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Formate Metabolism in the Vitamin B₁₂-Deficient Rat

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It was reported earlier (Fatterpaker, Marfatia & Sreenivasan, 1955; Lavate & Sreenivasan, 1956; Kasbekar, Lavate, Rege & Sreenivasan, 1959) that the protective action exerted by vitamin B₁₂ in experimentally induced hyperthyroidism in the rat is also observable in terms of a restoration of the uncoupling of oxidative phosphorylation caused by thyroxine. These studies were extended to ascertain whether the incorporation of formate into the purines of liver acid-soluble nucleotides is impaired in the thyrotoxic rat and whether vitamin B₁₂ protection is reflected in altered purine synthesis from administered formate.

METHODS

Animals. The basal diet employed was a modification of the corn-soya diet of Ericson, Harper, Williams & Elvehjem (1956) known to cause vitamin B₁₂ deficiency, and consisted of (percentages): corn meal 46.2, defatted pea-nut meal 46.2, salts (Ericson *et al.* 1956) 2, L-cystine 0.3, sesame oil 5.0, and vitamin mixture (Ericson *et al.* 1956) 0.3. Rats of either sex (Wistar strain) weighing 70–80 g. were divided into four groups and fed *ad lib.* on the basal diet either as such or with supplements of iodinated casein (Protomone: Cerophyll Laboratories, Kansas City, Mo., U.S.A.) or vitamin B₁₂ or both. Iodinated casein known to enhance vitamin B₁₂ deficiency (Lewis, Tappan, Register & Elvehjem, 1950) was added at 0.1 g./100 g. and vitamin B₁₂ at 20 µg./100 g. Feeding was continued for 7 weeks, during which period the animals on the basal diet with iodinated casein showed some 10% loss in weight when they were considered hyperthyroid (Du Toit, 1952).

Incorporation of formate into adenine and guanine of liver acid-soluble nucleotides. To animals from each group were administered intraperitoneally 0.5 ml. of a solution of

0.1M-sodium [¹⁴C]formate (2.25 × 10⁶ counts/min.). The animals were operated on under ether anaesthesia 2 hr. later and their livers perfused with cold aq. 0.09% NaCl, excised, weighed and extracted with 40 ml. of ice-cold 6% (w/v) trichloroacetic acid solution for 30 min. The protein precipitate was separated and washed by centrifuging in the cold at 5000 rev./min. The supernatant and washings were combined, neutralized and treated with 5 ml. of 20% (w/v) mercuric acetate solution to precipitate the acid-soluble nucleotides (Kerr, 1942). After separation and washing of the precipitate with 0.5% (w/v) mercuric acetate solution, the nucleotides were freed from Hg by H₂S and the excess of H₂S was removed by aeration. The nucleotides were then hydrolysed in N-H₂SO₄ for 1 hr. over a boiling-water bath. The liberated purines were precipitated at pH 1 with 1 ml. of M-AgNO₃ solution (Hayder, Chernigoy, Khatchadourian & Kerr, 1957) and extracted from their silver salts with hot N-HCl. The purine hydrochlorides were freeze-dried and dissolved in 0.2 ml. of N-HCl. The solutions were then chromatographed on Eaton-Dikeman (Mt. Holly Springs, Pa., U.S.A.) no. 613 paper strips, with 65% (v/v) propan-2-ol in 2N-HCl (Hayder *et al.* 1957) as the developing solvent. The separated purines were then identified as their mercury complexes made visible by ammonium sulphide (Vischer & Chargaff, 1948). Segments from the air-dried strips corresponding to the positions of the separated purines were directly eluted in 0.1N-HCl for adenine and N-HCl for guanine. They were estimated according to the procedure recommended by Vischer & Chargaff (1948) with a Beckman DUV spectrophotometer. Measured portions were evaporated to dryness on 25 mm. diameter Pyrex-glass planchets by heat lamps. Their activities were determined at infinite thinness in a helium-filled SC-16 Flow gas counter with an SC-51 Autoscaler (Tracerlab Inc., Waltham, Mass., U.S.A.) operating at 40–50% efficiency.

Folic acid and vitamin B₁₂. Other animals from each group were killed for determinations in liver of total folic

acid, vitamin B₁₂ and formate oxidation. For folic acid assays, 10% (w/v) liver homogenates were prepared in ice-cold water and autolysed overnight in 0.1M-phosphate buffer, pH 7.2, at 37° under toluene. The samples were then steamed, appropriately diluted, filtered and assayed microbiologically with *Streptococcus faecalis* (Mitbander & Sreenivasan, 1954).

Portions of the liver homogenates were incubated at 37° for 12 hr. under toluene with papain (25 mg./g. of fresh liver) in 0.1M-acetate buffer, pH 4.6. These samples were then analysed for vitamin B₁₂ with *Euglena gracilis* as the test organism according to the method of Hoff-Jørgensen (1954).

Formate oxidation. This was studied at 37° in Warburg vessels. Each flask contained 1.0 ml. of a 20% (w/v) aq. liver homogenate, 1.0 ml. of Krebs-Ringer phosphate buffer, pH 7.2, and 0.066M-formate in a total volume of 3.0 ml. When used, [¹⁴C]formate corresponded to a total activity of 1.25×10^4 counts/min./flask. The centre well contained 0.2 ml. of 10% (w/v) KOH solution. Oxygen uptake was measured for 1 hr. and the CO₂ evolved was trapped in the alkali and later precipitated as BaCO₃. The precipitate was washed, plated and counted at infinite thinness in the helium flow-gas counter.

Liver catalase. This was determined by a microcolorimetric method (S. P. Manjrekar, unpublished work). Aq. liver homogenate (1:2000) (2 ml.) was added to 10 ml. of buffered (5.0 mM phosphate buffer, pH 6.8) hydrogen peroxide solution (1.25 mM) at 0°. Portions (2 ml.) of the reaction mixture were pipetted out at different time intervals into 5 ml. of titanium sulphate reagent (Feigl, 1954). The colour developed for residual H₂O₂ was read at 420 mμ in a Klett-Summerson photoelectric colorimeter. Catalase activity was determined in terms of Kat.f. units (Euler & Josephson, 1927), the pseudo-first-order reaction constant *K* being equal to $D(\log x_0 - \log x_t)/t$, where *x*₀ and *x*_t are Klett-Summerson readings (corrected for proper blanks) at 0 and *t* min. and *D* is the dilution of tissue preparation used for assay.

All results reported are averages for determinations from at least four independent samples ± S.E.M.

RESULTS AND DISCUSSION

The protection by vitamin B₁₂ against weight loss caused by iodoprotein feeding, as well as the increased depletion of the vitamin under this condition (Table 1), are in conformity with earlier observations (Fatterpaker *et al.* 1955; Kasbekar *et al.* 1959). There is also a decrease in liver stores of folic acid in experimental thyrotoxicosis. Vitamin B₁₂ protection was not, however, observable in terms of a correction of this effect. 'Active formate', as it functions in the exchange and synthesis of C-2 and C-8 of the purine skeleton, is a reduced and formylated derivative of folic acid (Greenberg, Jaenicke & Silverman, 1955). It was therefore of interest to examine whether the decreased levels of folic acid in thyrotoxicosis are reflected in the lowering of [¹⁴C]formate incorporation into adenine and guanine of the acid-soluble nucleotides.

Table 1. Folic acid and vitamin B₁₂ levels, and incorporation of [¹⁴C]formate into acid-soluble nucleotides in livers of thyrotoxic and vitamin B₁₂-protected rats

Diet group	Wt. gain in 7 weeks (g.)	Vitamin B ₁₂ (μmg./g.)	Folic acid (μg./g.)	[¹⁴ C]Formate incorporation			
				Adenine (μm-moles/g.)	Guanine (μm-moles/g.)	Adenine (counts/min./g. of liver)	Guanine (counts/min./g. of liver)
Basal	116 ± 5	33 ± 4	3.74 ± 0.05	604 ± 27	43 ± 4	674 ± 10	428 ± 22
Thyrotoxic	95 ± 11	24 ± 4	2.96 ± 0.19	725 ± 36	54 ± 7	608 ± 12	442 ± 27
Basal + vitamin B ₁₂	124 ± 6	162 ± 8	3.70 ± 0.37	721 ± 35	63 ± 8	151 ± 10	110 ± 12
Thyrotoxic + vitamin B ₁₂	114 ± 5	108 ± 6	2.98 ± 0.15	798 ± 20	66 ± 9	211 ± 14	166 ± 13

Young adult rats were reared on a vitamin B₁₂-deficient basal diet with or without additions of iodinated casein and vitamin B₁₂. The animals were killed at the end of 7 weeks. The incorporation of formate into the adenine and guanine of liver acid-soluble nucleotides was studied 2 hr. after [¹⁴C]formate administration *in vivo*. For details and methods see text.

The observed decrease (Table 1) in specific activity (counts/min./ μ mole) of liver acid-soluble nucleotide adenine and guanine in the thyrotoxic rat is mostly a dilution effect, explainable in terms of increased endogenous synthesis of these nucleotides in thyrotoxicosis. When the total incorporation/g. of liver is considered, this difference levels off. Guggenheim & Halevy (1957) and Halevy & Guggenheim (1958) have shown that hepatic nucleic acid levels are not affected by various conditions adversely affecting folic acid metabolism, e.g. protein deficiency and incorporation of either succinylsulphathiazole or 4-aminopterine in the diet. Dinning, Cosgrove & Day (1957) have also shown in guinea-pig-liver homogenates that ascorbic acid deficiency depresses the utilization of formate in the synthesis of serine by 78% and in that of methionine by 85%. However, under identical conditions of folic acid metabolism, the incorporation of formate into acid-soluble nucleotides and nucleic acid purines remains remarkably unaffected. It would seem, therefore, that there may be differences in the requirements for 1-C transfer reactions and that, in mild folic acid-deficiency states, systems other than those involved in nucleic acid-purine synthesis are more susceptible.

The incorporation of vitamin B₁₂ into the basal or the iodocasein-supplemented rations resulted in decreased incorporation of [¹⁴C]formate into acid-soluble nucleotides (Table 1). This decreased incorporation cannot be attributed to dilution of activity due to increased synthesis of acid-soluble nucleotides on vitamin B₁₂ administration.

From Table 2 it would seem that oxidation of formate to CO₂ is significantly increased ($P < 0.05$) on vitamin B₁₂ administration, partially explaining the observed (Table 1) decrease in formate incorporation into acid-soluble nucleotides. This would suggest a role for vitamin B₁₂ in formate metabolism, directly or indirectly linked to formate oxidation. In a recent note Arnstein (1958) has proposed a similar role for vitamin B₁₂.

Since formate oxidation has been linked to catalase activity in rat liver (Nakada & Weinhouse, 1953), it is possible that the decreased formate oxidation in vitamin B₁₂ deficiency may be due to an effect of the vitamin on haem-protein synthesis. Catalase activity did not show any appreciable increase in the vitamin B₁₂-supplemented group, the Kat.f. values being $14\,220 \pm 1730$ units for the basal group and $14\,940 \pm 1460$ units for the vitamin B₁₂-supplemented group (C. Bhuvaneshwaran, unpublished work).

Other work from this Laboratory would also point to the possibility that the oxidation of rat-liver formate is not entirely linked to catalase activity. It was not enhanced when xanthine was included in the reacting system. Again it was inhibited to a less extent than was catalase by cyanide. For example, 3 mM-cyanide completely inhibits catalase activity, whereas a tenfold concentration of cyanide inhibited formate oxidation only to a negligible extent. Kruhoffer (1951) has shown formate oxidation in rat liver to be diphosphopyridine nucleotide-linked.

Formate oxidation is lowered, but to a less extent in the thyrotoxic rat (Table 2), even though vitamin B₁₂ deficiency is augmented. Similarly, the increase in formate oxidation on vitamin B₁₂ supplementation is less marked ($0.1 > P > 0.05$) in the iodoprotein-fed group. This would suggest that although administration of iodinated casein intensifies the severity of vitamin B₁₂ deficiency it is also accompanied by the development of other unknown metabolic complications. Adverse effects of prolonged iodocasein feeding have been demonstrated in other studies (Fatterpaker *et al.* 1959) with rats and mice, on blood haemoglobin and serum proteins, on the retention of vitamins and soluble sulphhydryl compounds in liver, as well as on the ability of liver enzymes to effect conjugation *in vitro* of *p*-aminobenzoic acid and glycine.

SUMMARY

1. An observed decrease in liver folic acid levels in the hyperthyroid rat is not corrected by vitamin B₁₂ administration.
2. It is also not accompanied by any clear-cut decrease in the incorporation of [¹⁴C]formate *in vivo* into the purines of liver acid-soluble nucleotides.
3. It is concluded that incorporation of formate into the purines of rat liver acid-soluble nucleotides is independent of mild disturbances in folic acid metabolism.
4. Vitamin B₁₂ administration to a vitamin B₁₂-deficient rat markedly decreased the incorporation of [¹⁴C]formate *in vivo* into liver acid-soluble nucleotides. This could be partly explained in terms of the

Table 2. *Oxidation of formate by liver enzymes in thyrotoxic and vitamin B₁₂-protected rats*

For details of grouping, see Table 1 and text. Oxidation of formate by liver enzymes was followed manometrically in conventional Warburg flasks with sodium [¹⁴C]formate, the ¹⁴CO₂ being recovered as carbonate in the centre well.

Diet group	O ₂ uptake (μ l./hr./flask)	Activity of total ¹⁴ CO ₂ (counts/min.)
Basal	16.0 ± 1.1	8470 ± 318
Thyrotoxic	18.4 ± 2.9	8750 ± 120
Basal + vitamin B ₁₂	25.5 ± 4.0	9530 ± 380
Thyrotoxic + vitamin B ₁₂	23.6 ± 1.2	9410 ± 320

accompanying increase in the oxidation of formate studied *in vitro*.

5. Vitamin B₁₂ has a role, directly or indirectly, in the oxidation of formate.

6. In rat liver, oxidation of formate may not necessarily be catalase-linked.

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