study were preserved in fixative. Thus, it was possible at a later date to have sections prepared. None out of 20 rats which started on the 30% lactose diet when aged 3–4 weeks and which were killed when aged 55–56 weeks showed any evidence of proliferative change in the adrenal medulla. Nor were such changes seen in any of the 20 corresponding control rats. However, five out of 20 of the 30% lactose-fed rats that started on the diet when aged 9 months and that were killed 9 months later showed adrenal medullary hyperplasia and in three of these animals the hyperplasia was florid (Figure 2). By comparison the adrenal medullae of all the 20 corresponding control animals appeared normal.

Possible mechanisms

Adrenal medullary tumours and multiple endocrine neoplasia

The first point that needs to be discussed is whether it is reasonable to be considering enzootic and epizootic adrenal medullary proliferative disease of rats in isolation. Both in rats and in humans the occurrence of adrenal medullary tumours is sometimes associated with the occurrence of neoplasms of other endocrine organs. In humans, phaeochromocytoma is seen in association with medullary thyroid carcinoma and parathyroid hyperplasia in Sipple's syndrome

Table 7 Incidence of proliferative changes in the adrenal medulla of male Wistar rats exposed to 0 or 20% lactose in the diet[a]

% lactose in the diet		0	20
No. of rats		50	50
Hyperplasia and/or neoplasia in either medulla	O	18	34
	E_2	24.95	27.04
	P		*
Benign or malignant phaeochromocytoma in either medulla	O	10	20
	E_2	15.13	15.87
	P		*
Malignant phaeochromocytoma in either medulla	O	3	9
	E_2	5.13	6.87
	P		NS
Bilateral hyperplasia and/or neoplasia of medullae	O	8	14
	E_2	10.28	11.72
	P		NS

[a]For key to abbreviations see Table 5 footnote

(also designated as 'multiple endocrine neoplasia, type II'). This is a familial disease that is inherited as an autosomal dominant trait (Sipple, 1961; DeLellis *et al.*, 1976; Carney *et al.*, 1975, 1976).

In rats, according to Russfield (1967), adrenal medullary tumours are commonly associated with pituitary adenomas, thyroid tumours, adrenal cortical tumours, islet-cell tumours of the pancreas and interstitial cell tumours of the testis. Also, Berdjis (1960) reported the coincident occurrence of tumours of these same sites in response to irradiation in rats. Although the coincident occurrence of these and other endocrine tumours is clearly not the expression of a simple genetic fault in the rat, there are, nevertheless, good reasons for suspecting the existence of links between the susceptibilities of certain endocrine glands to develop tumours. Embryologically the C-cells of the thyroid and the adrenaline- and noradrenaline-producing cells of the adrenal medulla belong to the same part of the APUD system in that they both originate from the neural crest (Whitehead, 1980). In view of this origin, it is perhaps not surprising that phaeochromocytoma cells grown in tissue culture have been observed to respond to nerve growth factor by developing neuritic processes (Tischler & Greene, 1978). Knowledge is presently beginning to accumulate on the role of regulatory peptides in the normal functioning of the adrenal medulla and on disturbances of regulatory peptide status that may occur in association with phaeochromocytomata (Adrian *et al.*, 1983). These workers reported large numbers of neuropeptide Y (NPY)-producing cells in tumour tissue from a series of 19 patients with phaeochromocytoma. Raised circulating levels of NPY were also observed in these patients. Other regulatory peptides found in the adrenal include neurotensin, enkephalin and other so-called opiate peptides (Polak & Bloom, 1983). To date, there has been no study of rats with adrenal medullary hyperplasia or neoplasia to assess whether these changes are associated with any disturbance of regulatory peptide status. If such disturbances are found it will be important to see whether they are primary or secondary in relation to the proliferative changes. Also, new knowledge in this area may throw light on the nature of the relation between increased incidence of adrenal medullary tumours and increased incidences of tumours of other endocrine glands.

Pending a better understanding of the relation between tumour risk in different parts of the endocrine system, we can do no more than note that, in rats, high incidences of adrenal medullary proliferative lesions are rarely seen in the absence of high incidences of tumours of other endocrine sites. This is true for the 'spontaneously' occurring adrenal lesions seen in untreated control rats (see Table 8, derived from Kociba *et al.*, 1978, 1979), also for lesions seen in rats exposed to various neuroleptic drugs (Table 9 illustrates the results obtained with one such drug). In the latter case, there are three points to note. First, there is the fact that the overall incidence of various endocrine tumours is very high in the untreated controls, particularly in the females. Secondly, the significant enhancement of adrenal medullary tumours in males is matched by increases in pituitary and pancreatic islet-cell tumours in males and in mammary and pancreatic islet-cell tumours in females. Thirdly, in this particular study, treatment was associated

with a highly significant reduction in C-cell tumours of the thyroid gland. This 'two-way' effect on incidence of different kinds of endocrine tumour is, in fact, not an uncommon phenomenon. Thus, as pointed out above, reserpine that increases adrenal medullary tumours in male rats decreases pituitary tumours in the same sex (see Table 2).

Relation between calcium and adrenal medullary proliferative disease in rats

A. B. Russfield (unpublished work, 1981) was the first to suggest that 'stimulation of medullary proliferation by prolonged feeding of sugar alcohols represents a

Table 8 Percentage of rats bearing adrenal medullary and certain other endocrine neoplasms among groups of 85/86 untreated Sprague–Dawley rats observed for up to 26 months (from Kociba *et al.*, 1978, 1979)

	1978 study		*1979 study*	
	Male	*Female*	*Male*	*Female*
Adrenal				
medulla	33	8	51	8
cortex	0	10	2	7
Mammary				
fibroadenoma	0	85	1	76
adenoma			0	12
adenocarcinoma	2	9	2	8
Pancreas				
acinar	18	0	33	0
islet-cell	16	6	16	9
Pituitary	34	57	31	63
Thyroid C-cell	13	21	8	8

Table 9 Percentages of control and treated rats bearing adrenal medullary and certain other endocrine tumours in a 2-year carcinogenicity study on a particular neuroleptic drug in Wistar rats

	Males		*Females*	
	Control	*High dose*	*Control*	*High dose*
Adrenal				
medulla	15	72***	19	24
cortex	7	6	16	4
Mammary	0	13*	52	78***
Pancreas endocrine	3	42***	3	28***
Pituitary	21	67***	73	82
Thyroid				
C-cell	26	8**	15	8
follicular	1	6	10	8

* Significantly higher than controls $P < 0.05$ ** Significantly lower than controls $P < 0.01$
*** Significantly higher than controls $P < 0.001$

non-specific response of the rat endocrine system'. In view of the evidence that sugar alcohols are not mutagenic, Russfield went on to suggest that their effect on the adrenal medulla may represent an 'example of response of a rodent endocrine gland to metabolic change rather than to a chemical carcinogen'. Subsequently, this suggestion has gained support from corresponding data for lactose as mentioned above.

Several of the agents which increase the incidence of adrenal medullary proliferative disease also enhance the absorption of calcium from the gut. This fact, which is true, for example, for lactose and for the polyols generally, suggested that, at least in the case of these compounds, changes in calcium homeostasis might be implicated in the aetiology of the adrenal medullary disease.

From numerous animal studies it is known that carbohydrates, generally, and starch and certain sugars, in particular, increase the absorption of calcium from the gut (Bergeim, 1926; Greenwald & Gross, 1929). Of many sugars and sugar alcohols tested by Dupuis & Fournier (1963), lactose had the greatest effect. There have been various theories regarding the mechanism whereby lactose and other sugars enhance calcium absorption. Vaughan & Filer (1960) noted that the more poorly a sugar is absorbed the more likely it is to reach the lower intestinal tract and that the presence of sugars in the ileum facilitates the absorption of calcium. The introduction of various monosaccharides, including glucose, directly into the ileum along with calcium chloride has been shown to increase the absorption of calcium (Lengemann & Comar, 1961). Thus the effects of large oral doses of lactose or other sugars that are poorly broken down to monosaccharides or poorly absorbed may be similar to those of introducing monosaccharides directly into the ileum.

As far as lactose is concerned the effect on calcium absorption of its presence in milk is thought to be particularly beneficial to the young growing mammal, who needs to absorb plenty of calcium to build its growing bones (Dupuis & Fournier, 1963). However, the fact that it is seemingly one of the most effective of all the carbohydrates tested in this regard should not be allowed to obscure the fact that the effect is a generic one. The list of carbohydrates known to act in the same way includes galactose, xylose, arabinose, mannose, sorbose, melebiose, cellobiose, raffinose, sorbitol, dulcitol, mannitol (Dupuis & Fournier, 1963), dextrin (Bergeim, 1926; Fournier, 1959), polydextrose (P. Estes, unpublished work, 1978), roasted dextrose (Fournier, 1959), and chemically modified starches (Hodgkinson *et al.*, 1982). In other words, the effect of lactose on calcium absorption is by no means unique.

Studies on the effect of lactose on calcium absorption in man have given somewhat conflicting results. In a recent study, in 12 subjects with 'normal' lactase levels in the gut, calcium absorption was enhanced by an average of 60%, by the administration of 50 g of lactose given as a single pulse dose, whereas in seven lactase-deficient subjects, calcium absorption was decreased by an average of 20% in a similar test. In the absence of dietary lactose, the two sets of subjects absorbed in rats fed on 30% lactose, the 60% enhancement of calcium absorption seen in the lactase-competent subjects was quite small. In the two studies reported by

Hodgkinson *et al.* (1982), for instance, the enhancement of calcium absorption was in the order of 2–3.5-fold.

There are now several indications of a link between increased calcium absorption and effects on the adrenal medulla. Two features of vitamin D deficiency are hypocalcaemia and hypofunctioning of the adrenal medulla. Brion & Dupuis (1980) reported that the oral administration of lactose to vitamin D-deficient rats corrected both the hypocalcaemia and the defect in adrenal function. Calcium has long been known to be involved in the secretion of catecholamines by chromaffin cells (Houssay & Molinelli, 1928; Douglas & Rubin, 1961; Perlman & Chalfie, 1977). According to Perlman & Chalfie (1977), whose substantial review is paricularly recommended to the reader, it is generally believed that an increase in intracelluar Ca^{2+} in chromaffin cells is a sufficient stimulus for catecholamine secretion. Although most of the studies linking calcium to catecholamine release are of an *in vitro* nature, there is also some evidence of such a link from studies *in vivo* (Sowers & Barrett, 1982). These investigators found that hypercalcaemia (induced in rats by the subcutaneous injection of a suspension of Leydig-tumour cells) led to catecholamine release from the adrenal medulla and hypertension. Pawlikowski (1982) listed calcium ions along with cyclic nucleotides and prostaglandins as possible external mediators capable of stimulating both hormonal secretion and cellular proliferation in the adrenal medulla.

An experiment on the effect of calcium homeostasis on adrenal medullary function has recently been carried out by one of us (A.B.). Four groups of rats were given a diet containing 20% xylitol. Control animals were fed the same diet with starch in place of the xylitol. As indicated above, xylitol at a level of 20% enhances calcium absorption in the same way as lactose. The diets given to the control (starch) group and to one of the 20% xylitol groups contained 0.4% calcium. The other three 20% xylitol diets had 0.2, 0.1 and 0.05% calcium respectively. A comparison of the 0.4% calcium/20% xylitol diet group with the 0.4% calcium/control diet group showed significantly higher adrenal medullary levels of adrenaline and dopamine in the xylitol group. These elevated levels fell to the control values when the level of calcium in the diet was reduced to 0.1%. This study provides evidence of the involvement of calcium in the pathogenesis of adrenal medullary changes in rats.

There is one further bit of evidence that points to their being a relation between hypercalcaemia and adrenal medullary proliferative disease in rats. In the course of reading sections derived from a carcinogenicity study in Wistar rats, one of us (F.J.C.R.) encountered, in male rats, high incidences of both adrenal medullary hyperplasia/neoplasia and metastatic calcification secondary to nephropathy and parathyroid hyperplasia. The substance under test had previously given negative results for mutagenicity and clastogenicity and had no effect on the incidence of any kind of neoplasm in the study concerned. It seemed of interest to see whether there was any association between the presence of metastatic calcification and adrenal medullary proliferative disease. An analysis of the data for 119 rats that survived for 64 weeks or more gave the following picture:

		Adrenal medullary hyperplasia or neoplasia	
		−	+
Metastatic calcification	−	55	26
	+	14	24

These values indicated that animals with metastatic calcification had a 3.25 greater chance ($P = 0.01$) of developing hyperplasia and/or neoplasia of the adrenal medulla than animals that had no metastatic calcification.

An association between the occurrence of nephrocalcinosis and pathological changes in the adrenal glands was also reported by Bleyl *et al.* (1980). However, in this experiment the changes in the adrenals were described as 'hypertrophy of the zona glomerulosa', the medulla apparently remaining intact.

Lack of any relation between hypercalcaemia and adrenal medullary proliferative disease in man

By contrast with the rat, there is no evidence of any involvement of calcium in the genesis of adrenal medullary lesions in man. Thus O. M. Wrong (personal communication) reviewed the records of 700 cases of hypercalcaemia associated with primary hyperparathyroidism that came to operation at University College Hospital, London. Only one of these subjects, which was probably a case of Sipple's disease, also had a phaeochromocytoma.

A report (Serfas *et al.*, 1983) of the dramatic improvement in symptoms in a woman with a noradrenaline-secreting phaeochromocytoma when she was given the calcium-channel blocking agent, nifedipine, indicates nothing more than that the release of catecholamines from the tumour in question was calcium-dependent. It does not imply that excessive calcium was involved in the formation of the tumour.

Significance of diet-related increased incidence of adrenal medullary tumours in rats in the safety assessment of foodstuffs for man

Common sense suggests that humans are at no increased risk of developing adrenal medullary neoplasia as a consequence of their intake of particular carbohydrates. If this were not so, then adrenal medullary hyperplasia and neoplasia would not be so rare in humans as they clearly are. Also, as pointed out above, enhancement of adrenal medullary proliferative disease in rats has only been seen against a high background incidence of other forms of endocrine neoplasia. This suggests that if there is any parallelism between the two species, it is likely to be confined to there being some sort of relation between the typical laboratory rat, with its high susceptibility to various endocrine neoplasms (particularly of adrenal medulla, thyroid C-cell and pancreatic islet-cell), and the rare cases of Sipple's disease in humans.

Elsewhere, one of us (Roe, 1981) has stressed the fact that current animal husbandry procedures, which include feeding, *ad libitum*, result in overnutrition,

which predisposes to multiple endocrine disease in rats. It is questionable whether carcinogenicity experiments conducted under conditions in which a majority of animals develop multiple tumours of endocrine origin are appropriate models for predicting health risks for man. Be this as it may, it seems very likely that the current high incidences of proliferative lesions of the adrenal medulla in rats are to a large extent due to overfeeding and/or of the provision of suboptimally constituted diets. Theoretically there are three factors that may be implicated: (i) excessive intake of food associated with feeding *ad libitum*; (ii) excessive intake of calcium and phosphate (NB the average commercial diet contains two to three times more calcium and phosphorus than young rats require); (iii) excessive intake of other food components (e.g. vitamin D and poorly absorbable carbohydrates) which predispose to increased calcium absorption.

It is interesting that, although excessive nutrition increases tumour incidence in mice (Tucker, 1979; Conybeare, 1980), the adrenal medulla is not a prominent target in this species. Similarly, it is interesting that, although dietary carbohydrates such as lactose (Feron *et al.*, 1978) and xylitol (Hunter *et al.*, 1978b) enhance calcium absorption in the mouse and give rise to nephrocalcinosis, these changes are not associated with any enhancement of proliferative disease of the adrenal medulla in this species. The difference may depend on ability of the mouse to handle excessive amounts of absorbed calcium more efficiently than the rat in so far as the maintenance of blood calcium levels is concerned. In all species calcium homeostasis is remarkably efficient so that even greatly enhanced or decreased calcium absorption from the gut is reflected in no more than marginal rises in blood calcium levels. The rat, however, particularly the overfed rat (Saxton & Kimball, 1941; Berg & Simms, 1960; Bras & Ross, 1964; Yu *et al.*, 1982), is highly susceptible to a form of progressive nephropathy which predisposes to parathyroid hyperplasia and metastatic calcification. The existence of these renal changes may make it more difficult for rats that are absorbing excessive amounts of calcium from the gut to avoid albeit short but physiologically significant excursions into hypercalcaemia which constitute an immediate cause of the adrenal medullary lesions. Further studies involving the frequent monitoring of blood calcium levels would be needed to substantiate this particular speculation.

Conclusions

There is no human equivalent to the epizootic adrenal medullary proliferative disease of rats but there may be some links between Sipple's disease of man and the form of multiple endocrine neoplasia which is commonly seen in overfed rats and which is a feature of the response of rats to various neuroleptic drugs.

With the exception of ionizing radiation, none of the agents referred to in this review, which enhance the risk of adrenal medullary proliferative disease, has been shown to be genotoxic. In seeking a non-genotoxic mechanism, two factors command special attention, first, genetically determined predisposition peculiar to some strains of rat and, secondly, a disturbance of physiological status in animals which, because of overfeeding and perhaps other inadequacies in animal husbandry, are already physiologically abnormal.

The position at present therefore seems to be that the enzootic and epizootic proliferative diseases of the adrenal medulla and of other endocrine organs of rats are dependent on abnormal endocrine status. In the case of certain carbohydrates, such as lactose, xylitol, sorbitol and other sugar alcohols, the effects on the adrenal medulla may be secondary to enhancement of calcium absorption and the resulting changes in the levels of hormones involved in maintaining calcium homeostasis. Excessive levels of calcium and phosphate in standard laboratory diets and progressive nephropathy associated with overfeeding may also play significant roles by making it more difficult for animals to maintain calcium homeostasis.

Obviously, there is an urgent need to devise ways of maintaining rats into old age without their developing major, and often multiple, manifestations of endocrine disease. Avoidance of overfeeding and excessive dietary levels of calcium and phosphate would help to achieve this end.

The authors gratefully acknowledge permission from the EEC-Commission to cite data for lactitol and lactose derived from studies supported under EEC contracts 723/78–B35, 2935/79–56 and 271/82–45. They are also grateful to the various manufacturers of lactose, sorbitol, xylitol and lactitol for permission to cite hitherto unpublished toxicological data.

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(Received 16 July 1984; accepted 24 July 1984)