SUMMARY

1. Revised curves relating activity of the enzyme to pH have been obtained for elastase, the optimum pH range for which has been shown to be 8.7-9.2.

2. Although, if adequately buffered, the enzyme is capable of dissolving elastic tissue from three sources at this pH equally efficiently, the products of the reaction are not universally the same.

3. Increasing amounts of sulphuric acid are released from aortic preparations especially above an initial pH of 9.8, smaller amounts are liberated from ox ligament.

4. Evidence is advanced that elastase consists of two enzymes which act on two components of an elastic tissue preparation which is itself dual in nature.

5. The optimum pH of the second enzyme which utilizes alkali-treated elastic tissue as substrate lies in the range 7.8-8.1.

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REFERENCES

- Balo, J. & Banga, I. (1949*a*). *Schweiz. Z. Path.* **12**, 350.
 Balo, J. & Banga, I. (1949*b*). *Nature, Lond.*, **164**, 491.
 Balo, J. & Banga, I. (1950). *Biochem. J.* **46**, 384.
 Banga, I., Balo, J. & Nowotny, A. (1949). *Z. Vitam.-Horm.-u. Fermentforsch.* **2**, 415.
 Banga, I. & Schuler, D. (1953). *Acta physiol. hung.* **4**, 13.
 Ewald, A. (1890). *Z. Biol.* **26**, 3.
 Hall, D. A. (1953). *Biochem. J.* **55**, xxxv.
 Hall, D. A. (1955). *Biochem. J.* **59**, 459.
 Hall, D. A., Reed, R. & Tunbridge, R. E. (1952). *Nature, Lond.*, **170**, 264.
 Lansing, A. I., Rosenthal, T. B., Alex, M. & Dempsey, E. V. (1952). *Anat. Rec.* **114**, 555.
 Vogel, A. I. (1943). *A Textbook of Quantitative Inorganic Analysis*, p. 808 *et seq.* London: Longmans Green and Co.

Observations on the Relationship Between Pteroylglutamic Acid and Nicotinamide Metabolism

By PREMA FATTERPAKER, URMILA MARFATIA AND A. SREENIVASAN

Department of Chemical Technology, University of Bombay, India

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Slightly depressed growth and fatty livers have been reported in rats fed moderate amounts of nicotinamide, together with a low protein diet (Handler & Dann, 1942; Handler & Bernheim, 1943). The fatty livers were susceptible to the action of choline, while methionine was found to alleviate the growth inhibition. Since excess of nicotinamide is detoxicated and eliminated as urinary *N*¹-methylnicotinamide, the observed toxicity of nicotinamide at unphysiological levels of feeding was attributed to forced methylation and consequent depletion of labile methyl reserves (Handler, 1944). In agreement with this, Handler observed no signs of toxicity as a result of ingestion of large quantities of nicotinamide by young rabbits or guinea pigs, neither of which species excrete *N*¹-methylnicotinamide (Handler, 1944). Production of fatty livers by feeding excessive amounts of glycocyamine, cystine or homocystine have also been reported (du Vigneaud, Chandler, Moyer & Keppel, 1939; du Vigneaud, Chandler, Cohn & Brown, 1940; Stetten & Grail, 1942). More recently, it has been shown that, in the pyridine-fed rat, there is a decreased urinary excretion of creatine owing to a reduction of available methyl groups, pyridine being detoxi-

cated as *N*-methylpyridinium hydroxide (Dinning, Keith, Parsons & Day, 1950).

The known mediation of pteroylglutamic acid in biological methylations including the methylation of nicotinamide (Fatterpaker, Marfatia & Sreenivasan, 1951, 1952*a*, 1954; Dietrich, Monson & Elvehjem, 1952) suggested the present studies on its influence on the extent of depletion of methyl reserves in rat livers consequent on ingestion of excessive amounts of nicotinamide. The studies included observations on a number of other substances and enzymes expected to be influenced by changes in pteroylglutamic acid or available labile methyl compounds. Pteroylglutamic acid deficiency was induced in the first set of experiments by dietary means, while in the second series aminopterin feeding was used. In the first series, additional groups with choline supplementation were included.

EXPERIMENTAL

Methods

Urinary excretions of creatine (Clark & Thompson, 1949), *N*¹-methylnicotinamide (Huff & Perlzweig, 1947) and total nicotinic acid (Swaminathan, 1946), liver N (Johnson, 1941),

methionine (Horn, Jones & Blum, 1946), choline (Horowitz & Beadle, 1943) and total (neutral) as well as phospholipids (Bloor, 1943) were determined by the methods cited.

Liver choline oxidase activity was followed according to the procedure of Dinning, Keith & Day (1949) using 1 mg./ml. of choline chloride as substrate and following oxygen uptake for 1 hr. Xanthine oxidase activities (Dhungat & Sreenivasan, 1954) were determined both in liver homogenates and for *in vitro* synthesis after incubation of slices for 3 hr. at 37° according to Dhungat & Sreenivasan (1952) in their medium 2. For determinations of enzyme activities, 1 ml. of liver homogenates prepared in 5 vol. of 0.01M phosphate buffer pH 7.4 in a chilled all-glass homogenizer were employed.

Liver pantothenic acid was estimated according to Skeggs & Wright (1944). Coenzyme A activity was followed by determination of extent of sulphanilamide acetylation (Lipmann & Kaplan, 1948) and is expressed as $\mu\text{g.}$ of sulphanilamide acetylated/g. of dry liver. Adrenaline was determined by modifications in the procedure of Ghosh, Deb & Banerjee (1951). Ascorbic acid, cysteine and glutathione were destroyed by treatment with 10% (w/v) NaHCO_3 . Further treatment with 5% (w/v) NaOH destroyed adrenaline without affecting uric acid and ergothionine. The colour developed by Folin's tungstic acid reagent was measured at 660 $\text{m}\mu$. Satisfactory values were obtained in recovery experiments. Allowance was made for any noradrenaline present by using the procedure of Euler & Hamberg, as adopted by D'Iorio & Plaut (1952); at pH 4, all adrenaline and only less than 10% of noradrenaline are destroyed by oxidation with I_2 while at pH 6 both are oxidized. Adrenaline was also tested for chromatographically, using Whatman no. 1 paper and acetone-benzyl alcohol- n-HCl (75:25:15, v/v) and spraying with ferric sulphate-ferricyanide solution. Adrenaline had an R_F value of 0.86, the value for noradrenaline being 0.77.

Animal experiments

Two sets of experiments were carried out. In the first one with simple pteroylglutamic acid deficiency, adult rats (Wistar strain, 100–120 g. body wt.) were reared on a pteroylglutamic acid-free, purified ration consisting of (g./100 g. diet) vitamin-free casein, 10; iodinated casein ('Protomone', Cerophyl Laboratories, Kansas City, Mo.), 0.5; sesame oil, 6; shark liver oil, 2; salt mixture (U.S.P. no. 2), 4; sucrose, 9.5; and maize starch, 68; with the following vitamin additions (in mg./kg. diet): thiamine hydrochloride, 6; pyridoxine hydrochloride, 6; calcium pantothenate, 20; riboflavin, 10; nicotinic acid, 30; biotin, 1; *p*-aminobenzoic acid, 100; choline chloride, 500; inositol, 500; vitamin K (Menadione, Merck), 10; α -tocopherol, 50; and vitamin B_{12} (Cobione, Merck), 0.15. The vitamin additions provided in the basal diet were considered adequate for the hyperthyroid condition. The animals were divided into six groups of four each, with due attention to details of weight, sex and litter, at the end of 5 weeks when leucocyte counts revealed definite signs of pteroylglutamic acid deficiency. The six groups were: (1) basal, pteroylglutamic acid-free, purified ration; (2) basal plus nicotinamide; (3) basal plus nicotinamide plus choline; (4), (5), and (6) same as (1), (2) and (3) respectively with pteroylglutamic acid (100 $\mu\text{g.}$ /day/animal intraperitoneally). Nicotinamide and choline were mixed in the diets at 0.5% levels. Urines were collected for 3 days after 3 weeks on the test diets;

values reported are averages of pooled samples. The animals were killed after 32 and 38 days on the experimental diets.

In preliminary trials, it was found that total daily food consumption was unaffected at this level of nicotinamide supplementation. Observations on daily food consumption over a fortnight revealed that the female rats took 7.7–8.7 g. (average 8.4 g.) of food/day each and the male animals 10.4–11.1 g. (average 10.6 g.). There was no difference in food intake specifically on account of pteroylglutamic acid or nicotinamide supplementation. Growth was, however, impaired in pteroylglutamic acid deficiency and as a result of nicotinamide feeding. The results obtained in this series of experiments are given in Tables 1, 3 and 4.

In the second series groups of rats (120 g.) that had been fed the laboratory stock ration were used; the diet consisted of (g./100 g. diet) whole wheat flour, 59; casein, 5; whole milk powder, 4; shark liver oil, 2; defatted peanut meal, 13; brewer's yeast, 4; wheat bran, 4; dried fish meal, 3; sesame oil, 2; and salt mixture (U.S.P. no. 2), 4. Pteroylglutamic acid deficiency was induced by daily subcutaneous injections of 25 $\mu\text{g.}$ of aminopterin for 6 days, and half this dose for a further 6 days. Nicotinamide (25 mg./day/animal) was given orally. Toxic symptoms (diarrhoea, swelling and bleeding around the paws, eyes and mouth) began to appear after 10–12 days. There were control groups on stock diet alone with and without nicotinamide supplementation. The animals were all killed on the 12th day. The results obtained are given in Tables 2 and 5.

All determinations were carried out in duplicate and the values reported are the averages for four animals in each group except for adrenaline where pooled adrenals from two animals were used for each assay.

RESULTS AND DISCUSSION

Weight of body organs. There was an increase in the liver weight to body weight ratio in pteroylglutamic acid deficiency (Table 1). This difference was somewhat more pronounced in the aminopterin-fed animals (Table 2) and was also seen in respect of kidney and spleen. Administration of nicotinamide resulted in larger liver weights in both series, the results being less noticeable in the pteroylglutamic acid supplemented groups. An observation of interest was the enlarged adrenals in the pteroylglutamic acid deficient or aminopterin-fed animals, especially in groups receiving nicotinamide. The changes in adrenaline are discussed later.

Keith, Broach, Warren, Day & Totter (1948) have reported on the ratio of chick liver weight to body weight being inversely related to the pteroylglutamic acid content of a purified diet and on the greatly enlarged condition of the gall bladders of the deficient birds. Similar observations on liver weight/unit body weight have been made by Williams, Sunde, Cravens & Elvehjem (1950). Induced dietary deficiencies evidently impose a stress on the animal organism, resulting in exhaustion, enlargement of glands and organs and hypofunction.

Urinary creatine and N¹-methylnicotinamide. The effect of pteroylglutamic acid (Fatterpaker *et al.* 1951, 1952a; Dietrich *et al.* 1952; Dinning & Day, 1949) is confirmed (Table 3). Increased methylation of the extra nicotinamide ingested could be seen and is much less in the pteroylglutamic acid deficient animals than in replete ones. Choline increased the excretion of N¹-methylnicotinamide in the deficient groups. Excretion of nicotinic acid bears the expected inverse relationship to N¹-methylnicotinamide excretion. Creatine excretion is not influenced in nicotinamide toxicity. Supplementation with both pteroylglutamic acid and choline enhances urinary creatine values.

Liver lipids. As reported by Handler (1944) and others, forced methylation as by the fed nicotinamide, causes increased liver lipids. There is also a definite decrease in phospholipids. The effect is

considerably augmented in pteroylglutamic acid deficiency (Table 1) or in the aminopterin-fed groups (Table 5) and is corrected by choline feeding.

Liver nitrogen, choline and methionine. A slight reduction in liver nitrogen (Table 1) is observable as a result of nicotinamide toxicity as well as in pteroylglutamic acid deficiency. Choline supplementation apparently helps in mobilization of proteins in the deficient livers.

Liver levels of choline and methionine are lowered in pteroylglutamic acid deficiency (Fatterpaker *et al.* 1954). Nicotinamide feeding results in a small but significant further reduction in these compounds only in the deficient group not receiving choline. A favourable influence of choline in the pteroylglutamic acid fed group is probably attributable to the low level of protein (10% casein) in the basal diet.

Table 1. *Changes in liver weight and composition*

For details, see Table 3 and text. The animals were killed at the end of 5 weeks on the test diets. The results are averages for four independent samples $\pm$ S.E.M.

Diet	Liver weight (g./100 g. body wt.)	Liver nitrogen (mg./g. dry liver)	Total lipids (g./100 g. fresh liver)	Phospho- lipids (g./100 g. fresh liver)	Methionine		Choline	
					(mg./g. fresh liver)		(mg./g. fresh liver)	
Basal (no pteroylglutamic acid)	4.68 $\pm$ 0.12	28.65 $\pm$ 0.70	3.83 $\pm$ 0.23	1.36 $\pm$ 0.07	4.5 $\pm$ 0.13		4.1 $\pm$ 0.16	
Basal + nicotinamide	5.50 $\pm$ 0.55	26.65 $\pm$ 0.33	4.79 $\pm$ 0.19	1.13 $\pm$ 0.04	4.2 $\pm$ 0.11		3.8 $\pm$ 0.11	
Basal + nicotinamide + choline	4.08 $\pm$ 0.38	32.29 $\pm$ 1.46	3.67 $\pm$ 0.27	1.27 $\pm$ 0.06	4.9 $\pm$ 0.14		4.3 $\pm$ 0.07	
Basal + pteroylglutamic acid	4.30 $\pm$ 0.18	32.29 $\pm$ 0.50	3.29 $\pm$ 0.14	2.08 $\pm$ 0.20	5.7 $\pm$ 0.20		4.8 $\pm$ 0.15	
Basal + nicotinamide + pteroylglutamic acid	4.49 $\pm$ 0.22	29.23 $\pm$ 0.98	3.70 $\pm$ 0.24	1.84 $\pm$ 0.06	5.5 $\pm$ 0.17		4.7 $\pm$ 0.24	
Basal + nicotinamide + choline + pteroylglutamic acid	4.00 $\pm$ 0.50	32.35 $\pm$ 1.31	3.59 $\pm$ 0.56	2.00 $\pm$ 0.11	6.1 $\pm$ 0.27		5.2 $\pm$ 0.20	

Table 2. *Changes in weight of body organs of aminopterin-fed animals*

Animals on the laboratory stock diet (see text) were administered 225 μ g. of aminopterin subcutaneously during 12 days, when they were killed. Nicotinamide was given orally at 25 mg./day/animal for this period. Results are the means from four animals in each group $\pm$ S.E. and are expressed as g./100 g. body wt.

Diet	Liver	Kidney	Spleen
Basal	4.02 $\pm$ 0.10	0.93 $\pm$ 0.01	0.30 $\pm$ 0.02
Basal + nicotinamide	4.57 $\pm$ 0.33	1.06 $\pm$ 0.04	0.36 $\pm$ 0.02
Basal + aminopterin	4.38 $\pm$ 0.12	1.11 $\pm$ 0.08	0.36 $\pm$ 0.03
Basal + aminopterin + nicotinamide	4.76 $\pm$ 0.14	1.18 $\pm$ 0.04	0.38 $\pm$ 0.03

Table 3. *Urinary excretion of nicotinic acid, N¹-methylnicotinamide and creatine*

Animals were grouped at end of 5 weeks on a pteroylglutamic acid-free purified ration (see text). Nicotinamide and choline supplementation were at 0.5% levels each, mixed in the diet. Pteroylglutamic acid (100 μ g./animal/day) was administered intraperitoneally. Urine collections were for 3 days, 3 weeks after grouping. Results are averages for the pooled samples from four animals in each group $\pm$ S.E.M. and are expressed as mg./100 g. body wt./day.

Diet	Leucocytes/mm. ³ ($\times$ 1000)	Total nicotinic acid	N ¹ -methyl- nicotinamide	Total creatine
Basal (no pteroylglutamic acid)	7.82 $\pm$ 0.45	0.31 $\pm$ 0.13	0.12 $\pm$ 0.03	1.1 $\pm$ 0.08
Basal + nicotinamide	6.56 $\pm$ 0.60	2.81 $\pm$ 0.20	0.48 $\pm$ 0.04	1.0 $\pm$ 0.04
Basal + nicotinamide + choline	7.81 $\pm$ 0.36	2.12 $\pm$ 0.23	0.73 $\pm$ 0.03	1.3 $\pm$ 0.14
Basal + pteroylglutamic acid	10.69 $\pm$ 0.78	0.25 $\pm$ 0.10	0.22 $\pm$ 0.02	1.4 $\pm$ 0.05
Basal + nicotinamide + pteroylglutamic acid	10.31 $\pm$ 0.49	1.68 $\pm$ 0.14	0.80 $\pm$ 0.04	1.2 $\pm$ 0.08
Basal + nicotinamide + choline + pteroylglutamic acid	10.62 $\pm$ 0.31	1.29 $\pm$ 0.25	0.83 $\pm$ 0.03	1.6 $\pm$ 0.04

That nicotinamide toxicity is caused by a depletion of labile methyl groups is thus inferable from the increased urinary excretion of N^2 -methyl-nicotinamide and decreased liver stores of choline and methionine. These changes also manifest themselves in increased liver lipids and decreased phospholipids. It is further seen that the nicotinamide effect is greater in animals rendered deficient in pteroylglutamic acid either by dietary means or by aminopterin feeding. Choline alleviates this toxicity. These results generally bear out recent reports on the mediation of pteroylglutamic acid and vitamin B_{12} in methyl metabolism (Arnstein & Neuburger, 1953; Stekol, Weiss, Smith & Weiss, 1953).

Choline oxidase. There is decreased choline oxidation in pteroylglutamic acid deficiency either by dietary means (Table 4) or by aminopterin (Table 5). A lowering in choline oxidase activity is also observable as a result of nicotinamide feeding in both the pteroylglutamic acid deficient and normal groups. Handler & Bernheim (1942) have observed a reduced choline oxidase activity in fatty livers. The activity of the oxidase is also known to

be influenced by pteroylglutamic acid (Williams, Sreenivasan, Sung & Elvehjem, 1953*b*) and vitamin B_{12} (Williams *et al.* 1953*a*). One of the reasons for the lowered choline oxidase activity in aminopterin-fed rats is stated to be a decreased synthesis of ascorbic acid and consequent impairment in the conversion of pteroylglutamic acid into folinic acid (Williams, 1951).

Xanthine oxidase. Several reports point to a relationship of pteroylglutamic acid to xanthine oxidation (Fatterpaker & Sreenivasan, 1951). The lability of the enzyme is also shown from observations on variations in its activity as a result of dietary changes (Litwack, Williams, Feigelson & Elvehjem, 1950; Dhungat & Sreenivasan, 1952). Since methionine is apparently essential in controlling xanthine oxidase activity, it was of interest to follow changes in this enzyme in the present work. Results (Tables 4 and 5) show that nicotinamide feeding reduces xanthine oxidase activity, presumably in part as a result of methyl depletion, and that *in vitro* synthesis of the enzyme is generally in proportion to the decrease in the liver xanthine oxidase activity (cf. Dhungat & Sreenivasan, 1952).

Table 4. *Changes in liver enzymes, pantothenic acid and adrenaline*

For explanation of table, see Tables 1 and 3 and text. Results are averages for four animals in each group $\pm$ S.E.M.

Diet	Choline oxidase (μ l. O ₂ /g. dry wt./hr.)	Xanthine oxidase activity (μ l. O ₂ /g. dry wt./2 hr.)	Xanthine oxidase synthesis <i>in vitro</i>	Pantothenic acid (μ g./g. dry liver)	Coenzyme A (μ g./sulphanilamide/g. dry liver)	Adrenaline* (μ g./100 mg. fresh adrenal glands)
Basal (no pteroylglutamic acid)	1530 $\pm$ 104	252 $\pm$ 18	348 $\pm$ 17	285 $\pm$ 18	260 $\pm$ 13	83 $\pm$ 4
Basal + nicotinamide	1250 $\pm$ 112	192 $\pm$ 17	307 $\pm$ 26	176 $\pm$ 12	202 $\pm$ 11	71 $\pm$ 3
Basal + nicotinamide + choline	1695 $\pm$ 126	258 $\pm$ 24	195 $\pm$ 15	264 $\pm$ 21	360 $\pm$ 24	97 $\pm$ 5
Basal + pteroylglutamic acid	2120 $\pm$ 130	249 $\pm$ 22	272 $\pm$ 28	247 $\pm$ 7	291 $\pm$ 19	111 $\pm$ 6
Basal + nicotinamide + pteroylglutamic acid	1835 $\pm$ 95	195 $\pm$ 25	215 $\pm$ 36	180 $\pm$ 10	205 $\pm$ 8	117 $\pm$ 4
Basal + nicotinamide + choline + pteroylglutamic acid	2170 $\pm$ 142	293 $\pm$ 16	202 $\pm$ 0	285 $\pm$ 26	362 $\pm$ 29	115 $\pm$ 1

* Pooled adrenals from two rats were used for assay in duplicate.

Table 5. *Nicotinamide toxicity in aminopterin-fed rats*

For explanation, see Table 2. All determinations were carried out in duplicate on four independent samples in each group. Results are mean values $\pm$ S.E.

Diet	Leucocytes/ mm. ³ ($\times 1000$)	Total lipids	Phospho- lipids	Choline oxidase (μ l. O ₂ /g. dry wt. in 2 hr.)	Liver xanthine oxidase	Xanthine oxidase synthesis <i>in vitro</i>	Adrena- line* (μ g./ 100 mg. fresh adrenals)
		(g./100 g. fresh liver)					
					(μ l. O ₂ /g. dry wt./2 hr.)		
Basal	10.78 $\pm$ 0.15	4.01 $\pm$ 0.06	1.78 $\pm$ 0.09	2820 $\pm$ 70	744 $\pm$ 66	716 $\pm$ 52	98 $\pm$ 6
Basal + nicotinamide	10.48 $\pm$ 0.12	4.15 $\pm$ 0.10	1.34 $\pm$ 0.03	2440 $\pm$ 115	466 $\pm$ 89	640 $\pm$ 32	89 $\pm$ 1
Basal + aminopterin	7.66 $\pm$ 0.16	4.95 $\pm$ 0.03	1.36 $\pm$ 0.07	2280 $\pm$ 65	288 $\pm$ 76	452 $\pm$ 41	72 $\pm$ 4
Basal + aminopterin + nicotinamide	7.81 $\pm$ 0.31	5.55 $\pm$ 0.24	1.14 $\pm$ 0.04	1880 $\pm$ 222	131 $\pm$ 69	318 $\pm$ 50	62 $\pm$ 3

* See footnote to Table 4.

Pantothenic acid and coenzyme A. These determinations were made in view of various observations correlating pantothenic acid and coenzyme A with pteroylglutamic acid and vitamin B₁₂. Yacowitz, Norris & Heuser (1951) had reported a sparing action by vitamin B₁₂ for pantothenic acid in chicks. That vitamin B₁₂ may also aid in the transfer of pantothenic acid from livers of chicks was suggested by Evans, Groschke & Butts (1951). More recently, it has been observed by Boxer, Ott & Shonk (1953) that there is a fivefold increase in coenzyme A concentration in livers of vitamin B₁₂-deficient chicks. A sparing action of methionine for pantothenic acid in antibody formation was noted by Ludovici, Axelrod & Carter (1951). According to Popp & Totter (1952), pteroylglutamic acid deficiency resulted in decreased liver stores of coenzyme A. An involvement of pantothenic acid in the control of adrenal function is recognized (Hurley & Morgan, 1952).

The pantothenic acid estimations in the present studies were carried out by a method (Skeggs & Wright, 1944) which has since been shown to give low results (Novelli, Kaplan & Lipmann, 1949) owing to incomplete liberation of bound forms. Nevertheless, a general lowering of pantothenic acid content is seen in nicotinamide toxicity (Table 4). This is also the case with coenzyme A. However, simple pteroylglutamic acid deficiency does not show itself in any significant changes in liver levels of pantothenic acid or coenzyme A. The effect of choline on coenzyme A stores is marked.

Adrenaline. An enlargement of the adrenals in the aminopterin-fed animals as well as due to nicotinamide toxicity was referred to earlier. This was followed by determination of the changes in adrenaline content of the adrenals in the different groups.

Keller, Boissonnas & du Vigneaud (1950) showed that phenylalanine and methionine are the precursors for adrenaline and that a transmethylation is involved. It is known that methionine deficiency can lower adrenaline synthesis in rats (Hutcheon & Parker, 1950). The present studies (Tables 4 and 5) show the relationship of dietary pteroylglutamic acid to adrenaline as well as the effect of nicotinamide toxicity on this hormone in pteroylglutamic acid deficiency. These results are consistent with earlier observations that pteroylglutamic acid is involved in other methylation reactions, such as creatine and N¹-methylnicotinamide formation (Fatterpaker *et al.* 1951, 1952*a*). In a recent report, D'Iorio & Plaut (1952) observed that in hyperthyroid rats the adrenaline content is decreased and that vitamin B₁₂ partially restores this condition. The potentiating effect of vitamin B₁₂ on pteroylglutamic acid in transmethylating systems has been discussed by Sreenivasan (1951).

Adrenaline secretion is known to be influenced by a number of experimentally uncontrollable stress and emotional factors. Hence the influence of pteroylglutamic acid on adrenaline metabolism has been confirmed indirectly in other studies on the reversible glycogen to glucose transformation in isolated rat diaphragms from normal and aminopterin-fed rats (to be published).

The involvement of pteroylglutamic acid in adrenaline biosynthesis is apparently indirect as with the creatine- and the N¹-methylnicotinamide-synthesizing system (Fatterpaker *et al.* 1952*b*, 1954).

SUMMARY

1. Incorporation of 0.5% nicotinamide in a pteroylglutamic acid-free purified ration for young albino rats resulted in forced methylation and consequent depletion of liver choline and methionine as well as to increase in neutral lipids and to decrease in phospholipids.

2. Pteroylglutamic acid deficiency or nicotinamide feeding also resulted in a decrease in liver choline oxidase activity; a similar but somewhat less pronounced effect on liver xanthine oxidase activity was also reflected in correspondingly increased ability of the liver slices for synthesis of the enzyme *in vitro*.

3. Adrenaline content of the adrenals is significantly decreased in nicotinamide-fed pteroylglutamic acid-deficient animals.

4. The foregoing effects of nicotinamide toxicity are corrected by pteroylglutamic acid (100 µg./day/rat) and/or 0.5% dietary choline.

5. There is no marked change in liver stores of pantothenic acid or of coenzyme A as a result of pteroylglutamic acid deficiency. However, ingestion of nicotinamide results in somewhat lowered values for both forms of the vitamin in the liver. Choline supplementation increases liver levels of coenzyme A.

6. The alleviation by pteroylglutamic acid of nicotinamide toxicity has also been shown in studies with aminopterin-fed rats.

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REFERENCES

- Arnstein, H. R. V. & Neuberger, A. (1953). *Biochem. J.* **55**, 259.
- Bloor, W. R. (1943). *Biochemistry of Fatty Acids*, pp. 46-9. New York: Reinhold Publishing Corporation.
- Boxer, G. E., Ott, W. H. & Shonk, C. E. (1953). *Arch. Biochem. Biophys.* **47**, 474.

- Clark, L. C. jun. & Thompson, H. L. (1949). *Analyt. Chem.* **21**, 218.
- Dhungat, S. B. & Sreenivasan, A. (1952). *J. biol. Chem.* **197**, 831.
- Dhungat, S. B. & Sreenivasan, A. (1954). *J. biol. Chem.* **208**, 845.
- Dietrich, L. S., Monson, W. J. & Elvehjem, C. A. (1952). *J. biol. Chem.* **199**, 765.
- Dinning, J. S. & Day, P. L. (1949). *J. biol. Chem.* **181**, 897.
- Dinning, J. S., Keith, C. K. & Day, P. L. (1949). *Arch. Biochem.* **24**, 463.
- Dinning, J. S., Keith, C. K., Parsons, J. T. & Day, P. L. (1950). *J. Nutr.* **42**, 81.
- D'Iorio, A. & Plaut, G. W. E. (1952). *Arch. Biochem. Biophys.* **41**, 153.
- Du Vigneaud, V., Chandler, J. P., Cohn, M. & Brown, G. B. (1940). *J. biol. Chem.* **134**, 787.
- Du Vigneaud, V., Chandler, J. P., Moyer, A. W. & Keppel, D. M. (1939). *J. biol. Chem.* **131**, 57.
- Evans, R. J., Groschke, A. C. & Butts, H. A. (1951). *Arch. Biochem.* **31**, 454.
- Fatterpaker, P., Marfatia, U. & Sreenivasan, A. (1951). *Nature, Lond.*, **167**, 1067.
- Fatterpaker, P., Marfatia, U. & Sreenivasan, A. (1952a). *Nature, Lond.*, **169**, 1096.
- Fatterpaker, P., Marfatia, U. & Sreenivasan, A. (1952b). *Nature, Lond.*, **170**, 894.
- Fatterpaker, P., Marfatia, U. & Sreenivasan, A. (1954). *Nature, Lond.*, **173**, 359.
- Fatterpaker, P. & Sreenivasan, A. (1951). *Nature, Lond.*, **167**, 149.
- Ghosh, N. C., Deb, C. & Banerjee, S. (1951). *J. biol. Chem.* **192**, 867.
- Handler, P. (1944). *J. biol. Chem.* **154**, 203.
- Handler, P. & Bernheim, F. (1942). *J. biol. Chem.* **144**, 401.
- Handler, P. & Bernheim, F. (1943). *J. biol. Chem.* **148**, 649.
- Handler, P. & Dann, W. J. (1942). *J. biol. Chem.* **146**, 357.
- Horn, M. J., Jones, B. & Blum, A. E. (1946). *J. biol. Chem.* **166**, 313.
- Horowitz, N. H. & Beadle, G. W. (1943). *J. biol. Chem.* **150**, 325.
- Huff, J. W. & Perlzweig, W. A. (1947). *J. biol. Chem.* **167**, 157.
- Hurley, L. S. & Morgan, A. F. (1952). *J. biol. Chem.* **195**, 583.
- Hutcheon, D. E. & Parker, R. H. O. (1950). *Brit. J. Pharmacol.* **5**, 604.
- Johnson, M. J. (1941). *J. biol. Chem.* **137**, 575.
- Keith, C. K., Broach, W. J., Warren, D., Day, P. L. & Totter, J. R. (1948). *J. biol. Chem.* **176**, 1095.
- Keller, E. B., Boissonnas, R. A. & du Vigneaud, V. (1950). *J. biol. Chem.* **183**, 627.
- Lipmann, F. & Kaplan, N. O. (1948). *J. biol. Chem.* **174**, 37.
- Litwack, G., Williams, J. N. jun., Feigelson, P. & Elvehjem, C. A. (1950). *J. biol. Chem.* **187**, 605.
- Ludovici, P. P., Axelrod, A. E. & Carter, B. B. (1951). *Proc. Soc. exp. Biol., N.Y.*, **76**, 665.
- Novelli, G. D., Kaplan, N. O. & Lipmann, F. (1949). *J. biol. Chem.* **177**, 97.
- Popp, E. M. & Totter, J. R. (1952). *J. biol. Chem.* **199**, 547.
- Skeggs, H. R. & Wright, L. D. (1944). *J. biol. Chem.* **156**, 21.
- Sreenivasan, A. (1951). *Curr. Sci.* **20**, 319.
- Stekol, J. A., Weiss, S., Smith, P. & Weiss, K. (1953). *J. biol. Chem.* **201**, 299.
- Stetten, D. & Grail, G. F. (1942). *J. biol. Chem.* **144**, 175.
- Swaminathan, M. (1946). *Ann. Biochem.* **6**, 69.
- Williams, J. N. jun. (1951). *J. biol. Chem.* **192**, 81.
- Williams, J. N. jun., Monson, W. J., Sreenivasan, A., Dietrich, L. S., Harper, A. E. & Elvehjem, C. A. (1953a). *J. biol. Chem.* **202**, 151.
- Williams, J. N. jun., Sreenivasan, A., Sung, S. C. & Elvehjem, C. A. (1953b). *J. biol. Chem.* **202**, 233.
- Williams, J. N. jun., Sunde, M. L., Cravens, W. W. & Elvehjem, C. A. (1950). *J. biol. Chem.* **185**, 895.
- Yacowitz, H., Norris, L. C. & Heuser, C. F. (1951). *J. biol. Chem.* **192**, 141.

Studies in the Biochemistry of Micro-Organisms

94. THE COLOURING MATTERS OF SPECIES IN THE *ASPERGILLUS NIDULANS* GROUP PART I. ASPERTHECIN, A CRYSTALLINE COLOURING MATTER OF *ASPERGILLUS QUADRILINEATUS* THOM & RAPER

BY B. H. HOWARD AND H. RAISTRICK

Department of Biochemistry, London School of Hygiene and Tropical Medicine, University of London

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One of the outstanding characteristics of the *Aspergillus nidulans* group of species of moulds is that they usually produce ascospores contained in dark-coloured perithecia. The ascospore members of the group consist of six recognized species or varieties (Thom & Raper, 1945) in cultures of which the colony reverse, the perithecial walls and the ascospores are usually all reddish purple to dark purple-red in colour. The ascospores are characterized by equatorial bands which are generally two in

number, but number four in one member of the group, *A. quadrilineatus* Thom & Raper—hence its specific epithet. We have investigated the colouring matters present in two highly pigmented strains of this species which were isolated in this Department from different samples of Australian soil, and have isolated from each of them in pure crystalline form a hitherto undescribed anthraquinone colouring matter for which we propose the name asperthecin. However, since the crude colouring matters isolated