PYRIDOXIN DEFICIENCY - AN EXPERIMENTAL STUDY

A thesis for the degree of Doctor of Medicine(M.D.) in the University of Glasgow

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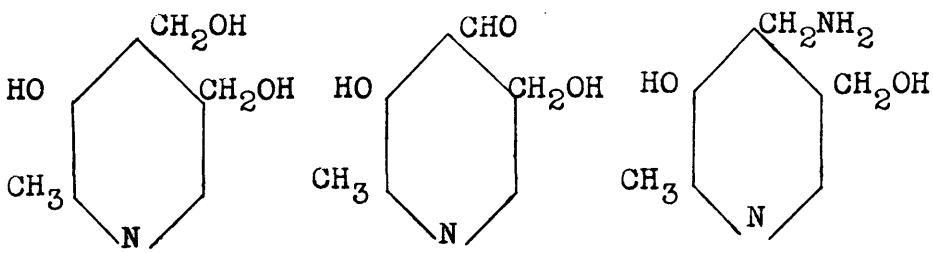
INTRODUCTION

The story of pyridoxin began in 1926 when Goldberger and Lillie reported that rats fed a diet deficient in what was regarded as the pellagra-preventing(P-P) factor developed a characteristic dermatitis. Once the multiple nature of the vitamin B complex was realised,the separate nature of Goldberger and Lillie's factor - which by this time had been variously called "factor Y"(Chick and Copping,1930),the "antidermatitis factor"(Hogan and Richardson,1936),"vitamin H" (Booher,1937),and "factor 1"(Lepkovsky,Jukes and Krause,1936)- was recognised,and Gyorgy's(1934) suggestion that it be called "vitamin B_6" was generally accepted. Vitamin B_6 was isolated as a pure crystalline organic compound in 1938,and the following year its structure was known and it was synthesized by Harris and Folkers(1939) in the United States, and by Kuhn,Westphal,Wendt and Westphal(1939) in Germany.

A shorter name than 2-methyl-3-hydroxy 4,5 dihydroxymethylpyridine was needed and biologists welcomed the suggestion of Gyorgy and Eckhardt(1939) that this new substance be called "pyridoxin". "Vitamin B_6" and "pyridoxin" are often regarded loosely as synonyms,but as two other substances - pyridoxal and pyridoxamine,an aldehyde and an amine derivative respectively of pyridoxin(Snell,1944) - possess the same biological activity in higher animals,Rabinowitz and Snell(1948) have suggested that the name "pyridoxin" be reserved for the

specific substance mentioned above,and that "vitamin B_6" should be used as a group term for pyridoxin,pyridoxal and pyridoxamine. The structure of these compounds is shown in Fig.1.



Pyridoxin Pyridoxal Pyridoxamine

Fig.1. Structure of Pyridoxin,Pyridoxal and Pyridoxamine

Pyridoxin is essential for mice,rats,chicks,dogs,swine and,possibly,monkeys; growth failure,owing to anorexia, has been reported in deficiency states in these animals. Also,in rats,pyridoxin deficiency causes dermatitis - the so-called "rat acrodynia"(Birch,Gyorgy and Harris,1935) - and, in long-standing cases,epileptiform fits(Chick,El Sadr and Worden,1940);whilst in dogs(Fouts et al.,1938) and swine (Wintrobe et al.,1943) hypochromic microcytic anaemia has been described.

Pyridoxin is important in protein metabolism. Normal tryptophan metabolism is interfered with in pyridoxin-deficient rats and metabolites such as xanthurenic acid are excreted in the urine(Lepkovsky,Koboz and Haagen-Smit,1943).

Bacteria capable of utilising members of the vitamin-B_6 complex(pyridoxin,pyridoxal and pyridoxamine) can convert them into amino acid decarboxylases. For example,pyridoxal can act as a coenzyme and catalyse the decarboxylation of amino acids such as tyrosine(Gunsalus and Bellamy,1944).

SCOPE OF THESIS

PART 1. Antibody Production in Pyridoxin-deficient Rats.

Stoerk and Eisen(1946) and Stoerk,Eisen and John(1947) reported that pyridoxin-deficient rats immunized with sheep erythrocytes developed serum-antibody(haemagglutinin) levels far below those of inanition control rats and of rats fed a complete diet ad libitum. They also reported a striking reduction in the number of fixed and circulating lymphocytes in pyridoxin-deficient rats. It seemed of interest to attempt to confirm and extend these findings,and the first part of this thesis is devoted to this work.

PART 2. Haematuria in Pyridoxin-deficient Rats.

During these experiments(to be described in Part 1) on antibody production in pyridoxin-deficient hooded rats, gross macroscopic haematuria was noted in several animals fed the pyridoxin-deficient diet but not in corresponding control rats. The second part of this thesis is concerned with a detailed study of this phenomenon.

PART 1. ANTIBODY PRODUCTION IN PYRIDOXIN-DEFICIENT RATS

As mentioned above, Stoerk and Eisen(1946) and Stoerk, Eisen and John(1947) reported that pyridoxin-deficient rats immunized with sheep erythrocytes developed serum-antibody (haemagglutinin) levels far below those of inanition control rats and of rats fed a complete diet *ad libitum*. Axelrod, Carter, McCoy and Geisinger(1947), using human erythrocytes as an antigenic stimulus, obtained similar results. It seemed of interest to attempt to confirm these findings and to use not only sheep erythrocytes but also a killed culture of a pathogenic organism, *Bacterium typhosum*. Further, Dimick and Schreffler(1939) observed "complete atrophy" of the thymus in pyridoxin-deficient rats; and as Stoerk *et al.* (1947) reported a striking reduction in the number of fixed and circulating lymphocytes in pyridoxin-deficient rats, it seemed worth studying the effect of pyridoxin deficiency on lymphoid tissue in the rat, particularly as the work of White and Dougherty(1946) indicated the importance of the lymphocyte in antibody production by virtue of the elaboration of gamma-globulin.

METHODS

Hooded Lister Institute rats(Rowett Institute strain) of both sexes were used. They were housed in individual cages with wide wire-screened bases to prevent coprophagy.

Unlimited access to water was allowed, and they were weighed daily.

DIET. The animals were weaned and maintained on the synthetic diet shown in Table 1. The B-complex vitamins (Table 1), except choline, were made up in a 'master mix' which was stored at 0-4°C. An appropriate amount of this powder was thoroughly mixed with the casein of the diet.

Table 1. *Composition of the experimental diet*

		Vitamin supplements added to casein		Other vitamin supplements	
Foodstuff	Content (%)	Vitamin	mg./100 g. diet	Supplement	Amount and method of feeding
Sucrose	73·0	*i*-Inositol	10·0	Choline chloride	15 mg./rat/day. 30 mg. in 0·1 ml. distilled water fed by pipette every alternate day
Vitamin-free casein (Glaxo Lab. Ltd.)	18·0	*p*-Aminobenzoic acid	10·0		
		Nicotinic acid	4·0		
Margarine	5·0	Ca-pantothenate	2·0		
Salt mixture (McCollum 185 +0·221 g. potassium iodide/kg.	4·0	Riboflavin	0·3	Radiostoleum (synthetic vitamin D, B.D.H. Ltd.)	5 ml./250 g. margarine
		Aneurin	0·3		
		Pyridoxin-HCl*	0·3	α-Tocopheryl acetate	2 mg./rat/week. Fed in arachis oil by pipette

* Pyridoxin-HCl was fed to control animals only.

DESIGN OF EXPERIMENT. Preliminary studies indicated that weanling rats fed the pyridoxin-deficient diet (Table 1) failed to grow as rapidly, owing to anorexia, as litter-mates receiving the same diet with pyridoxin. Any differences, e.g. in antibody titres or thymus weights, between a pyridoxin-deficient rat and a fully supplemented rat might thus be attributable not only to pyridoxin deficiency but also to inanition. Failure to make allowance for this in the design of the experiment makes it impossible or, at best, difficult to differentiate effects resulting from simple failure of the rat to grow, from effects specifically referable to lack

of the particular vitamin in question.

The preliminary experiments mentioned above indicated that "inanition controls" were essential,and two methods - paired-feeding and paired-weighing - were considered. Before discussing these methods it should be pointed out that inanition is not,of course,a specific feature of pyridoxin deficiency and occurs,for example,in deficiency of riboflavin,aneurin and pantothenic acid.

Paired-feeding consists in making another rat isocaloric with the rat fed the deficient diet by feeding this control rat the same amount of food,plus the specific vitamin,consumed by the animal fed the deficient diet.

Paired-weighing consists in mimicking in another rat the weight curve of the rat fed the deficient diet, and this is readily done by simple underfeeding. As in paired-feeding,the control rat receives the specific vitamin. This is the most exacting - and hence the most accurate - control available for nutritional experiments. Paired-feeding is not such a reliable control as it does not necessarily result in the weight curve of the paired-fed rat paralleling that of the rat fed the deficient diet. This only occurs in short-term experiments. At the end of a long-term experiment (Fig.2) with a quartet of litter-mate rats of the same sex

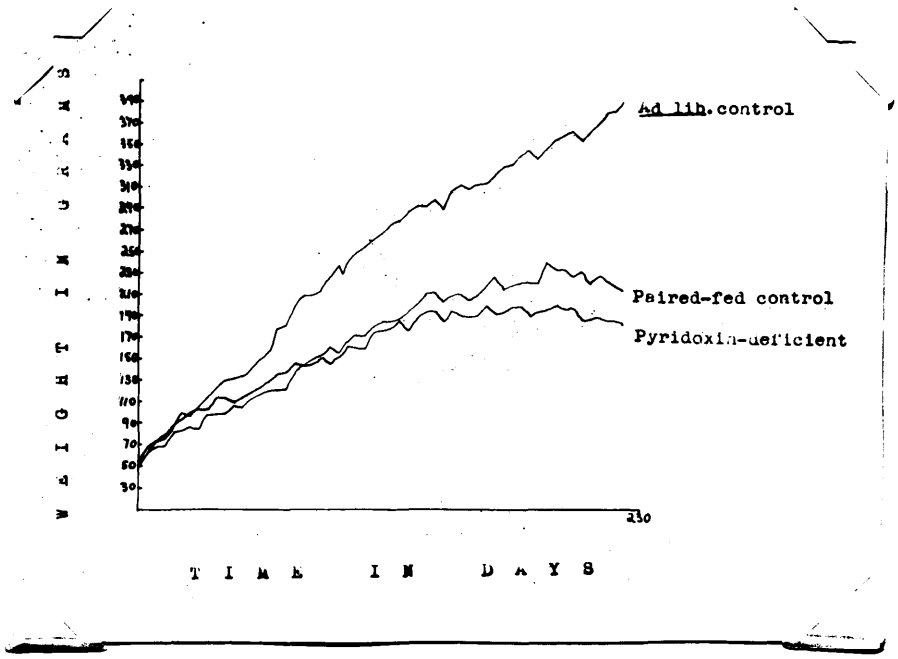


Fig. 2. Effect of paired-feeding and paired-weighing on weight curves of rats. The weight curve of the paired-weighed control follows that of the pyridoxin-deficient rat (see text).

and of the same initial weight,a difference of over 40 grams was found between the paired-fed control rat and its pyridoxin-deficient litter-mate. The growth curve of the paired-weighed rat was the same,with slight daily variations of not more than 5 grams,as the pyridoxin-deficient rat,and hence both growth curves are represented by one line in Fig.2. Presumably the paired-fed control rat,although isocaloric with the pyridoxin-deficient rat throughout the experiment, utilized its food more efficiently owing to supplementation with pyridoxin.

Throughout this work the paired-weighing technique was used,although in one experiment a few paired-fed controls were also used. Each experiment in the present series involved the use of several trios or quartets of litter-mate weanling rats of the same sex and approximately the same weight,the animals in each trio or quartet being arranged thus:

rat no.1,fed the experimental diet ad libitum with all vitamin supplements;

rat no.2,fed the diet ad lib.with all vitamin supplements except pyridoxin;

rat no.3,paired-weighed with rat no.2 but given pyridoxin;

rat no.4,if used,paired-fed with rat no.2 but given pyridoxin.

The importance of litter-mates in the design of nutritional experiments will be discussed in Part 2 with special reference to the statistical analysis of two typical experiments.

ANTIBODY EXPERIMENTS 1. Sheep Erythrocytes as Antigen

Thirty weanling rats were used in these studies, the animals being grouped in trios of litter-mates of the same sex and approximately the same weight as indicated above. On the 42nd or 43rd day of the experiment each rat received the first of three intraperitoneal injections, which were given on alternate days, of 0.5 ml. of a 5% saline suspension of sheep erythrocytes. Five days after the last injection the rats were bled from the axillary vessels under ether anaesthesia, and the haemagglutinin titre of each serum determined, twofold dilutions from 1 in 10 to 1 in 1280 being used.

2. Bacterium typhosum as Antigen

Twenty-one weanling rats were arranged in trios as indicated above. In three cases an additional inanition control(paired-fed) was used. On the 43rd day of the experiment each animal received an intraperitoneal injection of 2.0 ml. of a formolized suspension('H'antigen) of Bact.typhosum(strain Kasauli). The serums were set up in dilutions of 1 in 25, 1 in 50, 1 in 125, 1 in 250, 1 in 500,

1 in 1000,1 in 2500 and 1 in 5000,incubated for 2 hours at 37°C.,and allowed to stand overnight at room temperature before the agglutination titres were read.

EFFECT OF PYRIDOXIN-DEFICIENCY ON LYMPHOID TISSUE

Eighteen trios(twelve male,six female) of weanling rats,arranged as above,were used. Representative trios were killed after they had received the diet for periods ranging from 14 to 70 days. The animals were anaesthetized with ether,and blood was taken from the left axillary vein for total and differential white cell counts. The animals were then gassed and the thymus,spleen,submaxillary lymph nodes, and both adrenals carefully dissected out,weighed,and retained for histological examination. Care is particularly required in dissecting out the thymus because of the possibility of mistaking a mediastinal lymph node for part of the thymus(Agnew,1948). The adrenals were fixed in 4% (w/v) formalin(ten parts commercial formalin to ninety parts of water) and frozen sections were stained with Scharlach R or Sudan black B. The other organs were fixed in Zenker-formol or 2.0%(w/v) formol-saline(five parts commercial formalin to ninety-five parts of normal saline) and 4-6 μ paraffin sections stained with haematoxylin and eosin.

RESULTS

ANTIBODY EXPERIMENTS 1. Sheep Erythrocytes as Antigen

The detailed results are shown in Table 2. Eight of ten pyridoxin-deficient rats had titres less than those of their corresponding ad lib. and inanition controls, one had a titre the same as its inanition control, and only one had a higher titre than its inanition control. The titres of the inanition control animals were in four cases higher than those of the corresponding ad lib. controls, in five cases the same, and in only one case lower.

Table 2. *Reciprocals of haemagglutinin titres of rats in different dietary groups*

Sex	Trio no.	Dietary group: Pyridoxin-deficient	Paired-weighed control	*Ad lib.* control
M	1	10	80	80
M	2	10	80	80
M	3	0	40	20
M	4	10	20	20
M	5	40	40	20
F	6	0	80	40
F	7	0	80	20
F	8	40	80	80
F	9	40	20	20
F	10	10	20	40

2. Bacterium typhosum as Antigen

The detailed results are shown in Table 3. Five of seven pyridoxin-deficient animals had lower titres than their corresponding inanition controls, one had a titre the same as its inanition control, and only one had a

higher titre than that of its inanition control. Multiple

Table 3. *Reciprocals of agglutination titres of rats in different dietary groups immunized with a single injection of* Bact. typhosum, '*H*' *antigen*

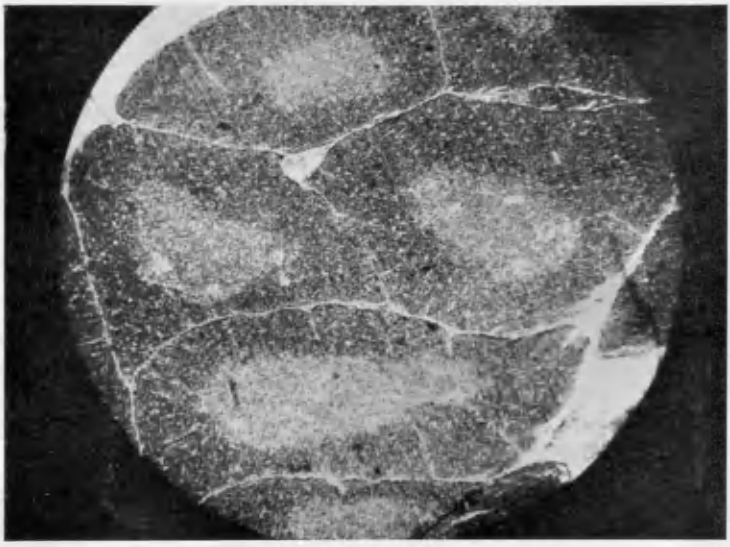
		Dietary group			
			Inanition control		
Sex	Trio no.	Pyridoxin-deficient	Paired-weighed	Paired-fed	*Ad lib.* control
M	11	50	250	...	250
M	12	50	500	...	50
M	13	25	2500	...	500
M	14	125	500	500	250
F	15	250	500	500	500
F	16	500*	250	250	500
F	17	250	250	...	1000

* Probably an anamnestic response ([illegible]).
... = No observation.

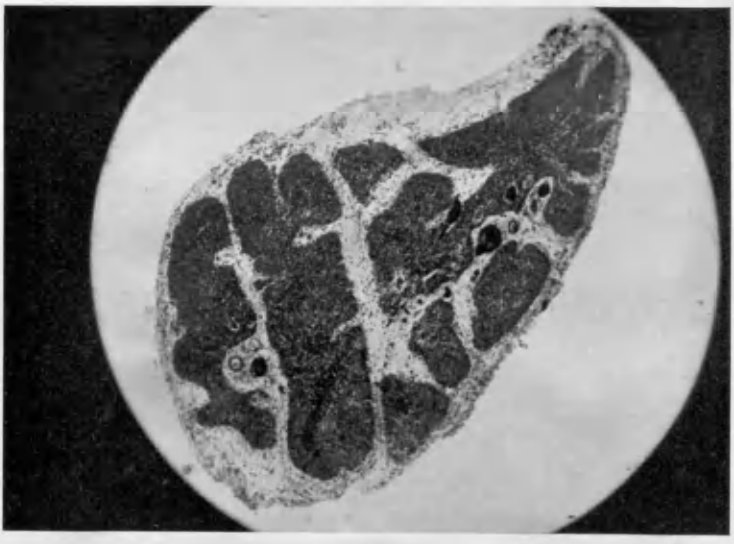
abscesses were observed in the right lung of the rat in question and Gram-positive diphtheroid bacilli were seen in pus from these lesions. The high titre obtained was probably an anamnestic response. In three cases the titres of the inanition control animals were higher than those of the corresponding ad lib. controls, in two cases the same, and in two cases lower.

EFFECT OF PYRIDOXIN-DEFICIENCY ON LYMPHOID TISSUE

Striking changes in lymphoid tissue were observed only in the thymus. Fig. 3 indicates that the thymus glands of the pyridoxin-deficient animals were smaller (g./100 g. body-weight) than those of the corresponding inanition and ad lib. control animals. Histologically,



1. Normal rat thymus showing cortico-medullary differentiation. × 30.



Thymus of pyridoxin-deficient rat. Note absence of cortico-medullary differentiation ar separation of the atrophic lobules by thickened trabeculas. × 30.

Fig. 4.

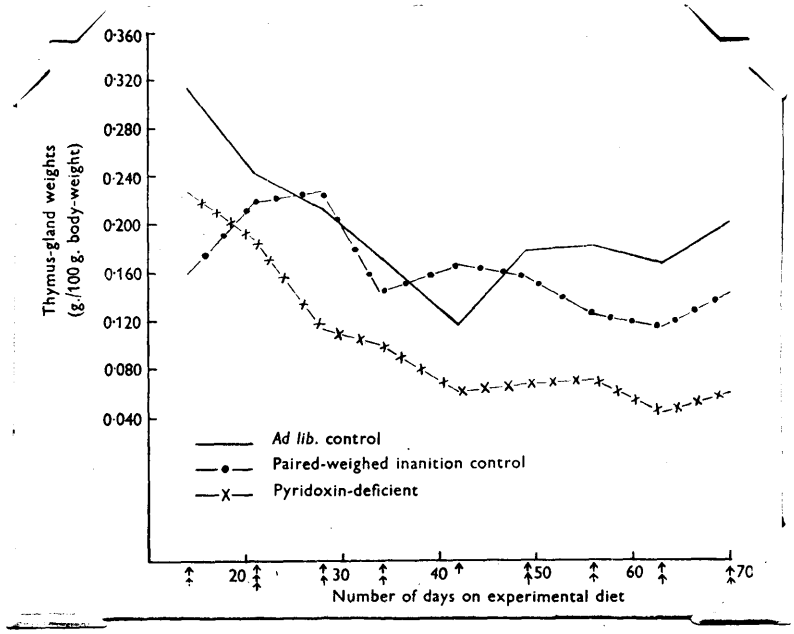


Fig.3. Thymus gland weights of pyridoxin-deficient rats and of ad lib. and paired-weighed controls, killed after 14-70 days on experiment. ↑ = Trio of rats

marked depletion of lymphocytes resulting in a disappearance of cortico-medullary differentiation (Fig.4) was a feature in most of the thymuses examined. The spleens of the pyridoxin-deficient rats were, if anything, larger (g./100 g. body-weight) than those of the corresponding inanition and ad lib. controls (Fig.5) and histologically slight depletion of lymphocytes was noted in only a few cases. The submaxillary lymph nodes of the pyridoxin-deficient animals did not appear to differ significantly in weight (g./100 g. body-weight) from those of the corresponding controls (Fig.6), although histologically these nodes usually showed some depletion of lymphocytes but not as severe as

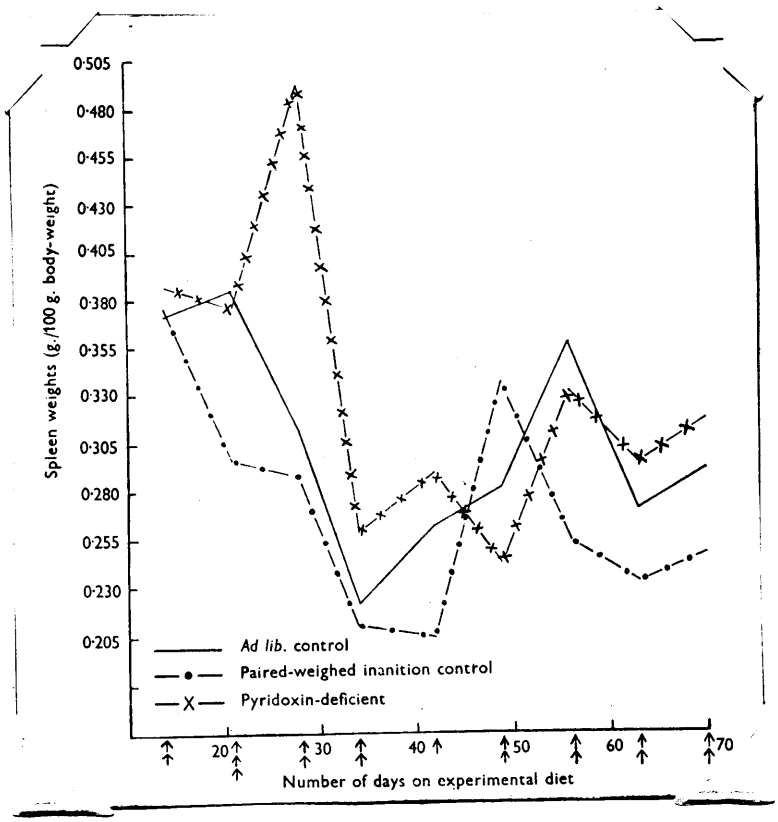


Fig.5. Spleen weights of pyridoxin-deficient rats and of ad lib. and paired-weighed controls, killed after 14-70 days on experiment.
↑ = Trio of rats

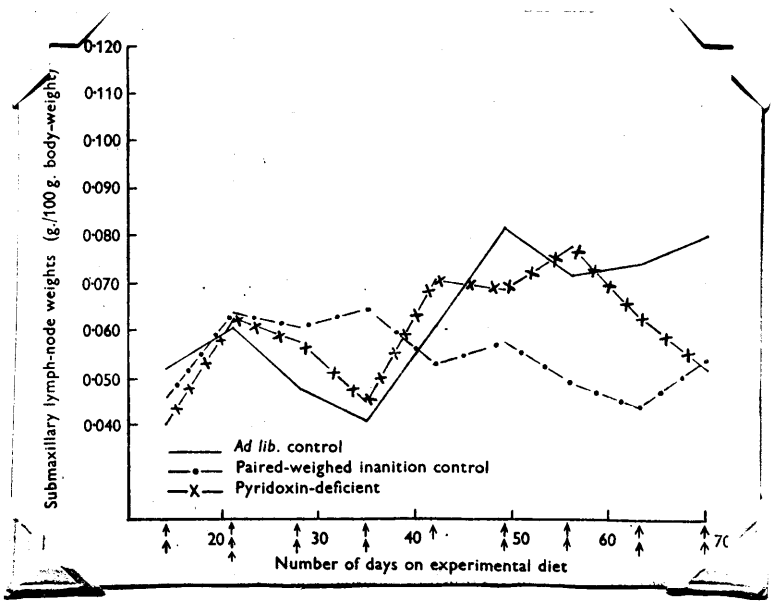


Fig.6. Cervical lymph node weights of pyridoxin-deficient rats and of ad lib. and paired-weighed controls killed after 14-70 days on experiment. ↑ = Trio of rats

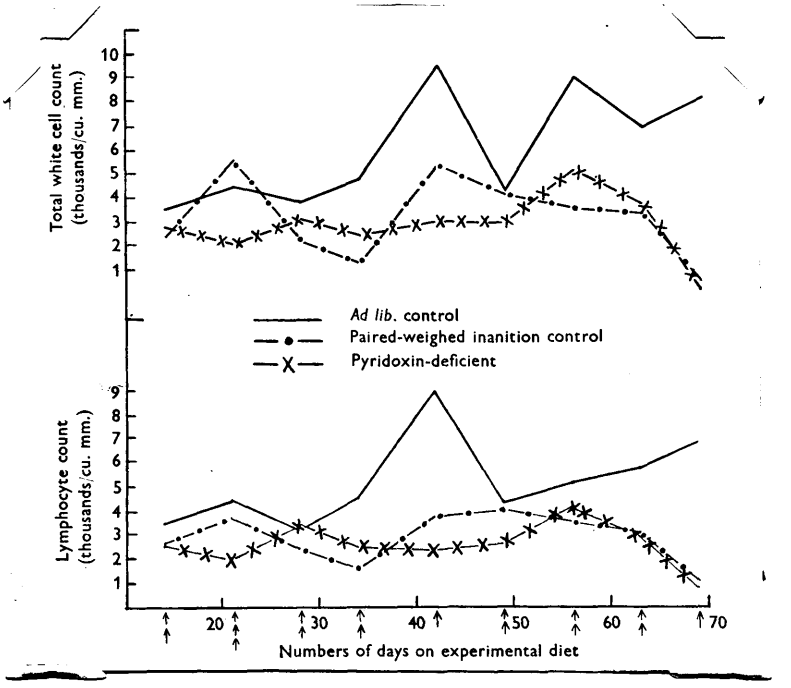


Fig.7. Total white cell counts(above)and lymphocyte counts(below)of pyridoxin-deficient rats and of ad lib.and paired-weighed controls killed after 14-70 days on experiment. ↑ = Trio of rats

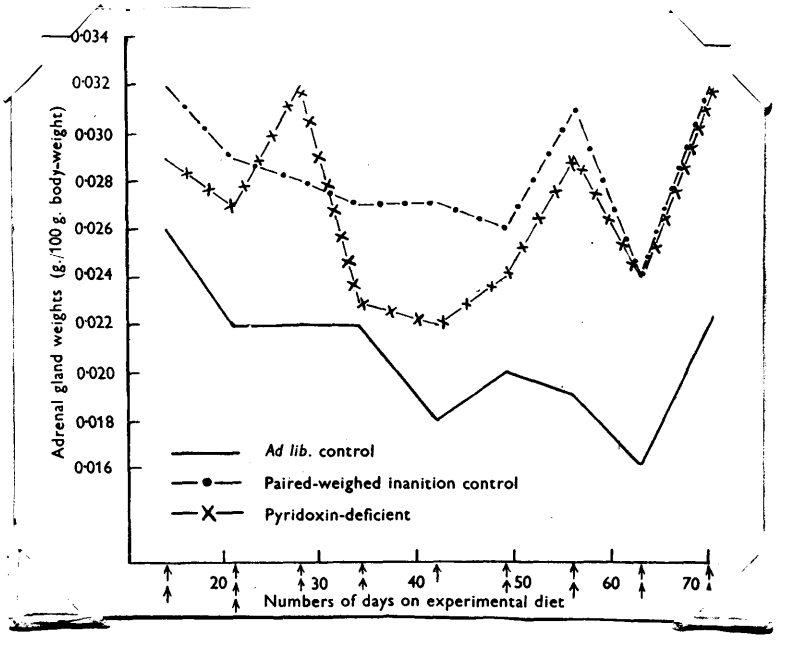


Fig.8. Adrenal gland weights of pyridoxin-deficient rats and of ad lib.and paired-weighed controls killed after 14-70 days on experiment. ↑ = Trio of rats

in the thymus. The total white cell counts and lymphocyte counts of the pyridoxin-deficient rats did not differ significantly from those of the corresponding inanition control animals, although the counts in both these groups appeared to be lower than those obtained in the ad lib. control animals (Fig.7).

Chronic starvation is known to cause a decrease in weight of lymphoid tissue (see, e.g., Jackson, 1929). Indeed, the thymus is so outstanding in this respect that it has been referred to as a "barometer of nutrition". It was expected that some evidence of the effect of inanition would be seen in the paired-weighed controls, and towards the end (70 days) of the experiment the thymuses (Fig.3), spleens (Fig.5) and cervical lymph nodes (Fig.6) became lighter than those of the corresponding ad lib. controls. A lowering of the total white cell count and lymphocyte count (Fig.7) was also observed, and this is in agreement with the recent work of Boutwell, Brush and Rusch (1948). Curiously enough, the red cell count and haemoglobin percentage were essentially unaffected, even in prolonged partial inanition (see Part 2, Table 10).

The weights of the adrenal glands of the pyridoxin-deficient rats were not significantly greater than those of the corresponding inanition control rats (Fig.8).

Further,histological examination of frozen sections of the adrenals stained with Sudan dyes did not reveal signs of depletion of cortical lipoids. These findings suggest that,whatever the mechanism of the thymic atrophy in pyridoxin-deficiency may be,pyridoxin deficiency does not act as a noxious stimulus and cause a modified 'alarm response'(Selye,1946) and thus initiate thymic atrophy by virtue of increased elaboration of adrenal cortical hormone secondary to augmented production of adrenocorticotrophic hormone by the anterior lobe of the pituitary. This confirms the work of Deane and Shaw(1947). Preliminary experiments on the effect of adrenalectomy on the weight of the thymus of pyridoxin-deficient rats have also tended to confirm these observations. For example,adrenalectomy had no apparent effect on the thymus weight of the pyridoxin-deficient rat in the experiment reported below:

Trio(see above) of rats fed synthetic diet from 3/6/47. Adrenalectomized on 27/7/47;and drinking water replaced by 0.9% saline. Killed 15 days later(11/8/47).

	Initial weight	Weight 27/7/47	Death weight	Thymus (g./100 g.b-w)
Ad lib. control	68 g.	192.8 g.	194.6 g.	0.287 g.
Inanition control	64 g.	129.4 g.	134.4 g.	0.220 g.
Pyridoxin-deficient	63 g.	134 g.	130.4 g.	0.117 g.

Technically,it was more convenient to adrenalectomize after the rats had been fed the experimental diet for

some time as in the experiment reported above. Adrenalectomy before feeding the diet was unsatisfactory as the food intakes and weight curves became grossly altered, and it was impossible to interpret the results as the animals refused food and died within a few days of the operation. These preliminary experiments were referred to in a paper on "Antibody Production in Pyridoxin-Deficient Rats"(Agnew and Cook,1949),but while this was in the Press, Stoerk(1948) reported that he had observed that adrenalectomy did not modify the extent of lymphoid atrophy in pyridoxin deficient mice and rats.

DISCUSSION

The data presented in Table 2 suggest that a deficiency of pyridoxin can cause a striking reduction in circulating haemagglutinins. These results thus confirm those of Stoerk and Eisen(1946) and Stoerk *et al.*(1947) who also used sheep erythrocytes,and of Axelrod *et al.* (1947) who used human erythrocytes as an antigenic stimulus. The experiments on the antibody response in rats after injection of a killed culture,'H' antigen,of *Bact. typhosum*(Table 3) indicate that pyridoxin deficiency caused a poor antibody response after injection of this antigen also. There is some doubt about the specificity of pyridoxin deficiency in causing an impaired antibody response.

Stoerk et al.(1947) observed impaired antibody production in pyridoxin deficiency alone,and not in deficiency of aneurin,riboflavin,or pantothenic acid. Axelrod et al. (1947),however,with human erythrocytes as antigen, reported just as poor an antibody response in pantothenic acid deficiency,and Carter and Axelrod(1948) noted a diminished antibody response in aneurin-deficient rats. These workers suggest that the reason for the differences between their results and those of Stoerk et al.(1947) may have been due to the difference in strength of the antigenic stimulus used.

The recent demonstration of the importance of the lymphocyte in antibody elaboration(see,e.g.White and Dougherty,1946) might suggest that the impaired antibody response in pyridoxin deficiency is secondary to the '...striking loss of fixed and circulating lymphocytes...' (Stoerk et al.,1947). But it was only in the thymus that changes were observed which could be described as 'striking'. Only slight depletion of lymphocytes was noted in the spleens and submaxillary lymph nodes and these organs(Figs.5 and 6) were not lighter than the corresponding organs of inanition and ad lib.controls. Indeed the spleens of the pyridoxin-deficient rats(Fig.5) were,if anything,heavier than those of the corresponding controls. Further,with regard to the number of circulating lymphocytes,no significant difference

was observed between the counts in the pyridoxin-deficient animals and their corresponding inanition controls. It may well be,however,that the technique of making only a single blood count in each animal just before death was not sufficiently representative,and that consecutive blood counts in the same animal during the period on the diet would have been more informative. Thymic atrophy is well marked in pyridoxin deficiency(Figs.3 and 4) although lesser degrees of atrophy have been noted,e.g. in deficiency of aneurin,riboflavin,and pantothenic acid. Stoerk et al. (1947) reported that histologically cortico-medullary differentiation was not lost in the thymus of an aneurin-deficient rat as it usually was in a pyridoxin-deficient animal(Fig.4),and that the effect of aneurin deficiency on lymphoid tissue was not so pronounced. Further,as mentioned above,despite the atrophy of lymphoid tissue,antibody production in the aneurin-deficient rat was unaffected. It would seem that the poor antibody response in pyridoxin-deficient rats is not secondary to atrophy of lymphoid tissue. Pyridoxin is of importance in protein metabolism, e.g.in tryptophan metabolism,and as a coenzyme in the decarboxylation of tyrosine,and it may be,as suggested by Stoerk et al.(1947),through interference with some phase of amino-acid metabolism that deficiency of pyridoxin causes an impaired antibody response and that the

'lymphoid atrophy occurring in pyridoxine deficiency could conceivably be a consequence of defective globulin synthesis rather than its cause'(Stoerk et al.1947,p.370).

That the inanition control rats exhibited antibody titres at least as high as those of the ad lib.fed rats (Tables 2 and 3) is of interest because of the fairly severe restriction of food intake. Stoerk et al(1947) reported a similar tendency in their animals. Cannon and his colleagues(e.g.Cannon,Chase and Wissler,1943) have stressed the importance of a low-protein diet in rabbits as a cause of diminished capacity to elaborate antibodies. The type of animal used and the degree of food restriction, particularly of protein,may well have been responsible for these conflicting results but further work on this problem is desirable.

Possible Clinical Applications of Above and Other Work.

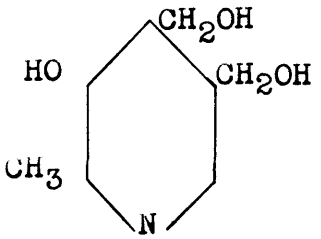
Evans(1948) tested the antibody response in patients with radiation-induced lymphopenia and in a series of reticulo-endothelioses comprising cases of myelocytic and lymphatic leukaemia,Hodgkin's disease,lymphosarcoma and lymphofollicular reticulosis. He observed a very poor agglutinin response response except in the myelocytic leukaemias,and attributed this to the degree of disorganization in the lymph glands in these conditions. Although administration of pyridoxin might be of value in such

cases the rationale for such therapy is not clear-cut. In the reticulo-endothelioses there is some evidence that administration of pyridoxin to improve the antibody response might even be harmful. Dubin(1947) found that generally there was a poor antibody response in patients with Hodgkin's disease. He had read Stoerk and Eisen's (1946) paper on the poor antibody response of pyridoxin-deficient rats injected with sheep erythrocytes,and not unnaturally thought that administration of pyridoxin might stimulate antibody production in one of his cases of Hodgkin's disease. Accordingly,he began treatment with 150 mg. of pyridoxin daily in addition to Röntgen therapy. At the outset he noted(p.910) that the patient's 'general condition was rather good'. Treatment continued until the patient died 2 months later. Dubin was struck(p.910) by 'the rapid worsening' of his patient,and the change in histological appearance of the lesions which showed transition from less to more active growth. He wondered if the pyridoxin had actually accelerated growth of the neoplastic tissue. Stoerk(1947) has reported that lymphosarcoma implants failed to develop in pyridoxin-deficient mice and that marked regression of lymphosarcoma implants occurred when mice were depleted of pyridoxin. Pyridoxin has been recommended for cases of 'radiation sickness',but until further studies are made on the effect

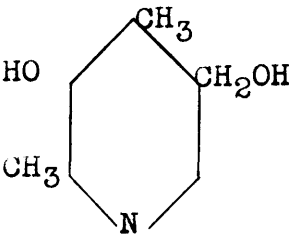
of pyridoxin on the reticulo-endothelioses it might prudent meantime to discontinue its use,and to accept radiation sickness,unless other treatment is available, as the lesser of two evils. Similarly,it seems unwise at present to attempt to improve antibody production in the reticulo-endothelioses by giving pyridoxin. Indeed it is possible,as Stoerk(1946) has suggested,that benefit might follow if the tissues were actually depleted of pyridoxin by administering an antivitamin(desoxypyridoxin). Studies on the human requirements of pyridoxin in the reticulo-endothelioses were therefore undertaken in collaboration with Professor H.W.Fullerton of Aberdeen University.

Although enough desoxypyridoxin was available for only one patient - a man aged 76 with chronic lymphatic leukaemia - it is proposed to give full details of this case as the problems involved were very similar to those being currently encountered in work with anti-folic acid compounds(e.g.aminopterin - 4-amino-pteroylglutamic acid).

During 1947 and 1948 about 30 grams of desoxypyridoxin were obtained for clinical trial through the courtesy of Dr Karl Folkers of Merck and Co.New Jersey and Dr F. Wrigley of Roche Products Ltd.. The structure of desoxy-pyridoxin(2,4-dimethyl-3-hydroxy-5-hydroxymethylpyridine) compared with that of pyridoxin(2-methyl-3-hydroxy 4,5 dihydroxymethylpyridine) is indicated in Fig.9.



Pyridoxin



Desoxypyridoxin

Fig.9. Structure of Pyridoxin and Desoxypyridoxin

The human requirements for pyridoxin are not known. A pure deficiency syndrome has not been reported and it is not known whether,even with such a potent antivitamin as desoxypyridoxin,such a syndrome could be induced for therapeutic purposes. However,as mentioned earlier, pyridoxin deficiency has been produced in monkeys,pigs, dogs,chicks,rats and mice and there is no reason - apart from endogenous biosynthesis of pyridoxin by intestinal bacteria - why a comparable deficiency state in man should not be induced by a pyridoxin-deficient diet plus desoxypyridoxin. Indeed Greenberg et al.(1949) have recently reported that human subjects fed a pyridoxin-deficient diet reacted similarly to a number of other animal species to pyridoxin deficiency,and developed a derangement of tryptophan metabolism which was manifested by the excretion of xanthurenic acid.

A synthetic diet of negligible pyridoxin content, based on the diet shown in Table 1,was devised;but personal trials indicated that lack of variety,to say nothing of the difficulty of getting the patient to consume vitamin-free casein,would probably make long-term experiments impossible. Anderson et al.(1946) have shown that aneurin deficiency could be induced in human subjects by feeding quite a wide range of palatable foodstuffs low or lacking in aneurin. Possibly an attractive pyridoxin-deficient diet could have been devised if the individual items of the diet had been microbiologically assayed. However,in the clinical trial of desoxypyridoxin carried out at Aberdeen Royal Infirmary it was decided to modify the ordinary hospital diet only in respect of protein. A high protein diet was given as a high protein intake has been shown to induce a more severe pyridoxin deficiency state.

The dosage of desoxypyridoxin for the human subject was,of course,unknown. However,in his experiments on lymphosarcomata in mice,Stoerk(1947) found an oral dose of 1 mg.per mouse(25 g.) per day suitable. This was used as a basis for calculation of the human dosage and an empirical figure of 3 g.per day for a 12 stone subject was arrived at. In an attempt to achieve and maintain an adequate concentration of the antivitamin in the tissues,

this dose was divided,1 gram being given orally thrice daily.

The assessment of the results of treatment was based on:-

1. Alteration in size of palpable swellings.
2. Radiographs - e.g.,of mediastinal glands.
3. Peripheral blood picture. Total and differential white cell counts,haemoglobin,and red cell count.

A biopsy specimen from an affected gland before and after treatment was also desirable but was,unfortunately,not obtained.

It was impossible to know how long to continue therapy with the antivitamin. As Stoerk(1947) says,of mice, 'obviously,continued pyridoxin deficiency,although acting more slowly,may ultimately be as fatal as lymphosarcoma'. This problem did not arise in the present case as only enough desoxypyridoxin was available for a trial of 10 days.

In addition to the observations mentioned above, the urine was examined daily for red cells and albumen (see Part 2 for rationale);the patient was weighed;and a careful examination of the nervous system was carried out. Finally,the following were regarded as indications for stopping treatment:-

1. Severe anaemia.
2. Haematuria(see Part 2).
3. Epileptiform fits or any untoward neurological signs or symptoms.
4. Anorexia of sufficient severity to result in weight loss.
5. Skin lesions.
6. Any type of bacterial infection.

The therapeutic effect of anti-folic acid compounds in acute leukaemia was discovered indirectly. Administration of folic acid to these patients made the disease more active,and from this it followed that administration of an anti-folic acid compound might be beneficial. It was decided to follow this indirect approach,and the clinical trial of desoxypyridoxin in a case of chronic lymphatic leukaemia was divided into 4 stages:-

Stage 1. Observation period of 18 days;no treatment.

Stage 2. Pyridoxin administration(200 mg.orally each day) for 10 days.

Stage 3. Rest period of 4 days;no treatment.

Stage 4. Desoxypyridoxin administration(3 g.orally each day) for 10 days.

No other treatment(e.g.radiotherapy) was given before or during the above trial.

The results(Table 4 and Fig.10) were disappointing; pyridoxin did not light up the activity of the disease, and desoxypyridoxin had no beneficial effect. No untoward signs or symptoms were observed with either substance. A few weeks after this trial Gellhorn and Jones(1949) reported essentially similar findings in 3 cases of acute leukaemia and 3 cases of lymphosarcoma. Signs of toxicity were observed in one patient of their series who was given 25 mg.per kg. of desoxypyridoxin;the dose was therefore reduced in all the cases to 2.5 mg.per kg.. As mentioned above,no untoward reactions occurred in the Aberdeen case of chronic lymphatic leukaemia although the dose given was 40 mg.per kg.. It is possible,however,that the previous treatment with pyridoxin was the reason why this high dose caused no toxic reactions.

No further supplies of desoxypyridoxin could be obtained for more extensive trials. Meantime,the negative results in the case reported here and in the cases of Gellhorn and Jones(1949) suggest that desoxypyridoxin has, at present,no therapeutic value.

Date (1948)	Hb % Haldane	R.B.C. ($x10^{-6}$)	W.B.C.	Neutrophils	Eosinophils	Lymphocytes	Monocytes	Basophils
9/11	50	2.64	176,000	2	1	97	0	0
16/11	64	3.15	220,900	5	0	95	0	0
18/11	-	-	205,000	4	0	96	0	0
19/11	62	3.12	201,000	4	0	96	0	0
20/11	-	-	204,000	4	0	96	0	0
22/11	-	-	247,000	4	0	96	0	0
23/11	68	3.41	224,000	5	1	94	0	0
24/11	-	-	236,700	4	0	96	0	0
25/11	-	-	232,300	7	0	93	0	0
26/11	58	2.94	236,800	1	0	99	0	0
27/11	-	-	268,000	9	0	91	0	0
	Pyridoxin administered from 27/11/48							
29/11	-	-	236,000	8	0	91	1	0
30/11	60	3.00	231,000	6	0	94	0	0
1/12	-	-	279,400	6	0	93	0	1
2/12	-	-	223,800	4	0	96	0	0
3/12	58	3.05	256,100	5	0	95	0	0
4/12	-	-	257,500	5	0	95	0	0
6/12	-	-	197,300	3	0	97	0	0
	Desoxypyridoxin administered from 10/12/48 after the following rest period							
7/12	64	3.06	246,500	3	0	97	0	0
8/12	58	-	212,000	3	0	97	0	0
9/12	62	-	177,300	3	0	97	0	0
10/12	60	2.99	218,000	3	0	97	0	0
11/12	58	-	257,500	5	0	95	0	0
13/12	58	-	243,000	2	0	98	0	0
14/12	62	3.00	266,000	4	1	95	0	0
15/12	58	-	274,100	4	0	96	0	0
16/12	56	-	256,000	3	0	97	0	0
17/12	58	2.84	258,000	5	0	95	0	0
18/12	54	-	250,000	3	0	97	0	0
	Trial finished on 18/12/48 and radiotherapy was started on 20/12/48							
20/12	64	-	276,000	3	1	96	0	0

Table 4. Haematological observations made during clinical trial of desoxypyridoxin(Henry Duncan,aet.76;chronic lymphatic leukaemia;Ward 1,Aberdeen Royal Infirmary)

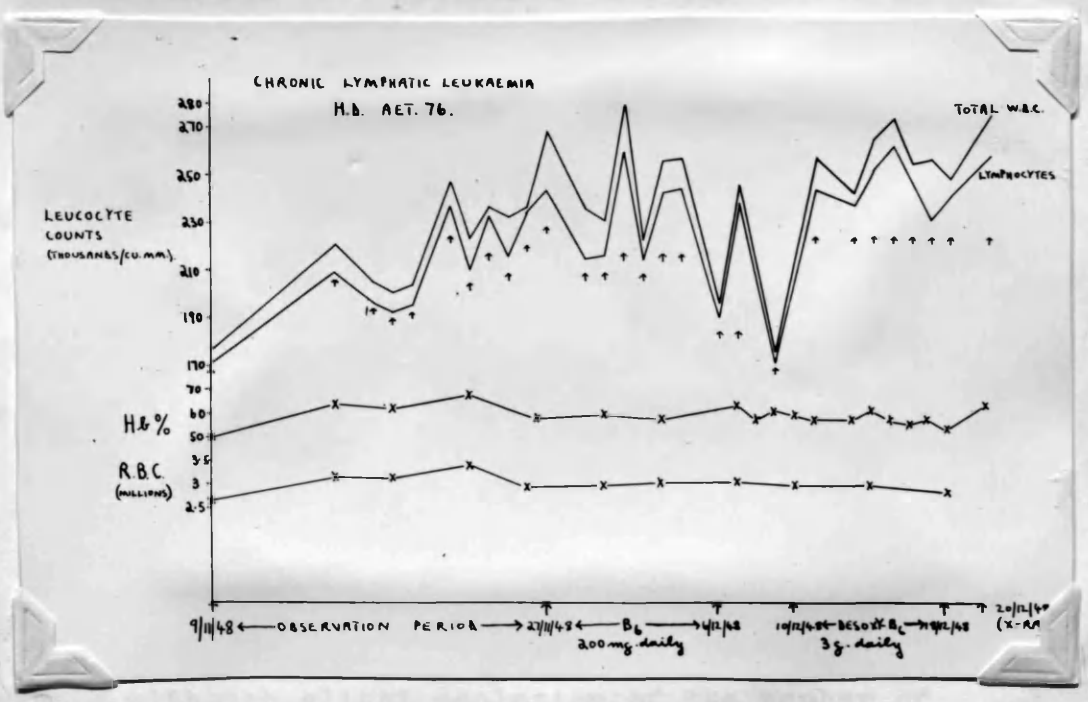


Fig.10. Graphical representation of data presented in Table 4.(Arrows and x's indicate points at which observations were made)

SUMMARY OF PART 1 OF THESIS

1. The antibody response of pyridoxin-deficient rats injected with sheep erythrocytes or a killed culture,'H' antigen,of Bact.typhosum,was significantly lower than that of similarly treated inanition and ad lib.control animals. Inanition control rats exhibited antibody titres at least as high as those of the corresponding ad lib.fed animals.

2. The thymus glands of pyridoxin-deficient rats were significantly lighter than those of corresponding inanition and ad lib.control rats and marked depletion of lymphocytes resulting in a loss of cortico-medullary differentiation was observed histologically.

3. No significant differences were observed in the weights of the spleens and submaxillary lymph nodes of the pyridoxin-deficient rats compared with inanition and ad lib. controls,although slight depletion of the number of lymphocytes,particularly in the lymph nodes,was observed.

4. No significant differences were observed in the total white cell count and the lymphocyte count between pyridoxin-deficient rats and their corresponding inanition controls, although both these counts were generally lower than counts obtained in the ad lib.control rats.

5. The effect of pyridoxin deficiency on lymphoid tissue did not appear to be mediated through a pituitary-adrenal mechanism.

6. The relationship between lymphoid tissue and antibody formation is briefly considered. The poor antibody response of pyridoxin-deficient rats did not appear to be secondary to atrophy of lymphoid tissue as striking changes in the lymphoid tissue of these rats were only observed in the thymus glands.

7. The possible clinical significance of pyridoxin and desoxypyridoxin is discussed. Desoxypyridoxin was found of no value in the treatment of a case of chronic lymphatic leukaemia.

8. Two types of inanition control are described, and the importance of this control in the design of nutritional experiments is stressed.

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References to papers cited in Part 1 appear at end of thesis

PART 2

HAEMATURIA IN PYRIDOXIN-DEFICIENT RATS

During the studies on antibody production in pyridoxin-deficient hooded rats(Part 1;and Agnew and Cook, 1949) gross macroscopic haematuria was noted in several animals fed the pyridoxin-deficient diet but not in corresponding litter-mate inanition(paired-weighed) control rats and rats fed the complete diet ad libitum. A preliminary account of this unexpected finding was given to the Nutrition Society in 1948(Agnew,1948-49) but as haematuria has not been reported in any of the numerous recent papers from the United States on pyridoxin deficiency,and only Birch(1938) appears to have published on this subject,it seemed of interest to make a more detailed study of the incidence,duration and severity of the haematuria that was so frequently observed. The effect of the haematuria on the peripheral blood picture(red cell count and haemoglobin)was also studied,as well as the effect of giving pyridoxin to rats with well-established haematuria. Finally,possible strain differences in susceptibility to haematuria were sought by observing the effect of pyridoxin deficiency in Wistar albino rats fed the same diet as the hooded Lister rats. Much of this work has been described in a recent paper(Agnew,1949a).

METHODS

The animals used were 1. hooded Lister rats(Rowett Institute strain),and 2. albino rats(originally derived by Glaxo Ltd. from brother-sister matings of Wistar rats) obtained from the Agricultural Research Council Field Station,Compton. Each experiment in the present series involved the use of several trios of litter-mate weanling rats of the same sex and approximately the same weight, each trio of animals being arranged as described in Part 1. Four experiments,each with 6 trios(3 male,3 female) of rats were set up as in Table 5. The synthetic diet used has already been described(Part 1). As before,the animals were housed in individual cages with wide wire-screened bases. The animals had unlimited access to water and were weighed daily except on Sundays. Growth curves for the various experiments are given in Figs.11-14.

The only readily available source of the linoleic acid used in experiment 3(Table 5) was B.D.H. technical linoleic acid,and it is possible that this substance is too impure for nutritional studies;this point is discussed later.

Rats,except those used for blood studies,were killed with coal gas alone. In all but two instances

Experiment	Strain of rat	Number of rat trios used	Amount and nature of fat in synthetic pyridoxin-deficient diet
1	Hooded Lister	6(3 male, 3 female)	5% margarine
2	Hooded Lister	6(3 male, 3 female)	5% lard
3	Hooded Lister	6(3 male, 3 female)	5% margarine plus 750 mg. linoleic acid/100 g.diet
4	Wistar Albino	6(3 male, 3 female)	5% margarine

Table 5. Plan of experiments to test the effects on the incidence and course of haematuria of adding various supplements to a synthetic pyridoxin-deficient diet

(Table 6,trio E;Table 10,trio F),when a rat died the remaining members of the trio were gassed. The heart and both kidneys were carefully dissected out. The heart was sliced and any blood removed from its chambers;thereafter the heart and both kidneys were weighed and placed in 2.0 per cent.(w/v) formaldehyde-saline(5 parts commercial formalin plus 95 parts 0.9 per cent.saline) for subsequent histological examination.

Rats of the trios selected for blood studies were anaesthetized with ether,and blood was obtained by cutting the left axillary vein.Red cell counts and haemoglobin estimations(Haldane-Gower method) were done. These animals were then killed with coal gas,and the hearts and kidneys dealt with as described above. The colour index was calculated from the formula given by Evans(1945,p.476):-

$$C.I. = \frac{\text{Haemoglobin \%(Haldane)} \times 5}{\text{Millions of cells/cu.mm.} \times 100}$$

This formula,based on human standards of normality,gave 0.59 for normal(i.e. ad lib.control) rats of this experiment(Table 10). Lower figures than this indicate the extent of the microcytosis and hypochromasia seen in the pyridoxin-deficient rats(Table 10).

COLLECTION OF URINE AND DETECTION OF HAEMATURIA.

A simple,rapid method for the examination of urine for red blood cells had to be devised as it was impracticable to provide a metabolism cage unit for each rat. A piece of white absorbent paper(Whatman no.1) was placed beneath each cage,usually every alternate day,and an hour or so later the urine splashes on the paper were examined for blood by the naked-eye and by the benzidine reaction. The benzidine reaction was used only as a screening test. If the reaction was negative,blood was assumed to be absent from the urine. A positive result,however,was interpreted as an indication that blood might be present,and that the urine should be examined microscopically. Sufficient urine for this purpose was usually obtained by approaching the rat gently,lifting it by the scruff of the neck,and chilling the abdomen with a gauze pad soaked with ether. Blood in the urine detectable by the naked-eye is referred to as "macroscopic haematuria" to distinguish it from "microscopic haematuria" detected by the benzidine reaction and microscopy.

RESULTS

1. INCIDENCE AND DURATION OF HAEMATURIA

Experiment 1.(Hooded rats;5 per cent.margarine).

The results are summarized in Table 6. Haematuria of

Trio	No. of days on diet	No. of days on diet before onset of haematuria: Inanition control	No. of days on diet before onset of haematuria: Pyridoxin-deficient	Duration of haematuria before rats died or were killed (days): Inanition control	Duration of haematuria before rats died or were killed (days): Pyridoxin-deficient	Remarks
A (♀)	147	No haematuria	24	No haematuria	123	All rats killed
B (♀)	175	No haematuria	28	No haematuria	147	All rats killed. Pyridoxin-deficient rat fed pyridoxin from 129th day onwards
C (♀)	104	No haematuria	34	No haematuria	7	All rats killed
D (♂)	146	No haematuria	27	No haematuria	119	Pyridoxin-deficient rat died; others killed
E (♂)	64 (pyridoxin-deficient rat) 188 (other members of trio)	5 (after receiving pyridoxin-deficient diet; see remarks column)	24	114 (see remarks column)	40	Pyridoxin-deficient rat died. Inanition-control rat fed pyridoxin-deficient diet and 5 days later developed haematuria (see text)
F (♂)	104	No haematuria	24	No haematuria	66	All rats killed. Pyridoxin-deficient rat had haematuria for 3 days at first; this stopped, then resumed 14 days later for 63 days
Arithmetic means (and ranges), excluding trio E	135 (104–175)	No haematuria	27 (24–34)	No haematuria	92 (7–147)	

Table 6. Incidence, time of onset, and duration of haematuria in hooded rats of Exp. 1 (5% margarine). No haematuria occurred in the *ad lib.* controls.

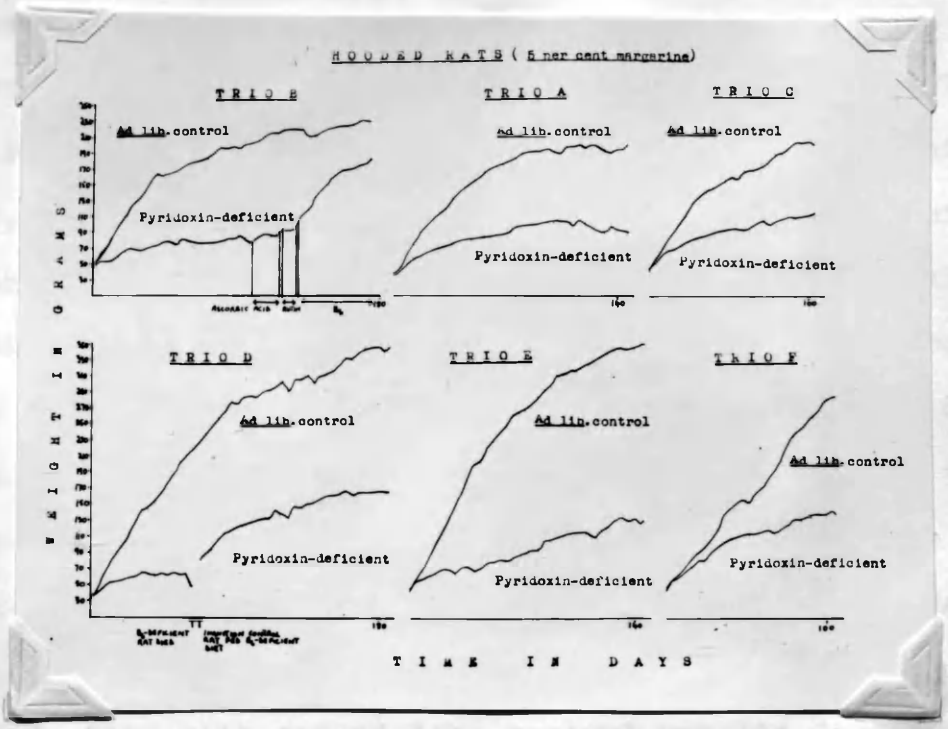

Fig. 11. Growth curves of trios of hooded rats used in Exp.1(5% margarine).

variable duration was observed in all the pyridoxin-deficient animals but not in any of the ad lib. or inanition controls. The mean time of onset of haematuria was 27 days (range 24-34) after the pyridoxin-deficient diet was first fed. The bleeding persisted in trios A,B and D until the animals died or were killed. The haematuria lasted 66 days in trio F and only 7 days in trio C. The pyridoxin-deficient rat of trio E died after 64 days on the experimental diet,having had haematuria for the last 40 days of this period. The remaining members of this trio were not killed at this time and for this reason figures from this trio were omitted in calculating the means given in Table 6. As a matter of interest,the inanition control rat of this trio was given the pyridoxin-deficient diet from five days after the pyridoxin-deficient rat died. Within five days it developed haematuria,which persisted until the animal was killed,along with the ad lib. control rat,114 days after it first showed haematuria.

An attempt was made to see if ascorbic acid and rutin,a flavonol glycoside,which are thought to counteract bleeding tendencies,could influence the haematuria. The pyridoxin-deficient rat of trio B had severe haematuria and was given a course of ascorbic acid(50 mg.daily by mouth) from the 101st day on the deficient diet,for 17

days. Two days after,60 mg.of rutin was fed daily for 9 days. Neither substance modified the severity of the haematuria in any way. Pyridoxin was then fed to this rat and the bodyweight at once increased(Fig.11,trio B) although haematuria continued as before until the rat, along with the control animals of the trio,was killed 28 days later.

Experiment 2.(Hooded rats;5 per cent.lard).

The results are summarized in Table 7. As in experiment 1,haematuria of variable duration was observed in all the pyridoxin-deficient animals but not in any of the ad lib. or inanition controls. The mean time of onset was 26 days(range 23-34). In trios A,B,C,D and F the bleeding persisted until the animals died or were killed. The pyridoxin-deficient rat in trio E had haematuria for only 8 days at first;bleeding resumed 6 days later and persisted until the animal was killed. Pyridoxin was fed to the pyridoxin-deficient rats,which had severe haematuria,of trios B and C from the 105th day until the animals were killed. Although bodyweight at once increased markedly(Fig.12) no modification of the duration or severity of the haematuria was observed,and bleeding persisted until the animals were killed.

Trio	No. of days on diet	No. of days on diet before onset of haematuria	Duration of haematuria before rats died or were killed (days)	Remarks
A ♀	130	23	107	All rats killed
B ♀	140	25	115	All rats killed. Pyridoxin-deficient rat fed pyridoxin from 105th day until death
C ♀	134	25	109	All rats killed. Pyridoxin-deficient rat fed pyridoxin from 105th day until death
D ♂	124	25	99	Pyridoxin-deficient rat died; others killed
E ♂	111	34	71	All rats killed. Pyridoxin-deficient rat had haematuria for 8 days at first; this stopped, then resumed 6 days later for 63 days
F ♂	110	25	85	All rats killed
Arithmetic means (and ranges)	125 (110–140)	26 (23–34)	98 (71–115)	

Table 7. Incidence, time of onset, and duration of haematuria in the pyridoxin-deficient hooded rats of Exp. 2 (5% lard). No haematuria occurred in the ad lib. and inanition controls.

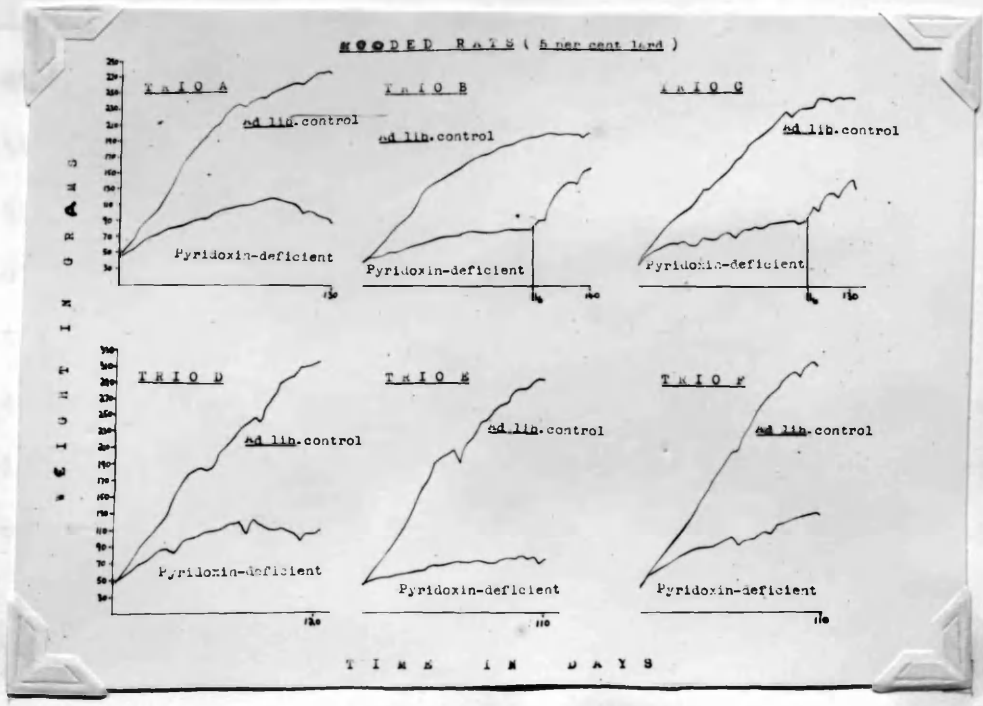

Fig.12. Growth curves of trios of hooded rats used in experiment 2(5% lard).

Experiment 3.(Hooded rats:5 per cent.margarine plus 750 mg.linoleic acid per 100 g.diet).

The results are summarized in Table 8. Haematuria of variable duration was observed in all the pyridoxin-deficient rats and,most unexpectedly,in 5 of the six corresponding ad lib. control rats. One inanition control rat(trio F) died 49 days after the start of the experiment,but haematuria was not observed in this or in any of the other inanition control rats. Haematuria appeared later in the pyridoxin-deficient rats than in experiments 1 and 2,the mean time of onset being 76 days(range 41-105). The haematuria was intermittent in the pyridoxin-deficient rats of trios C and F. The mean time of onset of the haematuria observed in the ad lib. control rats was 60 days(range 30-147). The bleeding was intermittent in two cases(trios C and F),and in three cases(trios A,E and F) continued until the rats died or were killed. Weight curves for this experiment are given in Fig.13.

Experiment 4.(Albino rats:5 per cent.margarine).

The results are summarized in Table 9. Haematuria was observed in only 2 animals. Microscopically - never with the naked-eye - blood was noted intermittently in the urine of the ad lib. control rat and the inanition control rat of trio B 94 and 91 days respectively after the start

Trio	No. of days on diet	No. of days on diet before onset of haematuria		Duration of haematuria before rats died or were killed (days)		Remarks
		Ad lib. control	Pyridoxin-deficient	*Ad lib.* control	Pyridoxin-deficient	
A (♀)	154	35	100	119	9	All rats killed
B (♀)	114	No haematuria	105	No haematuria	2	Pyridoxin-deficient rat found dying, therefore all rats killed. Post-mortem examination of pyridoxin-deficient rat revealed multiple lung abscesses
C (♀)	156	147	53		49	All rats killed. Pyridoxin-deficient rat had two remissions of 33 and 23 days after development of haematuria
D (♂)	153	30	92	13	61	All rats killed. *Ad lib.* control rat had two remissions of 95 and 14 days after development of haematuria
E (♂)	105	34	41	71	53	Pyridoxin-deficient rat died; others killed
F (♂)	150 (Inanition control died after 49 days)	24	66	126	35	*Ad lib.* control and pyridoxin-deficient rats killed. Latter had haematuria with remission of 49 days
Arithmetic means (and ranges)	139 (105–156)	60 (30–147)	76 (41–105)	86 (2–126)	35 (2–61)	

Table 8. Incidence, time of onset, and duration of haematuria in hooded rats of Exp. 3 (5% margarine plus 750mg. crude linoleic acid/100g. diet). No haematuria occurred in the inanition controls.

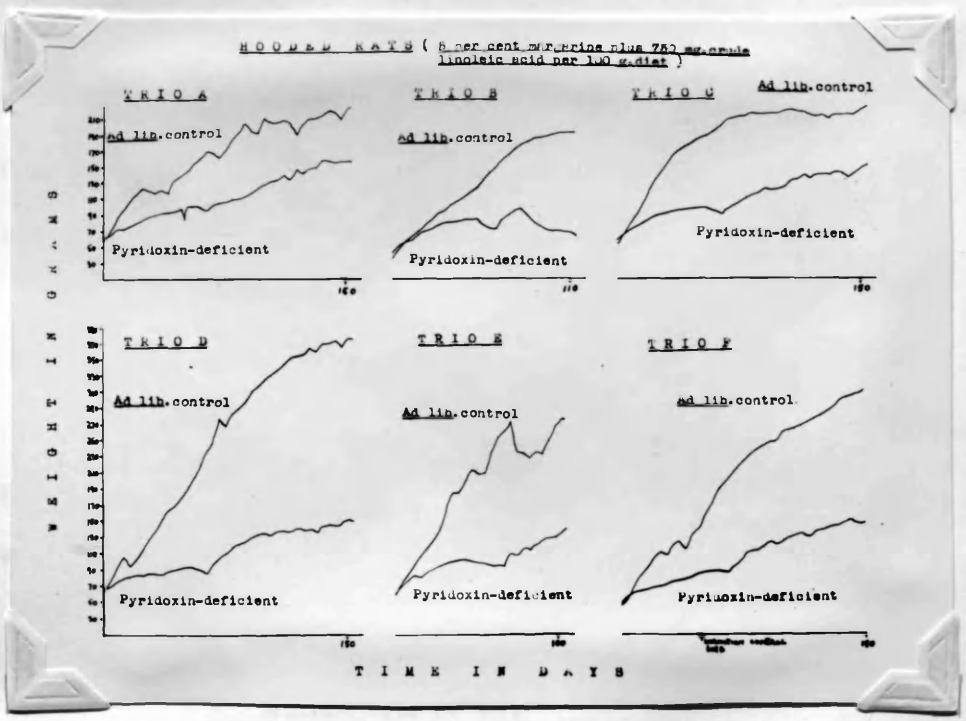

Fig.13. Growth curves of trios of hooded rats used in Exp.3(5% margarine plus 750mg.crude linoleic acid/100g. diet).

of the experiment. The ad lib. control rat had haematuria for a total of 15 days,and the inanition control rat for 4 days. Unfortunately,these rats were accidentally killed during an attempt to stimulate urine secretion by carbachol. However,these rats would have been killed a day or two later in any case as it is doubtful if,for reasons discussed below,the pyridoxin-deficient rat of the trio would have lived much longer. Weight curves for this experiment are given in Fig.14.

2. SEVERITY OF HAEMATURIA

In experiments 1,2 and 3 the haematuria was generally severe and a blood-stained paper was very often found below the cages(Fig.15). In an occasional animal

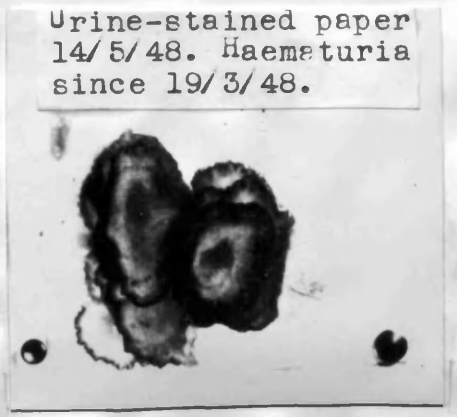

Fig.15. Photograph of filter paper from beneath cage of pyridoxin-deficient rat(experiment 1,trio F)

(e.g.experiment 1,trio C) bleeding was detected only by the benzidine reaction and confirmed by microscopic

Trio	No. of days on diet	No. of days on diet before onset of haematuria		Duration of haematuria before rats died or were killed (days)		Remarks
		Ad lib. control	Inanition control	*Ad lib.* control	Inanition control	
A (♀)	132	No haematuria	No haematuria	No haematuria	No haematuria	All rats killed, as pyridoxin-deficient rat would not have lived more than 2–3 days
B (♀)	120	94	91	15	4	Control rats accidentally killed (see text). Haematuria noted in both control rats was intermittent and microscopic
C (♀)	98	No haematuria	No haematuria	No haematuria	No haematuria	Pyridoxin-deficient rat died; others killed
D (♂)	79	No haematuria	No haematuria	No haematuria	No haematuria	Pyridoxin-deficient rat died; others killed
E (♂)	85	No haematuria	No haematuria	No haematuria	No haematuria	Pyridoxin-deficient rat died; others killed
F (♂)	126	No haematuria	No haematuria	No haematuria	No haematuria	All rats killed, as pyridoxin-deficient rat would not have lived more than 2–3 days
Arithmetic means (and ranges)	107 (79–132)	94	91	15	4	

Table 9. Incidence, time of onset, and duration of haematuria in albino rats of Exp. 4 (5% margarine). No haematuria occurred in pyridoxin-deficient rats.

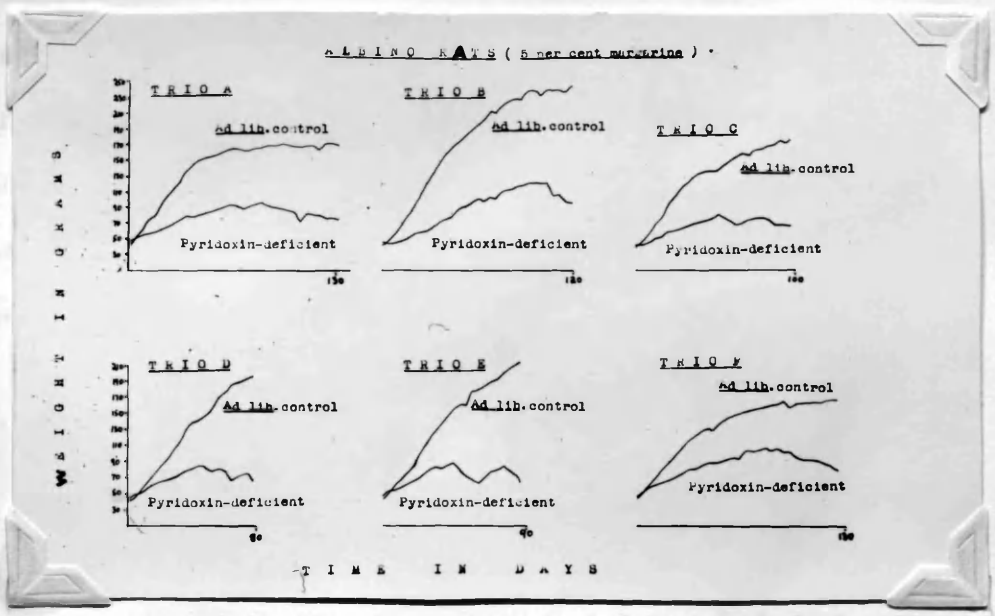

Fig.14. Growth curves of trios of albino rats used in Exp.4(5% margarine).

examination of the urine. Severe macroscopic haematuria was often preceded and succeeded by a variable period of bleeding detectable only microscopically. The duration of macroscopic haematuria was extremely variable, ranging from only one day to several weeks. In an earlier experiment (Agnew, 1948-49) the usual duration of macroscopic haematuria in 7 pyridoxin-deficient rats was 4-6 days (range 1-13 days) but the duration of haematuria was probably far longer since, in these experiments, the benzidine reaction and microscopy were not employed to detect non-macroscopic bleeding.

3. HAEMATOLOGY

Table 10 summarizes the observations made of the red cell count and haemoglobin percentage of eight trios (4 male, 4 female) of rats that had been fed the experimental diet for periods ranging from 104 to 156 days. The results indicate that pyridoxin deficiency caused a significant ($P < 0.001$) increase in the red cell count, a significant ($P < 0.02$) decrease in haemoglobin percentage, and a significant ($P < 0.0001$) lowering of the colour index. Microcytosis has been reported in pyridoxin-deficient rats by Carpenter and Kodicek (1948), and the present results confirm their findings.

It is of interest to note the effect of

Trio	Days on diet	Red cell count (millions)			Haemoglobin (%)			Colour index		
		Ad lib. control	Inanition control	Pyridoxin-deficient	*Ad lib.* control	Inanition control	Pyridoxin-deficient	*Ad lib.* control	Inanition control	Pyridoxin-deficient
1 A (♀)	147	7·04	8·31	11·75	83	90	82	0·59	0·54	0·35
1 F (♂)	104	9·20	7·76	9·93	92	91	95	0·53	0·61	0·36
2 A (♀)	130	6·84	8·11	9·16	80	88	70	0·59	0·54	0·38
2 E (♂)	111	6·66	8·26	9·15	96	91	74	0·72	0·55	0·40
2 F (♂)	110	9·83	4·12	8·84	90	68	73	0·46	0·83	0·41
3 A (♀)	154	7·38	8·06	9·22	85	95	71	0·58	0·59	0·39
3 C (♀)	156	6·97	8·01	10·42	89	96	73	0·64	0·60	0·35
3 D (♂)	150	7·00	7·55	11·58	90	91	95	0·64	0·60	0·41
Arithmetic means		7·62	7·52	10·01	88·1	88·8	79·1	0·59	0·61	0·38
S.E. of differences between means*			0·702			3·82			0·042	

* These standard errors are based upon a joint error mean square calculated by the analysis of variance.

Table 10. Red cell count, haemoglobin percentage and colour index in untreated pyridoxin-deficient hooded rats and their controls.

Trio	Total no. of days on diet	No. of days on diet before pyridoxin given to deficient rats	Red cell counts (millions)			Haemoglobin (%)			Colour index		
			Ad lib. control	Inanition control	Previously pyridoxin-deficient	*Ad lib.* control	Inanition control	Previously pyridoxin-deficient	*Ad lib.* control	Inanition control	Previously pyridoxin-deficient
1 B (♀)	175	129	8·16	7·55	7·01	92	91	95	0·56	0·60	0·68
2 B (♀)	140	105	7·25	7·84	7·08	77	89	79	0·53	0·57	0·56
2 C (♀)	134	105	8·63	7·20	6·73	86	84	82	0·50	0·58	0·61
Arithmetic means			8·01	7·53	6·94	85	88	85	0·53	0·58	0·62
S.E. of differences between means*			0·448			3·62			0·023		

* These standard errors are based upon a joint error mean square calculated by the analysis of variance.

Table 11. Red cell count, haemoglobin percentage and colour index of pyridoxin-deficient hooded rats after administration of pyridoxin.

administration of pyridoxin on the blood picture of deficient animals(Table 11). Statistical analysis of Tables 10 and 11 indicated that differences due to sex were negligible as were differences due to litter effect, and thus comparison of the red cell count,haemoglobin percentage and colour index of these trios was permissible. As mentioned above,the duration and severity of haematuria in the trios in Table 11 were not influenced by pyridoxin administration. It would seem,therefore,that the severe haematuria observed in these rats had not affected the blood picture as administration of pyridoxin caused a return of the red cell count and haemoglobin percentage to normal and,curiously,a just significant($P < 0.05$) elevation of the colour index above normal. As already noted the colour index of the rats in Tables 10 and 11 is calculated from a formula for the human and is thus to be regarded only as a relative measurement. Compared with 0.59 for normal rats(Table 10) the figure of 0.38 indicates that the red cells of the pyridoxin-deficient rats were hypochromic and microcytic.

4. ORGAN WEIGHTS

Experiment 1.(Hooded rats:5 per cent.margarine).

The results are summarized in Table 12,from which

trio E(see Table 6) has been omitted. Although the pyridoxin-deficient rat of trio B received pyridoxin(see Tables 6 and 11),this trio is included in Table 12 as this treatment had no effect on the haematuria. The kidneys of the pyridoxin-deficient rats were not significantly heavier than those of the inanition control rats although the observed differences were suggestive,and just reached significance($P < 0.05$) when the kidney weights of the ad lib. and inanition control rats were combined.

The heart weights of trios C and F were not recorded. The hearts of the pyridoxin-deficient rats of trios A,B and D were significantly($P < 0.05$) heavier than those of the corresponding ad lib. and inanition controls.

Experiment 2.(Hooded rats;5 per cent.lard).

The results are summarized in Table 12. The pyridoxin-deficient rats of trios B and C had received pyridoxin (see Tables 7 and 11),but these trios are included in Table 12 as this treatment had no effect on the haematuria. The kidneys of the pyridoxin-deficient rats were significantly($P < 0.01$) heavier than those of the ad lib.and inanition control rats.

The heart weights of trios E and F were not recorded. The hearts of the pyridoxin-deficient rats of trios A,B,C and D just failed to be significantly heavier

Exp. no.	Trios used	Organ: Kidneys (g./100 g. body-weight)			Organ: Heart (g./100 g. body-weight)		
		Ad lib. control	Inanition control	Pyridoxin-deficient	*Ad lib.* control	Inanition control	Pyridoxin-deficient
1. Hooded rats (5% margarine)	A–D and F (heart weights not recorded for C and F)	0·849	0·878	1·037	0·341	0·372	0·506
S.E. of differences between means*			0·098			0·060	
2. Hooded rats (5% lard)	A–F (heart weights not recorded for E and F)	0·764	0·874	1·233	0·360	0·424	0·518
S.E. of differences between means*			0·089			0·052	
3. Hooded rats (5% margarine plus 750 mg. crude linoleic acid/100 g. diet)	A–E	0·818	0·865	1·150	0·331	0·373	0·489
S.E. of differences between means*			0·098			0·046	
4. Albino rats (5% margarine)	A–F	0·865	1·080	1·611	0·384	0·473	0·645
S.E. of differences between means*			0·089			0·042	

* These standard errors are based upon a joint error mean square calculated by the analysis of variance.

Table 12. Arithmetic means of kidney and heart weights of rats in different dietary groups.

than those of the inanition control rats,but this might be ascribed to one chance high value in the latter group(trio C).

Experiment 3.(Hooded rats:5 per cent.margarine plus 750 mg.linoleic acid per 100 g.diet).

The results are summarized in Table 12,from which trio F(see Table 8) has been omitted. The kidneys of the pyridoxin-deficient rats were significantly($P < 0.01$) heavier than those of the inanition and ad lib. control rats. The hearts of the pyridoxin-deficient rats were also significantly($P < 0.05$) heavier than those of the inanition and ad lib. controls.

Experiment 4.(Albino rats:5 per cent.margarine).

The results are summarized in Table 12. The kidneys of the pyridoxin-deficient rats were significantly($P < 0.01$) heavier than those of the inanition and ad lib. controls. The inanition control kidneys were significantly($P < 0.01$) heavier than the ad lib. control kidneys. The hearts of the pyridoxin-deficient rats were significantly($P < 0.01$) larger than those of the inanition and ad lib. controls.

Statistical analysis indicated that in all the above experiments sex did not influence the organ weights. No significant differences in heart and kidney weights of comparably treated rats in experiments 1,2 and 3 was observed,but the albino rat kidneys and hearts were

significantly($P < 0.01$) heavier than those of comparably treated rats in any of the other experiments.

The results summarized in Table 12 provide a striking example of the importance of using litter-mates in the design of these and similar experiments. For the heart weights the use of litter-mates produces a 35 per cent. increase in accuracy, while for the kidney weights a 152 per cent. increase in accuracy is attained. In other words, if litter-mates had not been used, 35 and 152 per cent. more animals would have been necessary to achieve the same accuracy. These figures for the improvement due to the use of litter-mates are derived as follows:-

A common measure of variability in statistics is the variance or mean squared error. The variance of a mean or a difference between means indicates its accuracy. This quantity decreases as the number of observations increases, so that, for example, the variance of the mean of ten observations is 1/10th the variance of each observation. If, therefore, two experiments are carried out and the variance in the first is twice that in the second, twice as many observations would be required in the first to give as accurate a result in the second. In the present experiment (Table 12) there are two estimates of variance, or variability,

1. the variance between animals from different litters, and 2. the variance between animals from the same litters. The ratios of these take the values 1.35 and 2.52 for the heart and kidney respectively. This shows that 100 litter-mates give the same accuracy as 135 or 252 unrelated animals. Naturally, as these figures are determined from this one experiment they are not to be regarded as absolute, but merely as indicative of an improvement due to the use of litter-mates.

5. RENAL PATHOLOGY

Interesting renal lesions, ranging from gross macroscopic pitting and scarring to changes visible only microscopically, were observed in most of the rats that had had haematuria (Tables 6, 7 and 8). A detailed account of these findings is in preparation and only a few general observations can, at present (December, 1949), be made.

Material Examined.

1. The kidneys of the rats mentioned in Tables 6, 7, 8 and 9.

2. The kidneys of the rats used in the antibody production experiments with a killed culture of *Bacterium typhosum* as antigen (Part 1, Table 3). Macroscopic haematuria was observed in 4 of the 7 pyridoxin-deficient rats shortly before these and their corresponding controls were killed on the 53rd day of the experiment.

Findings: 1. Macroscopic.

No abnormality was observed in any of the kidneys of the rats killed on the 53rd day of the deficiency(see 2 above). Gross scarring,however,was noted in the kidneys of several pyridoxin-deficient rats that had had protracted haematuria(Tables 6,7 and 8). Scarring was also observed in the kidneys of 2 of the 5 ad lib. control rats that had developed haematuria after receiving the synthetic diet plus crude linoleic acid(Experiment 3,Table 8). Scarring did not occur in all the rats that had had haematuria;the incidence in the hooded pyridoxin-deficient rats was as follows:-

Experiment	Incidence of scarring in pyridoxin-deficient rats
1. (Table 6)	4 out of 5 rats(Trio E excluded)
2. (Table 7)	1 out of 6 rats
3. (Table 8)	4 out of 6 rats
Total:	9 out of 17 rats

Scarring was not observed in the kidneys of any of the albino rats(Experiment 4,Table 9).

Fig.16 shows a typical pair of kidneys from a pyridoxin-deficient rat. One kidney was grossly scarred, but the other had only a few small,shallow pits.

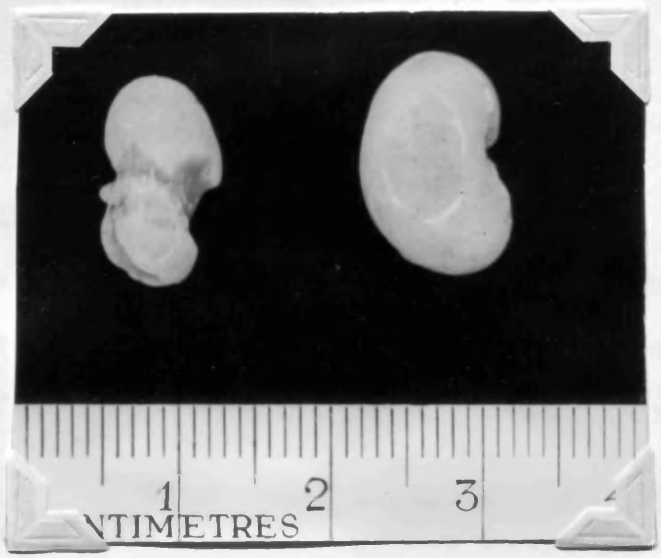

Fig.16. Kidneys of pyridoxin-deficient hooded rat (Table 8,trio C) showing gross scarring of one kidney and only slight damage(a few small, shallow pits) of the other.

Deposition of yellowish cretaceous material at the tip of the renal papilla was noted in a few cases,and this was verified histologically(see below). Hydronephrosis - possibly the result of partial obstruction of the ureter by a piece of this cretaceous material - was observed in one case(Table 7,trio D). The affected kidney was enlarged and pale,and clear fluid escaped from the grossly dilated pelvis when the kidney was sliced.

2. Microscopic.

Examination of the scarred and pitted kidneys confirmed the suspicion that the naked-eye appearance was the result of gross fibrosis. In several cases,early fibrosis without macroscopic scarring was noted,and Fig.17 shows an example of early subcapsular fibrosis. More severe scarring,with tubular dilatation,is seen in Figs. 18 and 19. Occasionally,tubular dilatation without obvious fibrosis was seen,and Fig.20 shows this in the medulla and Fig.21 in the renal papilla. In some cases, tubular dilatation without associated fibrosis appeared to be secondary to distal obstruction,e.g. at the tip of the papilla as in Fig.22. Calcification and destruction of the papilla tip was seen in several cases(Figs.22 and 23; compare with appearance of normal papillae in Figs.24 and 25). This lesion has been reported in the kidneys of

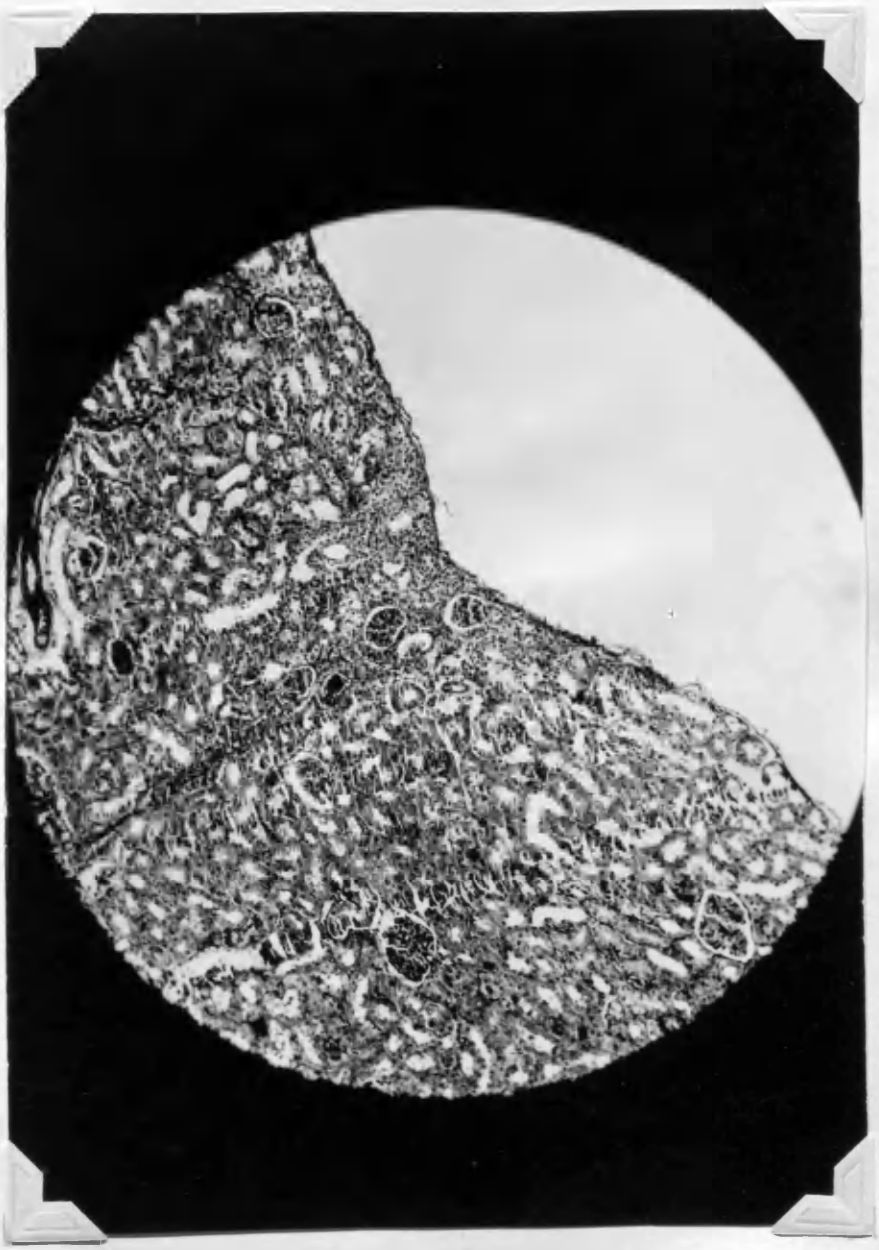

Fig.17. Puckered scar caused by early wedge-shaped subcapsular fibrosis.(Table 7,trio C). Masson trichrome. x 90.

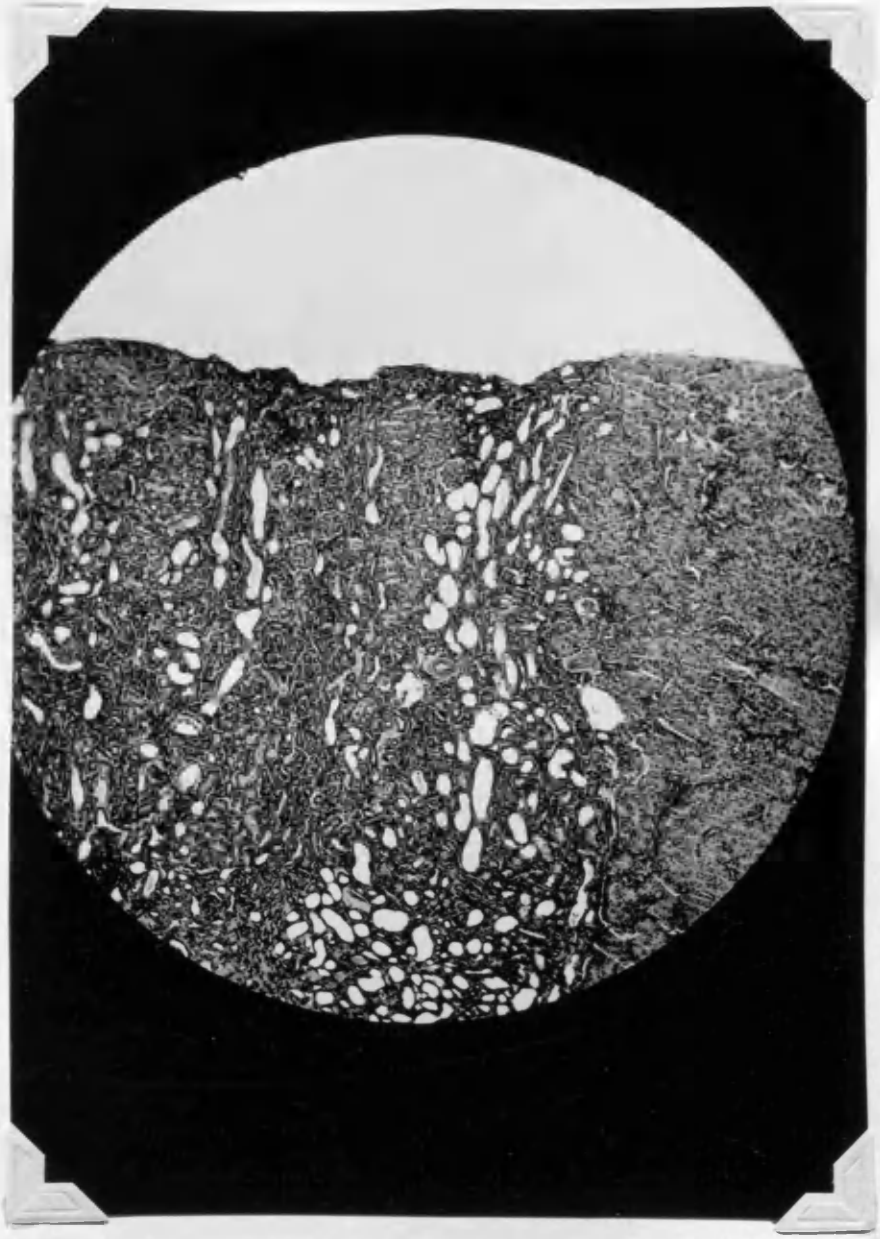

Fig.18. Alternating areas of normal renal cortex with areas of fibrosis and tubular dilatation. Cortical surface puckered over affected areas. (Table 6,trio A). Haematoxylin and eosin. x 65.

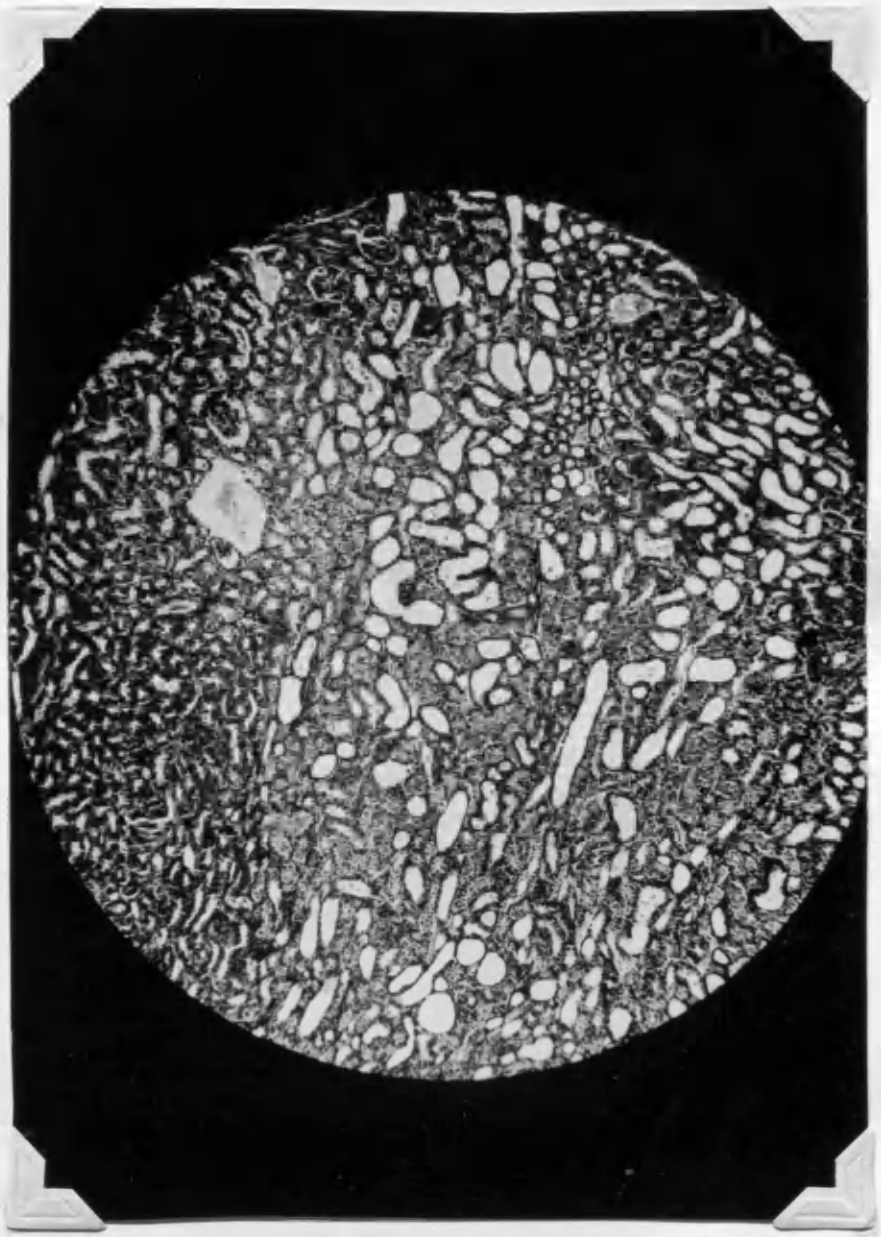

Fig.19. Cortical fibrosis and tubular dilatation. This ad lib. control rat(Table 8,trio D) received crude linoleic acid and developed haematuria. Scarred kidneys were observed at post mortem. Haematoxylin and eosin. x 65.

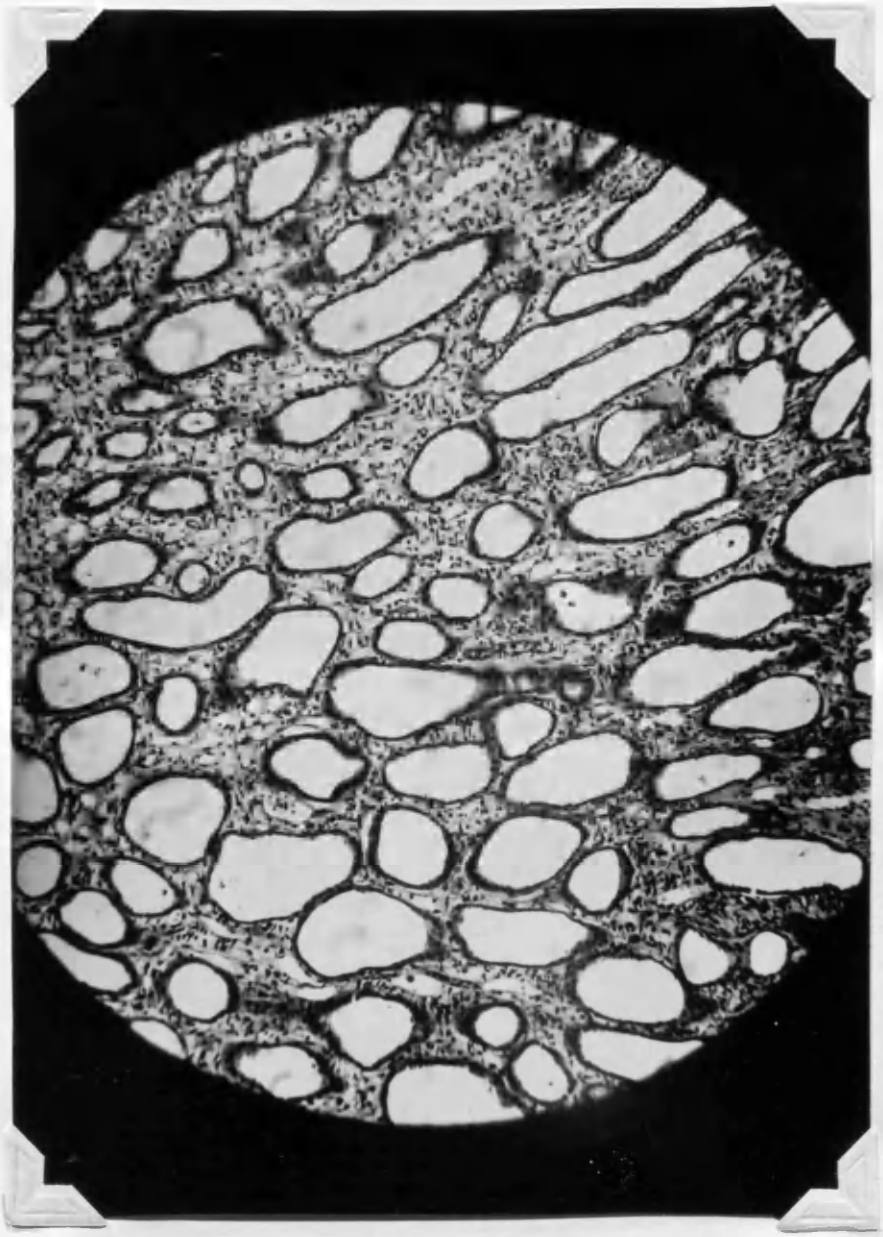

Fig.20. Tubular dilatation at junction of papilla and medulla. Well-marked flattening of tubular epithelium.(Table 6,trio A). Haematoxylin and eosin. x 110.

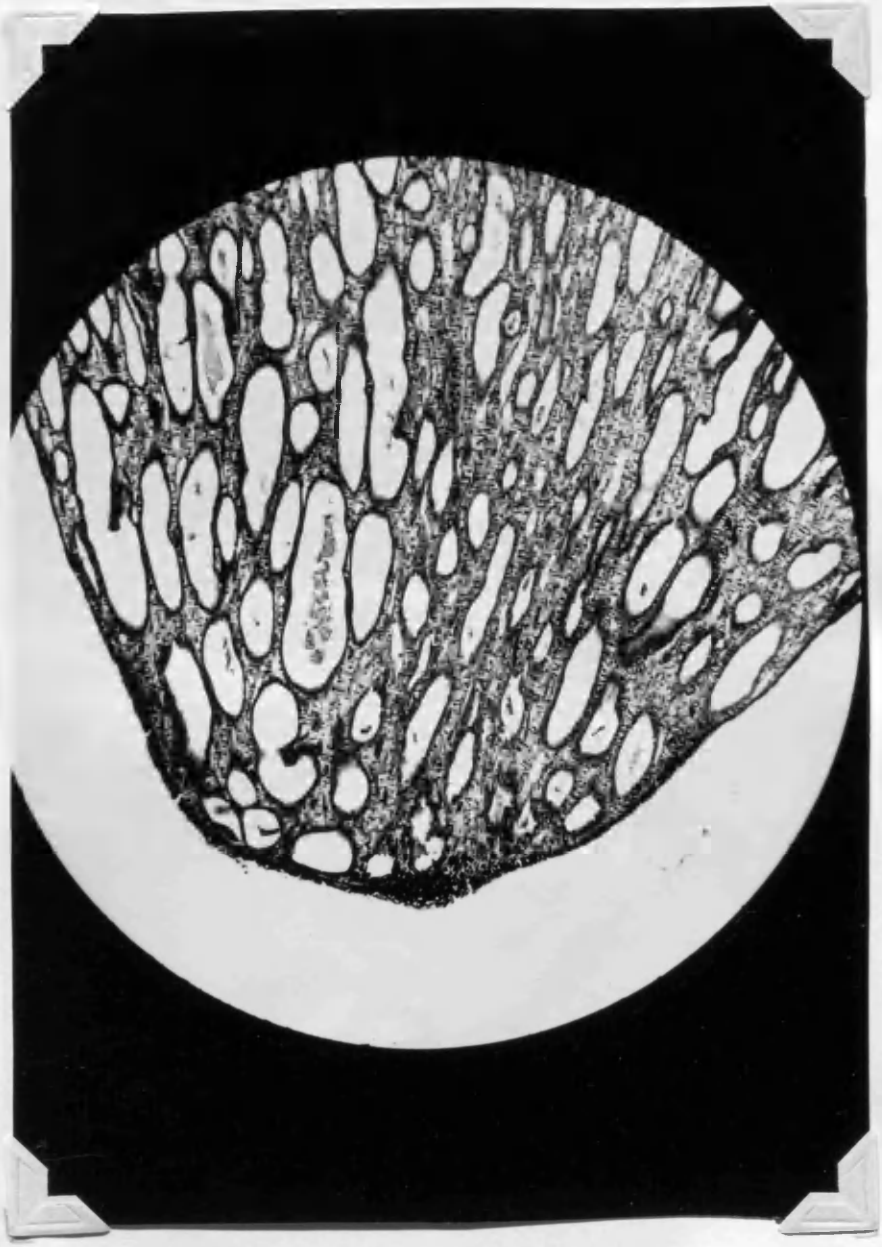

Fig.21. Dilatation of tubules in renal papilla. Cf. Figs.24 and 25. (Table 6,trio A). Haematoxylin and eosin. x 65.

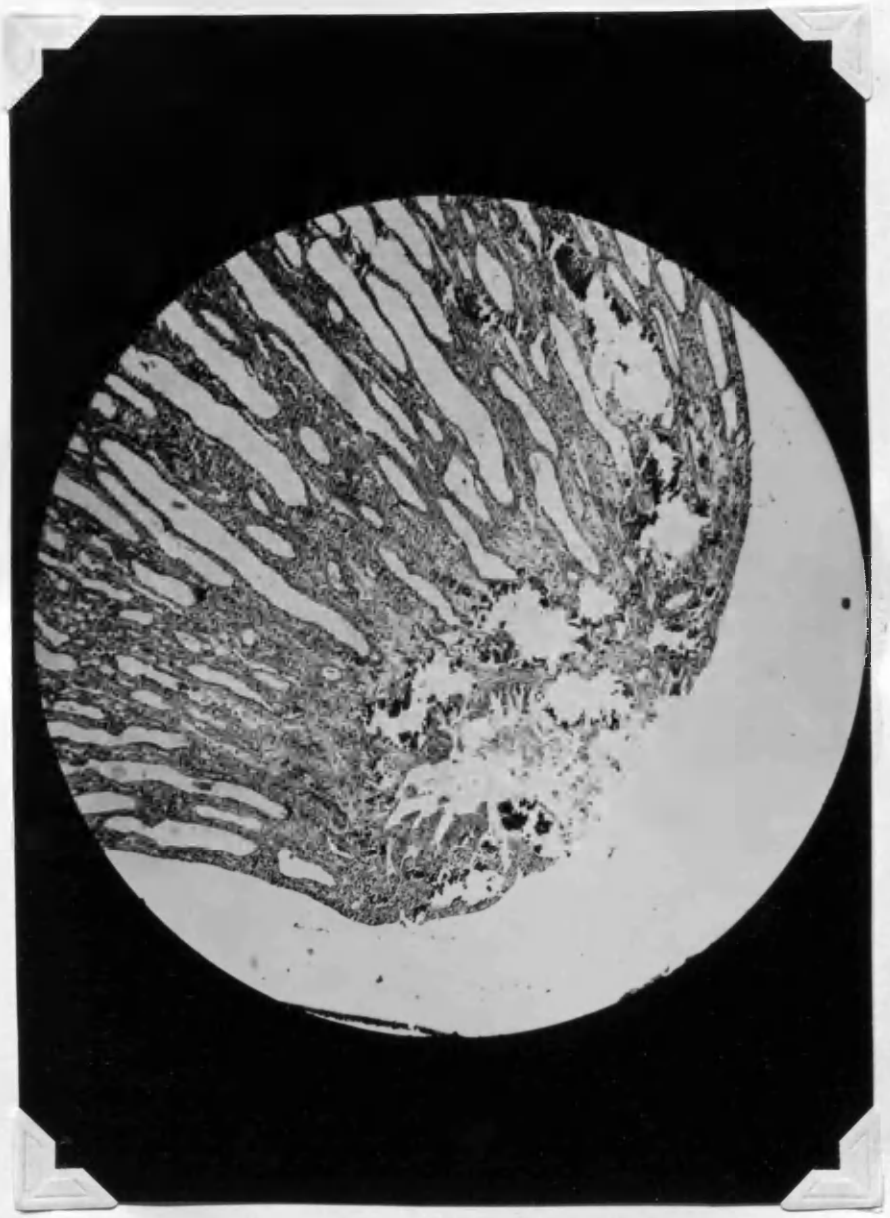

Fig.22. Destruction and "calcification" of papilla tip. Cf. Figs. 24 and 25. (Table 6,trio B). Haematoxylin and eosin. x 65.

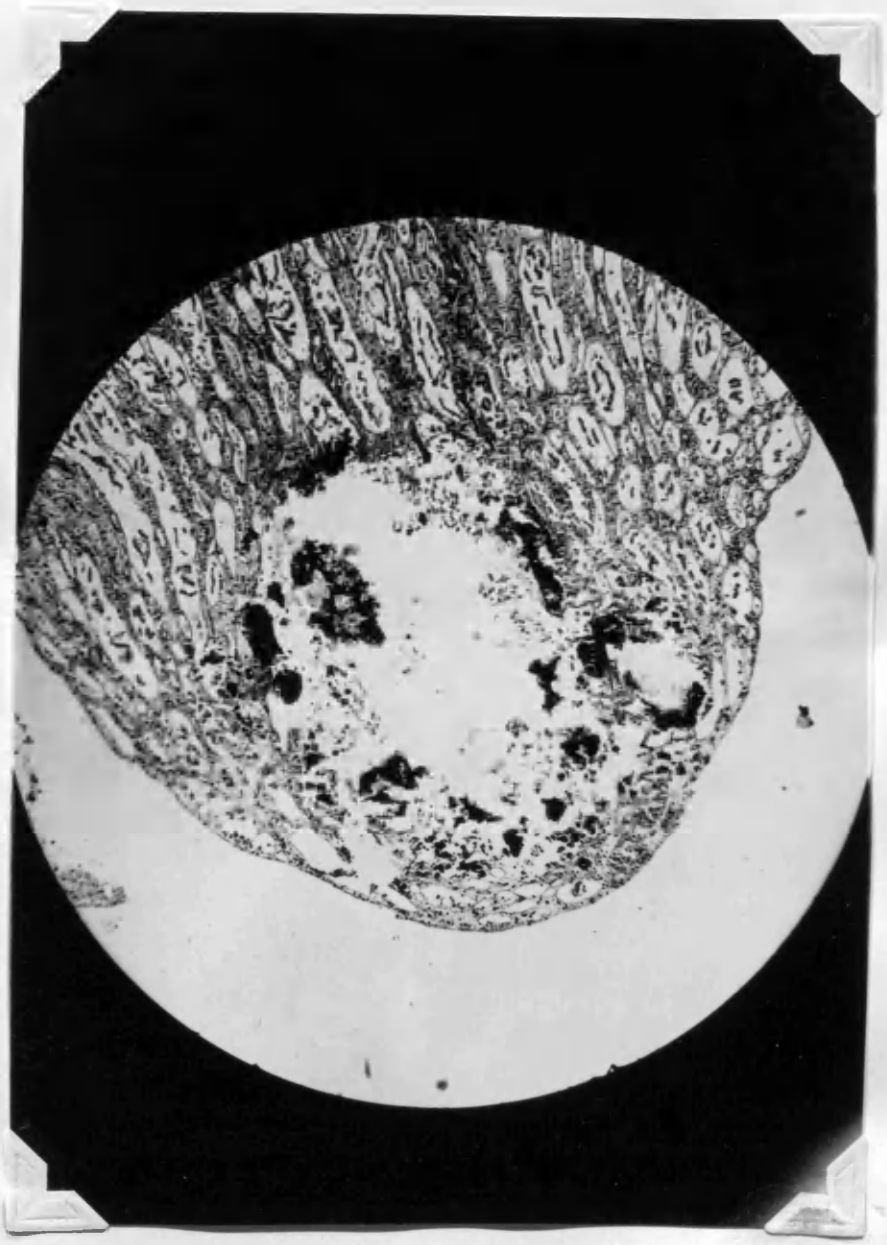

Fig.23. Destruction and "calcification" of papilla tip. Desquamated epithelial cells are present in most of the tubules, but this is probably the result of post mortem change as the rat died during the night, and the kidneys were not fixed until the next day. (Table 6, trio D).
Haematoxylin and eosin. x 65.

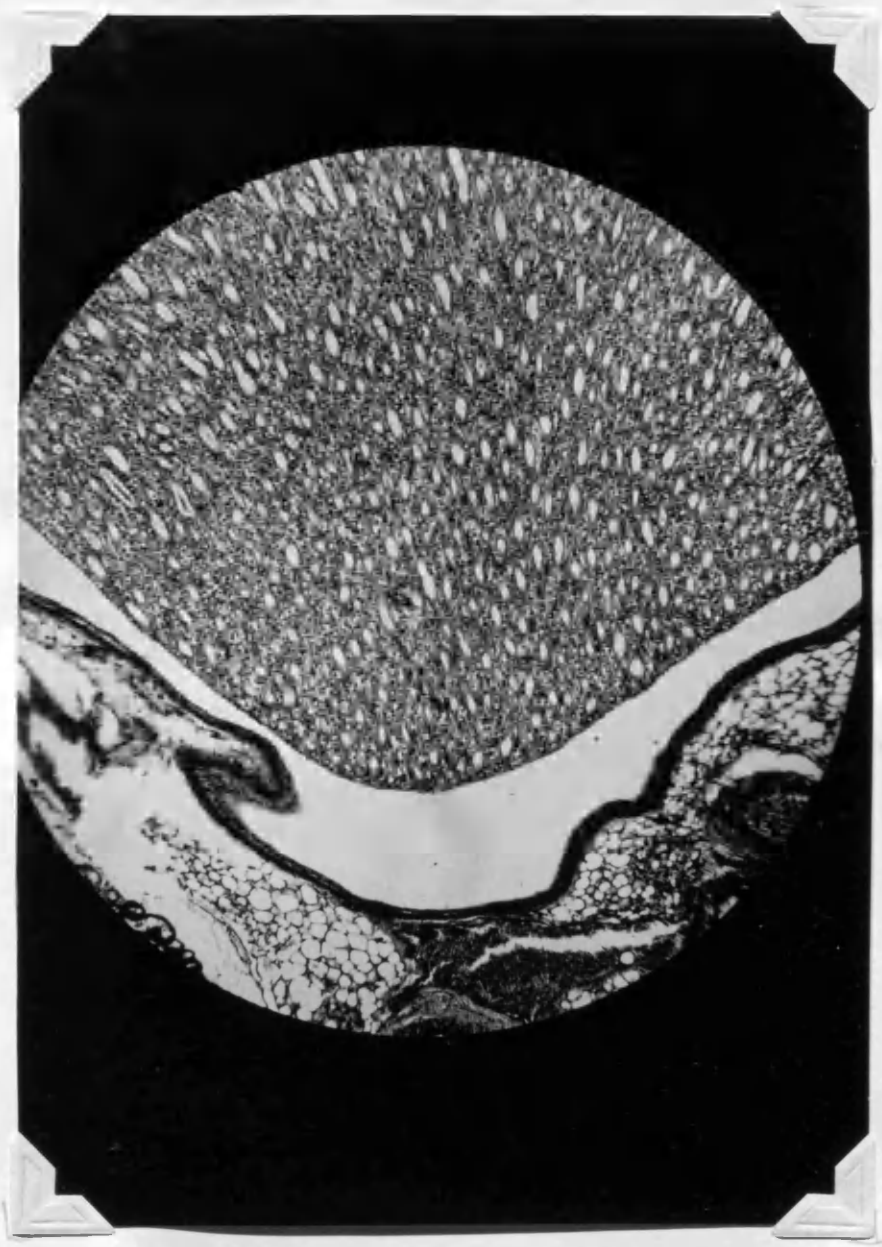

Fig.24. Longitudinal hemisection of normal papilla. Cf.Figs.21-3 and 28. Inanition control rat (Table 6,trio F).
Haematoxylin and eosin. x 65.

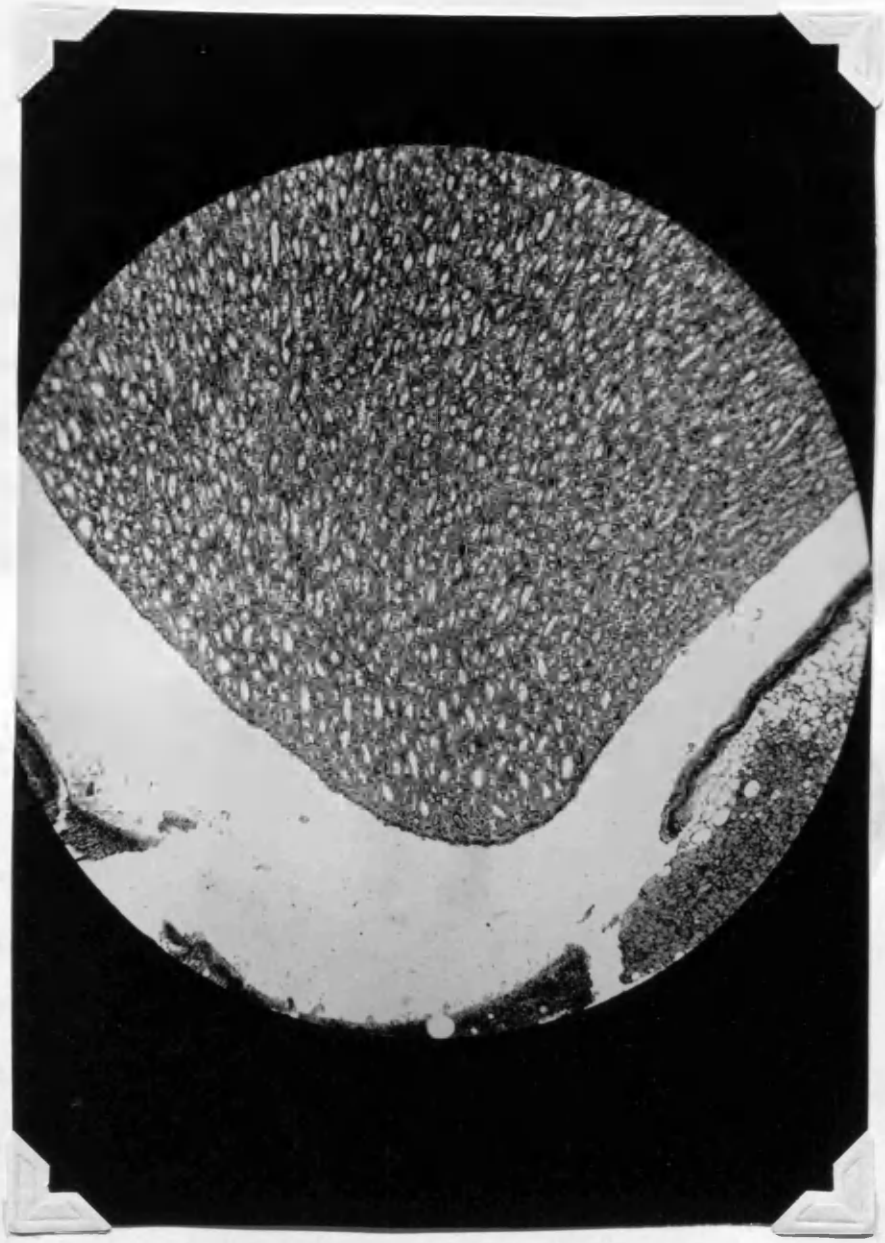

Fig.25. Longitudinal hemisection of normal papilla. Cf.Figs.21-3 and 28. Ad lib. control rat. (Table 6,trio C).
Haematoxylin and eosin. x 65.

essential fatty acid-deficient rats(Borland and Jackson, 1931),and as haematuria has often been noted in such rats(e.g.by Burr and Burr,1930,and Birch,1938) it seemed possible that fatty acid deficiency might have been a factor in the present work. However,as none of the inanition or ad lib. control rats used in Experiments 1 and 2(Tables 6 and 7) developed haematuria,and as 5 per cent.margarine and 5 per cent.lard had been fed respectively in these experiments,it is unlikely that an essential fatty acid deficiency could have occurred. Gross calcification - i.e.deposition of cretaceous material in the kidney tubules and characteristically stained by haematoxylin and rendered black by the von Kossa silver nitrate technique - was often observed,and was usually cortico-medullary in distribution(Fig.26). Calcium deposition in the rat kidney has,however,to be interpreted with care as it may occur in quite normal control or stock animals. Calcium deposition was frequently observed in many of the control rats(e.g.see Fig.27) of the present series but,with the exception of one animal,was never as marked as in Fig.26.

Severe pyelonephritis was observed post-mortem in two pyridoxin-deficient rats(Table 8,trio E;Table 9, trio E). Fig.28 shows gross erosion,destruction and

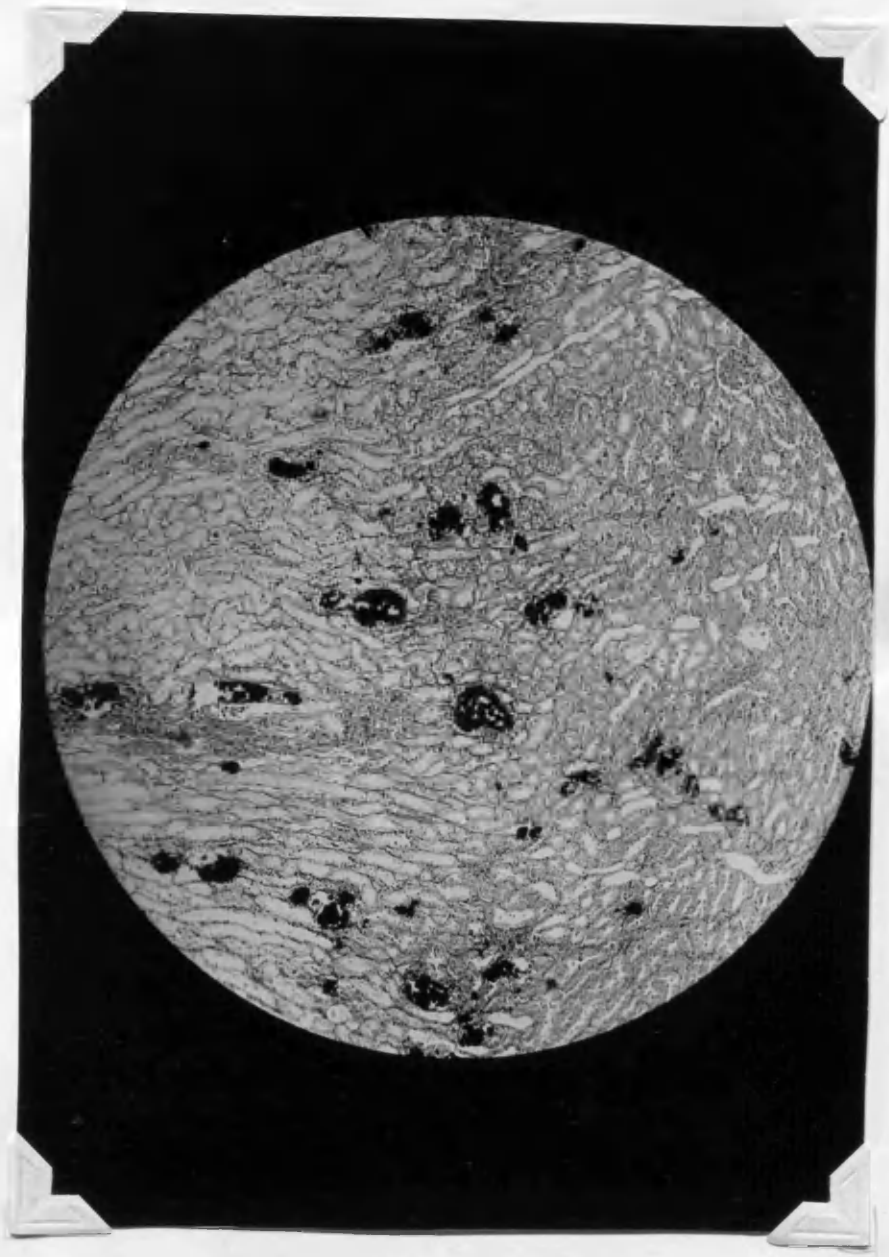

Fig.26. Calcium deposition in tubules in cortico-medullary region of kidney of pyridoxin-deficient rat(Table 7,trio F).
Von Kossa.
x 65.

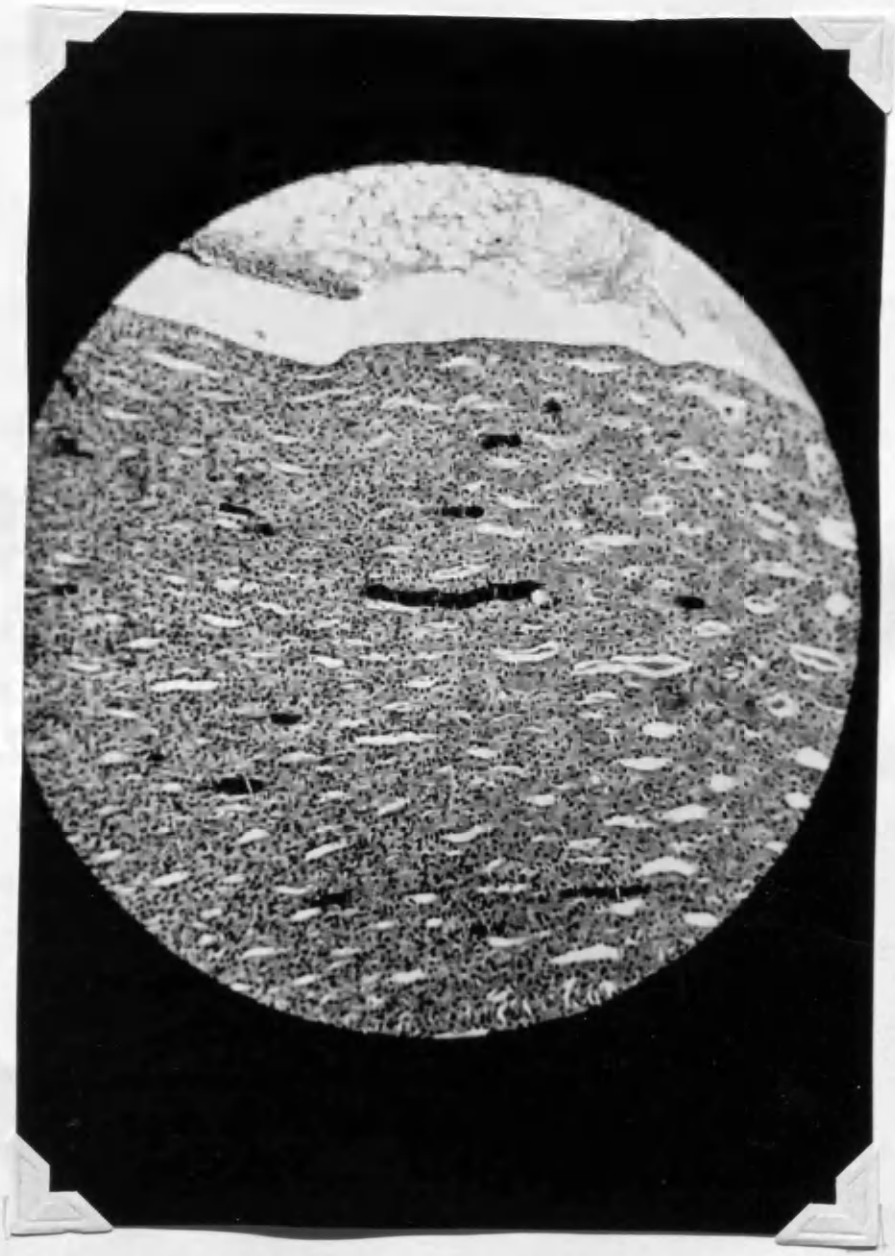

Fig.27. Calcium deposition in tubules of inanition control rat(Table 8,trio C).
Haematoxylin and eosin. x 90.

neutrophil infiltration of the papilla in this condition. Fig.29 shows an enlargement of a tubule in Fig.28 to show one of the many neutrophil casts. The appearance of the papilla is quite different from those shown in Figs.22-5. In two other pyridoxin-deficient rats(Table 6,trio A; Table 7,trio D) a few neutrophils were seen in occasional tubules(e.g.see Fig.30) and a diagnosis of mild pyelonephritis was made. The possibility that the haematuria and renal lesions observed in the pyridoxin-deficient hooded rats might have resulted from pyelonephritis of varying severity had therefore to be considered,especially as the work reported in Part 1 confirmed and extended the work of Stoerk and Eisen(1946),which showed impaired antibody production in pyridoxin-deficient rats. Although spontaneous abscess formation has occasionally been observed in pyridoxin-deficient rats(e.g.Table 8,trio B; and Part 1,Table 3,trio 16),the poor antibody response of pyridoxin-deficient rats to such antigens as sheep erythrocytes(Stoerk and Eisen,1946;Part 1,Table 2) or a killed culture of Bacterium typhosum(Part 1,Table 3) does not necessarily mean that such animals would be more susceptible to infection. Urine cultures from other pyridoxin-deficient rats with haematuria failed to reveal organisms(e.g. Salmonella enteritidis,see Smadel,1937) commonly associated with outbreaks of infective

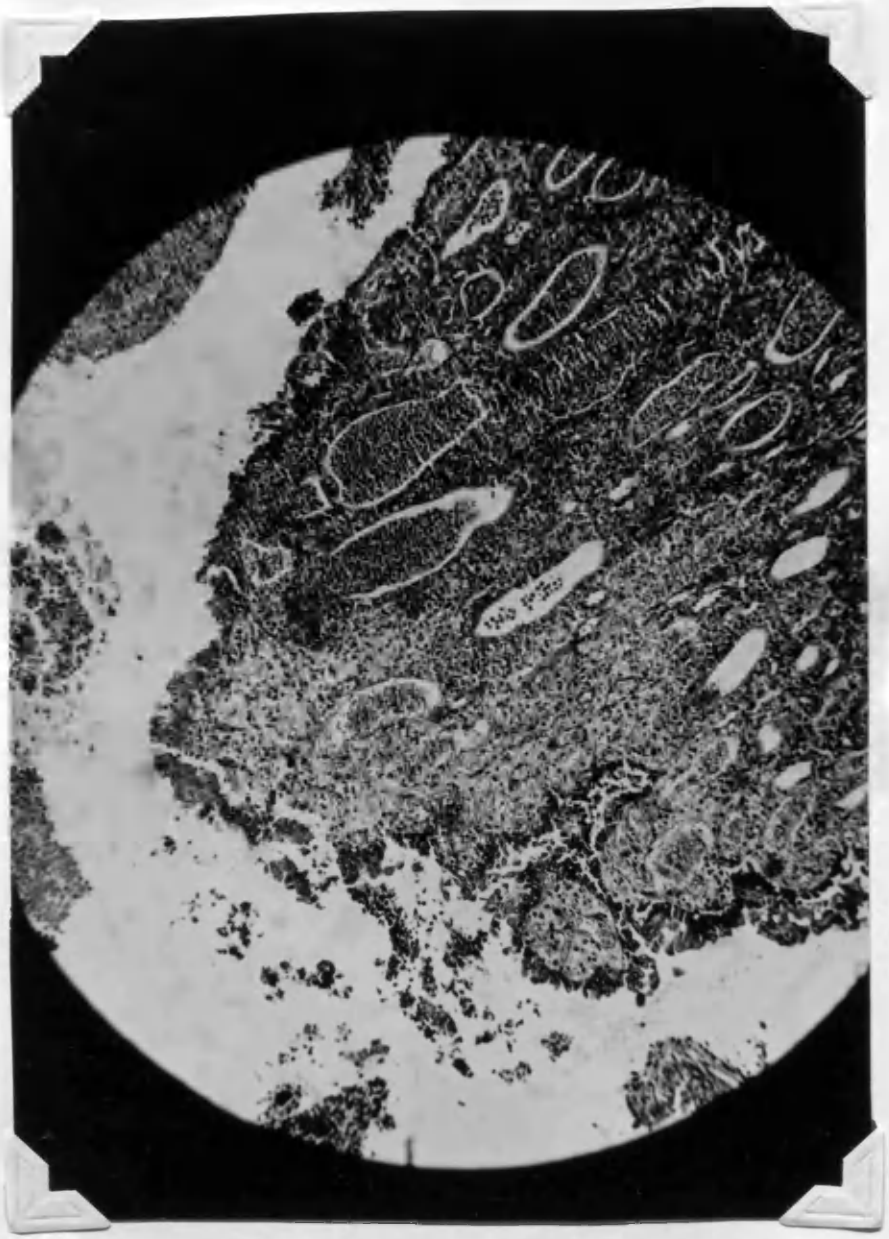

Fig.28. Gross erosion, destruction and neutrophil infiltration of papilla in a case of pyelonephritis. (Table 8, trio E).
Haematoxylin and eosin. x 110.

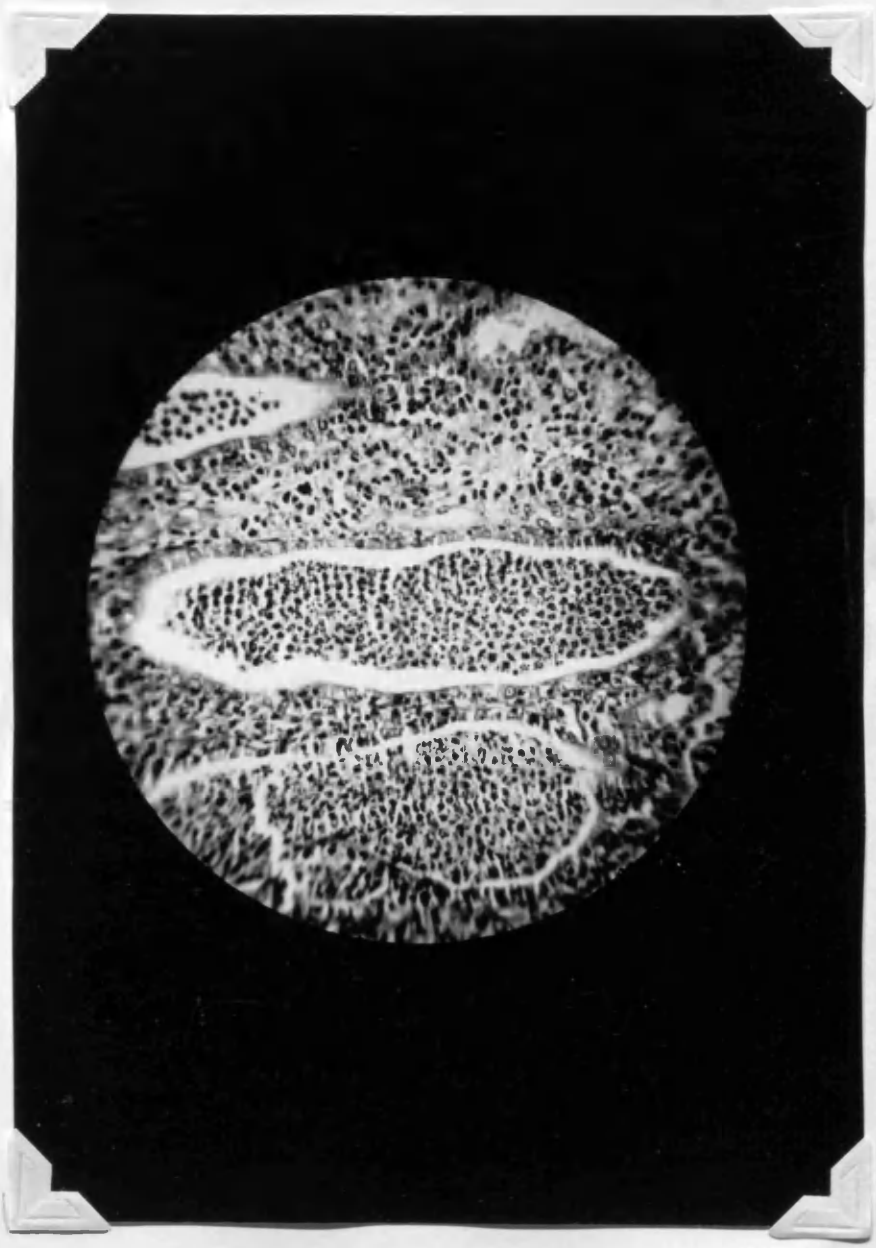

Fig.29. Enlargement of tubule from Fig.28 to show neutrophil cast.
Haematoxylin and eosin. x 220.

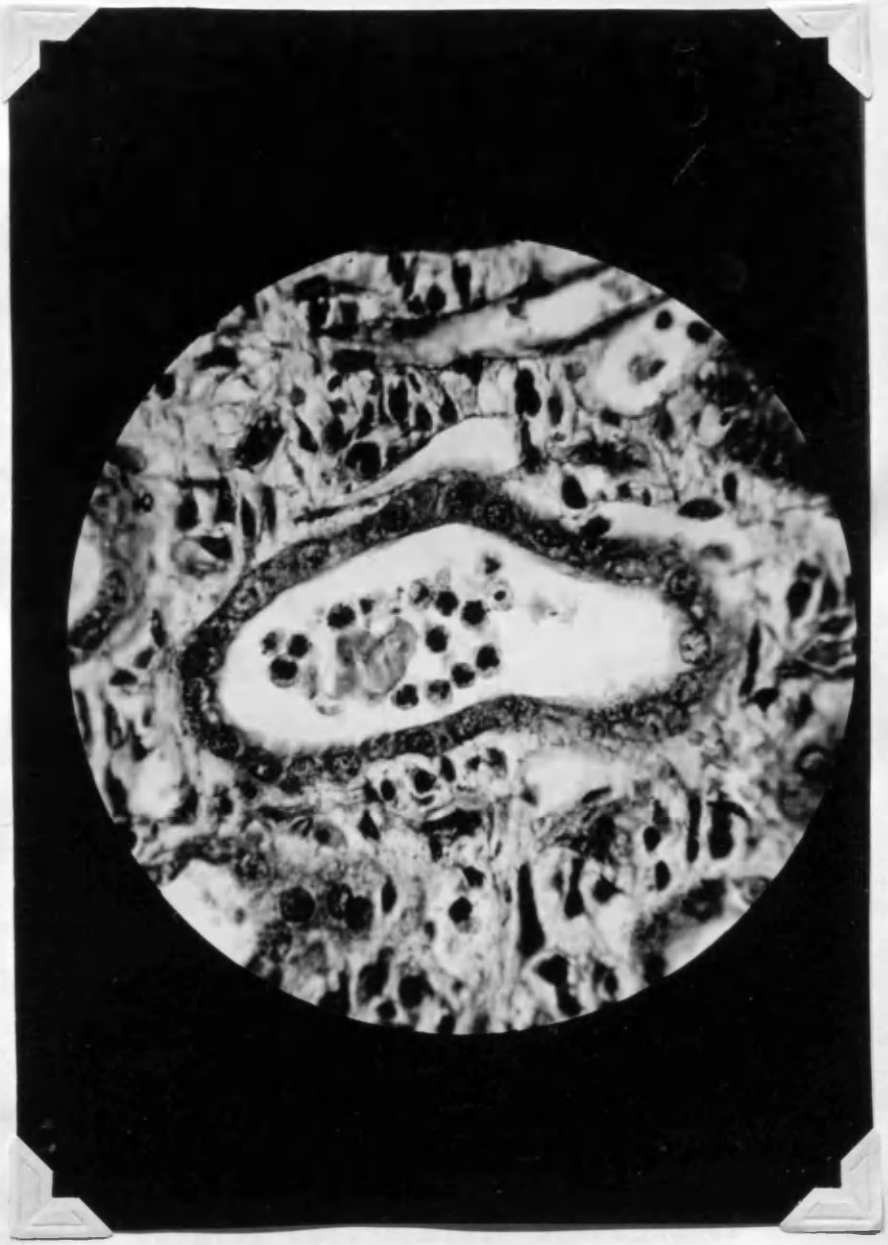

Fig.30. Mild pyelonephritis showing leucocytes - mainly neutrophils - in tubule.(Table 7,trio D). Haematoxylin and eosin. x 620.

pyelonephritis in stock rats. In earlier experiments, examination of the kidneys of rats killed shortly after the onset of haematuria did not reveal signs of early pyelonephritis and bacteria were not seen in Gram-stained sections. Further,pyelonephritis was noted in one of the kidneys of one of the pyridoxin-deficient albino rats but haematuria was not observed in any of these rats, although antibody production was probably impaired. It would seem unlikely that,with the exception of the four rats mentioned above,increased susceptibility to infective pyelonephritis by virtue of pyridoxin-deficiency could have accounted for the haematuria and renal lesions observed in these experiments.

Apart from the four cases of pyelonephritis just discussed,the severe lesions described above are presumably the late stages of pathological changes which must have been going on for many weeks during the deficiency. The earliest lesions observed were in rats killed within a few days of the onset of haematuria(2.above). In such cases,amorphous faintly eosinophilic subcapsular deposits were often seen. Figs.31 and 32 show typical deposits. In two rats,about 50 per cent. of the subcapsular spaces in the sections examined were involved. Figs.31 and 32 suggest strongly that the deposits result from the

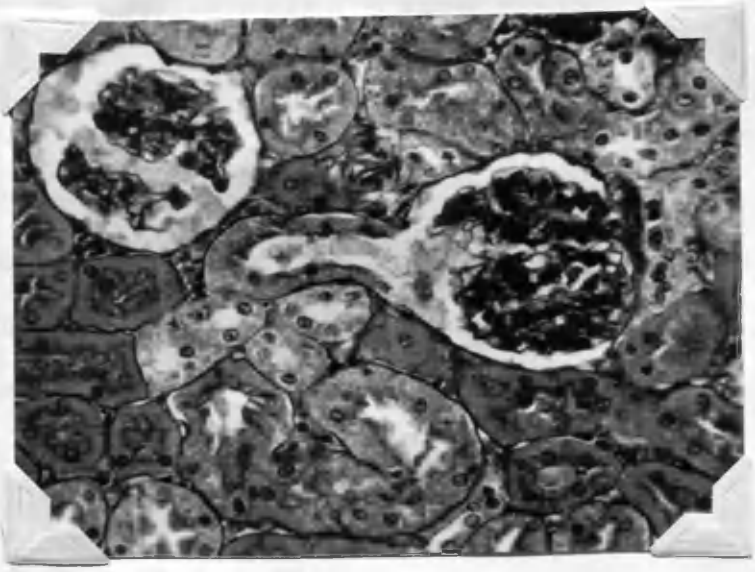

Fig.31.

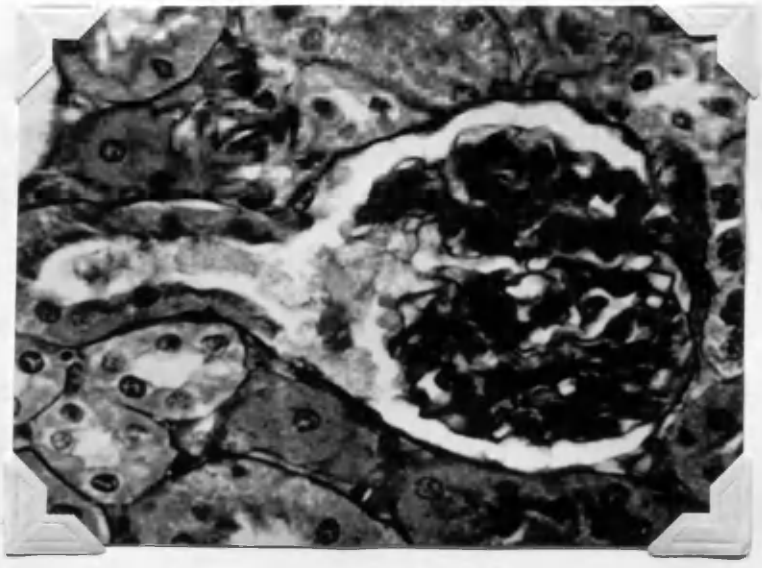

Fig.32. Enlargement of glomerulus seen on right above.

Figs.31 and 32. Subcapsular deposits(see text). Haematoxylin and eosin. Fig.31 x 390,Fig.32 x 660.

leakage of some material - possibly blood,which would explain the haematuria conveniently - through the glomerulus. Red cells were not,however,observed in the subcapsular spaces and staining of one or two sections of affected kidneys with Weigert's fibrin stain indicated that the deposits were not fibrin. The deposits were examined in polarized light and were not birefringent. The periodic acid - Schiff technique(McManus,1948) was found to give the best staining contrast for photomicrography(Figs.31 and 32).

Although cardiac hypertrophy was observed in the hooded and albino pyridoxin-deficient rats(Table 12), vascular lesions(e.g. arterial or arteriolar medial myohypertrophy) were not seen in the hearts and kidneys of these rats. If hypertension was responsible for the cardiac hypertrophy - and this seems the most likely explanation - it may be that the rats died or were killed before the vessels became noticeably involved or,less likely,that the degree of hypertension was not sufficient to cause vessel changes. It is unlikely that the cardiac hypertrophy was secondary to hypertension caused by renal damage,as the hearts of the albino rats were enlarged and haematuria and severe renal lesions were not observed in these rats.

6. STRAIN DIFFERENCES

The most striking difference between pyridoxin-deficient rats of the hooded and albino strains, apart from the failure of the albinos to develop haematuria, lay in the rate of development and severity of acrodynia-like skin lesions. Hooded rats fed the deficient diet did not develop skin lesions until after 4-5 months, the skin changes then observed being dry scaling of the dorsa of the hind paws and, later, dry necrosis of the ear tips. Usually the fur became rather unkempt and in one animal the fur of one side of the nose became denuded. Fits were noted in a few of the animals that had been at least four months on the deficient diet.

Within 50 days, however, 4 of the 6 pyridoxin-deficient rats developed severe acrodynia-like lesions (Figs. 33 and 34). The paws became red, hot and swollen; the fur was unkempt and occasionally "spiky"; a bloody exudate covered the snout (Fig. 34); and there was denudation of fur about the eye. Once started, these acrodynia-like lesions persisted. In 2 of the 6 pyridoxin-deficient albino rats these skin lesions did not become marked until the deficient diet had been fed for about 100 days. Three of the more severely affected animals died

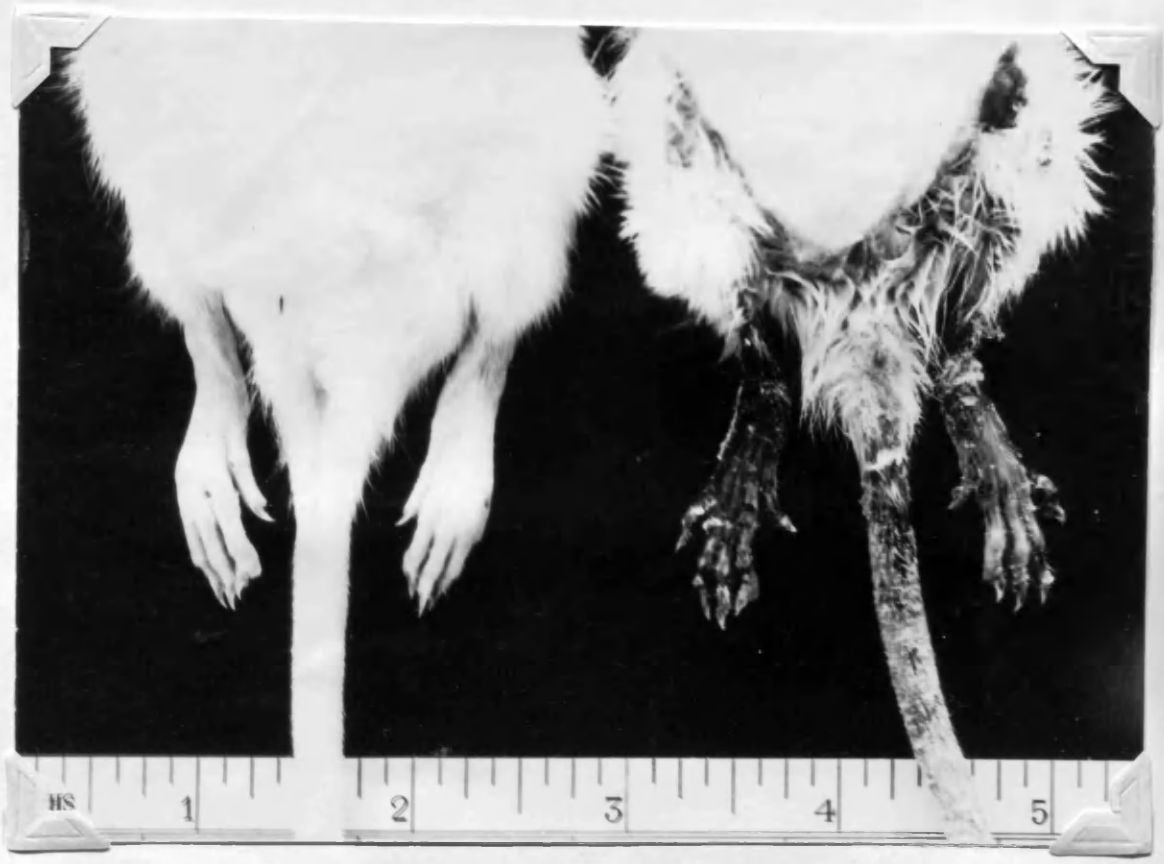

Fig.33. Acrodynia of hind paws of pyridoxin-deficient albino rat. Paired-weighed control rat on left.

Fig.34. Pyridoxin-deficient albino rat showing spiky fur, bloody exudate over snout, and swollen fore paws. Inanition control rat on right.

after relatively short periods on the diet(Table 9). The remaining three trios were killed(one accidentally), but in each case it was doubtful if the pyridoxin-deficient rat would have survived longer than another day or two. As indicated above,haematuria was not observed in any of the pyridoxin-deficient albino rats,although noted for variable periods in all of the pyridoxin-deficient hooded rats. The possible significance of the mild microscopic haematurias that were observed in two of the albino control rats(Table 9) will be discussed below.

No striking differences were noted between the growth curves of the albino and hooded pyridoxin-deficient rats(Figs.11-14). However,it seemed possible that the hooded rats did not develop severe acrodynia because their intestinal bacteria synthesised enough pyridoxin to protect them against this effect. Table 13 summarises the results of an experiment designed to test one aspect of this hypothesis. Nine male littermate hooded rats were divided into trios in the usual way. The pyridoxin-deficient rats of trios 2 and 3 were given orally 5mg. of desoxypyridoxin,the potent antivitamin of pyridoxin,by tube in 0.5ml distilled water on 10 occasions for trio 2 and 7 occasions for trio 3(see Fig.35),50mg. and 35mg. being given over periods of 18 and 14 days respectively.

The ad lib. and inanition control animals of the trios were given 0.5ml distilled water orally by tube on the occasions when desoxypyridoxin was given to the pyridoxin-deficient animals. The rats receiving desoxypyridoxin lost weight(Fig.35) and severe acrodynia-like lesions, particularly of the fore-paws,developed which were comparable to those hitherto noted only in the albino rats. Unkempt fur and a blood-stained exudate about the snout were also noted. Both rats died,and their kidneys and hearts were significantly($P < 0.001$,and $P < 0.02$ respectively) heavier than the kidneys and heart of the control pyridoxin-deficient rat of trio 1(Table 13). Haematuria was not observed in any of these rats. No skin lesions were observed in the control pyridoxin-deficient rat of trio 1.

It would seem,therefore,that administration of desoxypyridoxin to hooded rats already receiving a pyridoxin-deficient diet can cause,possibly by destruction of pyridoxin synthesised in the intestine,a striking increase in severity of acrodynia-like skin lesions. Carpenter,Harris and Kodicek(1948) appear to have obtained a similar result by feeding hooded rats 1 per cent. succinylsulphathiazole incorporated in a pyridoxin-deficient diet.

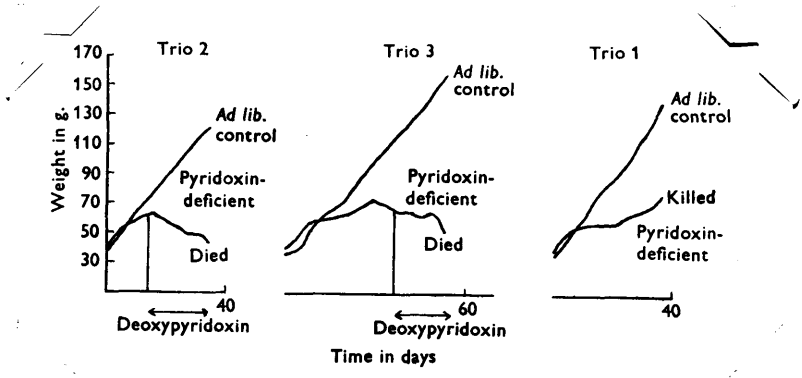

Fig.35. Growth curves of pyridoxin-deficient rat given desoxypyridoxin, of pyridoxin-deficient litter-mate, and of litter-mate ad lib. controls.

		Organ						
		Kidneys (g./100 g. body-weight)			Heart (g./100 g. body-weight)			
Trio	No. of days on diet	*Ad lib.* control	Inanition control	Pyridoxin-deficient	*Ad lib.* control	Inanition control	Pyridoxin-deficient	Remarks
1 ♂	36	0·940	1·173	1·263	0·429	0·501	0·442	Control trio (no deoxypyridoxin given)
2 ♂	36	1·074	1·328	2·504	0·437	0·549	0·640	Deoxypyridoxin tube-fed to pyridoxin-deficient rat
3 ♂	54	0·953	1·164	2·400	0·436	0·463	0·731	Deoxypyridoxin tube-fed to pyridoxin-deficient rat
Arithmetic means (trios 2 and 3)		1·014	1·246	2·452	0·437	0·506	0·686	

Table 13. Effect of desoxypyridoxin administration on kidney and heart weights of hooded rats on pyridoxin-deficient diet.

Studies on vitamin biosynthesis and similar microbiological problems in the rat have been hampered by failure to obtain ready access to the caecal contents in order to study changes in the flora and vitamin content over long periods of time. Although the rat has been so frequently employed in nutritional investigations it does not appear to have been subjected to studies with the fistula technique. A one-stage permanent colostomy operation was therefore devised(Agnew,1949b).and in such rats ready access to the caecal contents is possible at any time. Also,each rat could be said to act as its own control as regards changes in,for example,caecal flora. Apart from their use in microbiological problems such as refection,these rats can be used in many other ways. Full details of the operative technique and of the uses of these animals are given in the reprint at the end of the thesis.

DISCUSSION

Haematuria was observed for periods of variable duration in all the pyridoxin-deficient hooded rats (Tables 6,7 and 8). Replacement of 5 per cent. margarine with 5 per cent lard as the source of fat in the diet (experiments 1 and 2) did not significantly influence the mean time of onset or the severity of the

bleeding(Tables 6 and 7),but in rats fed 5 per cent. margarine plus crude linoleic acid(750mg./100g.diet) the mean time of onset was later(Table 8). The reason for this is unknown,but the animals in this experiment (experiment 3) were initially rather heavier(Fig.13) than those of experiments 1 and 2(Figs.11-12) and "storage" of pyridoxin(Cerecedo and Foy,1942) rather than any possible protective effect of linoleic acid,may have determined the later onset of haematuria. Five of the 6 ad lib. control rats fed linoleic acid developed haematuria(Table 8). Although Kratzer and Williams(1948) observed growth reduction,curable by pyridoxin,in chicks fed linseed-oil meal at a level of 10 per cent. or higher in the diet,and linseed-oil meal is a rich source of linoleic acid,there is no evidence that the ad lib. control rats of experiment 3 developed pyridoxin deficiency. Growth was unsatisfactory in only one ad lib. control rat(Fig.13,trio A) and this was probably due to severe haematuria and not to pyridoxin-deficiency. Further,none of the inanition control rats developed haematuria although the effect of depriving an inanition control rat of pyridoxin(Table 6, trio E) suggests that these animals might be especially sensitive to lack of pyridoxin. As mentioned above,the linoleic acid used(B.D.H.technical) may have been unsuitable for nutritional studies. However,a direct

toxic action is unlikely as the inanition control rats fed the complete diet plus linoleic acid did not develop haematuria. For the same reason it is unlikely, although it is possible, that one or more of the vitamin supplements might have been inactivated by contact with the linoleic acid when the diet was made up in bulk, and that this could have been a contributory cause of the haematurias in the ad lib. control rats. Gyorgy et al. (1942 have reported that crude linoleic acid incorporated in a synthetic diet and fed at the high level of 16 per cent. proved toxic for rats. Loss of weight, progressive anaemia and leucopenia were observed in their rats but haematuria is not mentioned. Of interest was their finding that these toxic manifestations could be neutralised preventively and therapeutically by administration of yeast. In this connection it is possibly significant that pathological changes were seen in the kidneys of the above ad lib. control rats that had had haematuria that were similar to those observed in pyridoxin-deficient rats.

Tables 6,7 and 8 show that the incidence of haematuria in the pyridoxin-deficient hooded rats was 100 per cent. (18 of 18 animals). However, there may be great variation in the incidence and time of onset of

the bleeding. For example,haematuria was observed in 4 of 7 pyridoxin-deficient rats of trios used in studies of antibody production(Part 1,Table 3) and killed after 53 days on the deficient diet. Again,haematuria was noted in 7 pyridoxin-deficient rats after the diet had been fed for 34,39,40,62,63,74 and 75 days respectively(Agnew,1948-49);the true incidence is not known as trios of rats were killed throughout the experiment after different periods on the diet,but was probably less than 50 per cent..

Haematuria was not observed in any of the pyridoxin-deficient albino rats(Table 9) although red cells were detected microscopically in the urine of the ad lib. and inanition control rats of one trio(Table 5,trio B). The rat normally excretes protein in the urine and possibly a greater degree of patency of the glomerular membrane than usual might allow the escape of an occasional red cell. Occasionally,a few red cells may be observed in the tubules of quite normal rats(Fig.36). Microscopic haematuria of variable duration has also been reported in apparently normal rats by Cavelti and Cavelti(1945) and by Humphrey(1948),and no significance is attached to the microscopic haematuria in the above albino control rats. Presumably strain differences were responsible for the failure of the pyridoxin-deficient albino rats to develop

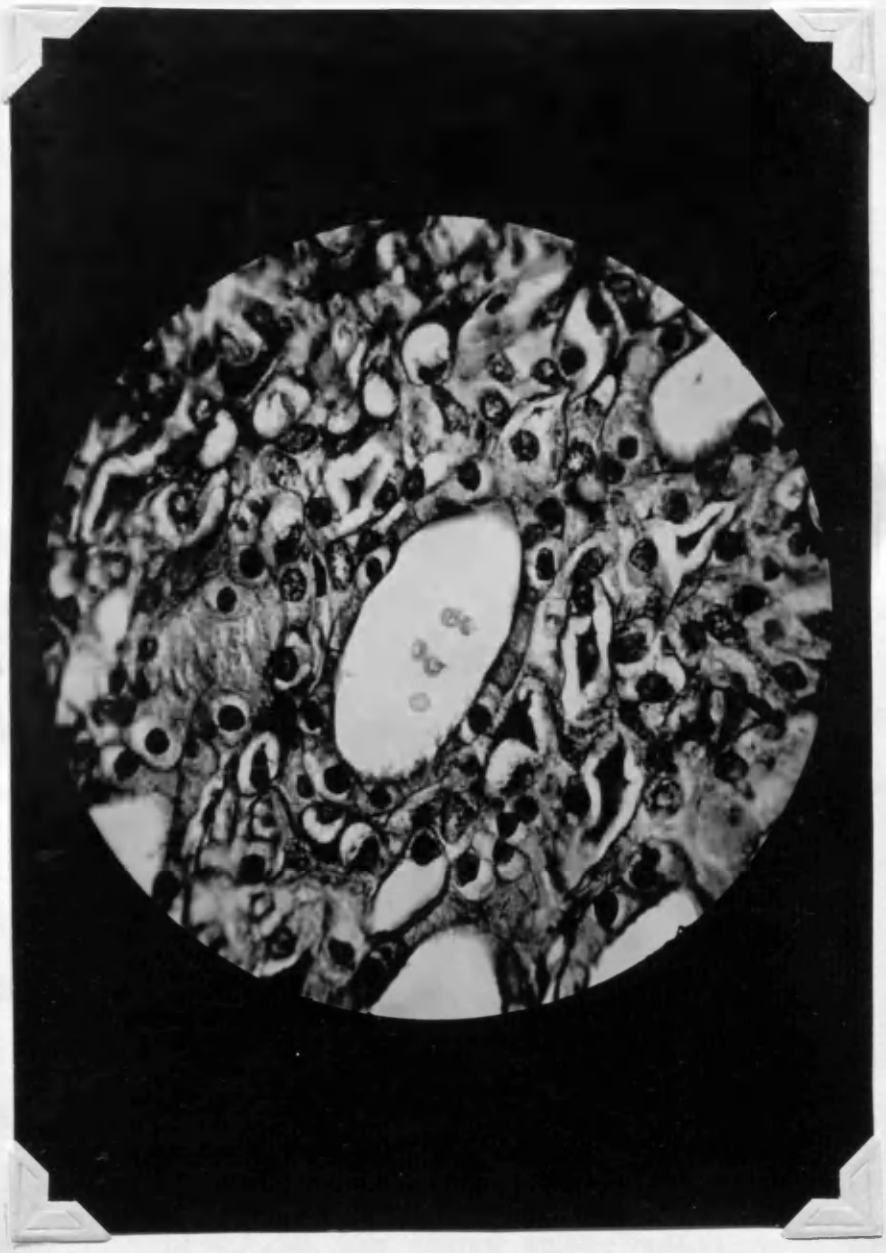

Fig.36. 5 red blood cells in tubule of ad lib. control rat(Table 9,trio C). Haematoxylin and eosin. x 620.

haematuria. Smadel and Swift(1941) studied the effect of administration of anti-kidney serum to three different strains of rat and found that Wistar albino rats were less susceptible than Whelan(usually hooded) or Evans (usually hooded) rats,and it may well be that the factors which determined these differences in susceptibility to anti-kidney serum were operative in the present experiments. Although haematuria was not observed in the pyridoxin-deficient albino rats the kidneys of these rats were,like those of the pyridoxin-deficient hooded rats,significantly heavier(g./100g.body-weight) than those of the corresponding inanition and ad lib. control rats(Table 12). The hearts also of the pyridoxin-deficient albino and hooded rats were generally significantly heavier(g./100g. body-weight) than those of the corresponding inanition and ad lib. control rats,presumably because of some degree of hypertension. Recent work by Hartroft and Best(1949) on the development of hypertension in choline-deficient rats with residual kidney damage is of interest in this connection;however,in the above experiments,severe renal lesions were not observed in the pyridoxin-deficient albino rats although cardiac and renal hypertrophy were noted.

Only Birch(1938) appears to have observed

haematuria in pyridoxin-deficient rats,but bleeding appeared as a terminal symptom after his animals had been about 16-20 weeks on the deficient diet. Also,it is possible that he was not dealing with uncomplicated pyridoxin deficiency.

The failure of administration of pyridoxin to influence the duration or severity of the haematuria (Table 6,trio B;Table 7,trios B and C) is interpreted as an indication that irreversible renal damage had occurred. Administration of pyridoxin shortly after the onset of haematuria might have been more effective but it would then be impossible to be sure that the bleeding would not have ceased in any case(see Table 6,trio C).

The effect of administration of desoxypyridoxin to hooded rats fed the pyridoxin-deficient diet suggests that there may be a direct relationship between the severity of pyridoxin depletion and the weight of the kidneys(Table 13). If the rate of development and severity of acrodynia-like skin lesions are taken as an index of the severity of pyridoxin depletion,and the experiments with desoxypyridoxin seem to justify this assumption, then the albino rats were exposed to a more severe depletion of pyridoxin than the hooded rats. But haematuria

was not observed in the pyridoxin-deficient albino rats. It would seem, therefore, that the degree of severity of pyridoxin depletion was not a factor in the present experiments in determining the observed strain differences in susceptibility to the development of haematuria.

A direct relationship is known to exist between the degree of severity of pyridoxin deficiency and the percentage of protein fed in the diet. In all the experiments reported here the level of casein in the diet was 18 per cent.. Kidney hypertrophy has been reported in rats fed high protein diets(Reader and Drummond,1926;Hartwell, 1928;MacKay,1933). These workers have also shown that the increase in kidney weight could be prevented(Reader and Drummond,1926;Hartwell,1928) or greatly lessened(MacKay, 1933) by increasing the amount of yeast in the diet. It is possible that pyridoxin deficiency, produced indirectly by an increase in the percentage of protein in the diet, was responsible for the kidney hypertrophy described by these workers. This hypothesis would conveniently explain the beneficial action of the yeast and also why, in the experiments of Reader and Drummond(1926), poor growth resulted when a diet containing 70 per cent. of protein was supplemented with only the same proportion(4 per cent.) of yeast extract that sufficed for good growth with 20 per cent. of protein.

SUMMARY OF PART 2 OF THESIS

1. Severe haematuria of variable duration was observed in all of 18 hooded rats fed a pyridoxin-deficient diet with 5 per cent. margarine,5 per cent. lard,or 5 per cent. margarine plus 750mg./100g. diet of crude linoleic acid. Haematuria was unexpectedly observed in 5 of 6 of the ad lib. control hooded rats receiving the crude linoleic acid supplement,and possible reasons for this are discussed. Haematuria was not observed in any of the paired-weighed control hooded rats.

2. Haematuria was not observed in albino pyridoxin-deficient rats although these rats developed far more severe acrodynia-like skin lesions than pyridoxin-deficient hooded rats. The degree of severity of pyridoxin depletion, as gauged by the rate of development and severity of acrodynia-like skin lesions,did not appear to be the factor determining the observed strain differences in susceptibility to the development of haematuria.

3. Preliminary experiments suggest that the failure of pyridoxin-deficient hooded rats to develop acrodynia-like skin lesions as severe as those observed in albino rats was due to endogenous biosynthesis of pyridoxin,as administration of desoxypyridoxin to hooded rats already

receiving a pyridoxin-deficient diet resulted in the development of severe acrodynia-like skin lesions similar to those hitherto seen only in albino rats.

4. The severity of haematuria did not influence the blood picture, essentially that of microcytosis, of the pyridoxin-deficient hooded rats.

5. Pyridoxin administration did not ameliorate the haematuria once established; possible reasons for this apparent anomaly are discussed. Ascorbic acid and rutin, which were tried in one case, were similarly ineffective.

6. Spontaneous infective pyelonephritis resulting from the impaired antibody production in pyridoxin deficiency is not thought to account for the development of haematuria in these experiments.

7. Irrespective of strain, and hence irrespective of the presence of haematuria, the hearts and kidneys of the pyridoxin-deficient rats were usually significantly heavier (g./100g. body-weight) than those of corresponding paired-weighed and ad lib. control rats. Statistical analysis of these heart and kidney weights indicated that a striking increase in experimental accuracy had been obtained by the use of litter-mate rats.

8. A brief description is given of renal lesions, ranging from gross macroscopic pitting and scarring to changes visible only microscopically, which were observed in most of the hooded rats that had had haematuria.

9. A one-stage permanent colostomy operation has been devised for the rat, and the value of this procedure in microbiological and other problems is briefly indicated.

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