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A Study of Vitamin B_{12} Protection in Experimental Thyrotoxicosis in the Rat

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Occurrence of vitamin B_{12} deficiency in animals is not common. The maternal carry-over of the vitamin to the young and a substantial contribution to the host by the intestinal microflora render it difficult to induce experimental vitamin B_{12} deficiency even on strictly vitamin B_{12}-free diets. A widely practised method of producing such a deficiency is by the use of thyroid-active materials like iodinated casein. Young growing animals fed on diets supplemented with thyroprotein were therefore used by early investigators (Zucker & Zucker, 1950) for assays of liver preparations. In the presence of an ample supply of other nutrients, iodinated casein induces retardation of growth and subsequently a high mortality, which are partially counteracted by supplementation of the ration with vitamin B_{12} (Emerson, 1949; Cuthbertson, 1949; Sure & Easterling, 1950; Graham, Reichstein, Watson & Hier, 1952).

Relatively little is known, however, about the mode of action of thyroxine and the counteraction of its effects by vitamin B_{12} in intermediary metabolism. Recent work of Maley & Lardy (1955) suggests that the thyroid hormone probably acts by impairing the efficiency of oxidative phosphorylation. However, the hormone had no direct effect on the enzymes of oxidative phosphorylation, as shown by Cooper & Lehninger (1956) with digitonin preparations from mitochondria. It is possible therefore that damage to the mitochondrial morphology may be one of the reasons for the observed derangements. Fatterpaker, Marfatia & Sreenivasan (1955) studied certain model conjugation systems, such as acetylation of *p*-aminobenzoic acid and benzoylation of glycine, and observed that in the hyperthyroid animal there is a marked reduction in the efficiency of coupling of the energy-generating and -utilizing mechanisms. The counteraction of this condition by vitamin B_{12} led them to suggest that the primary manifestation of thyrotoxicosis is a deficiency of vitamin B_{12} and that vitamin B_{12} probably acts by restoration, in part at least, of oxidative phosphorylation.

The work presented here deals with the vitamin B_{12} reserves in tissues of animals fed on diets supplemented with iodinated casein. A pronounced lowering of liver and blood vitamin B_{12} is shown to be paralleled by a major derangement in the metabolism of soluble sulphydryl compounds in the liver. Observations on centrifugally separated cell fractions also indicate damage to the mitochondria. All these derangements are corrected by vitamin B_{12}, which suggests that vitamin B_{12} probably exerts protection through the maintenance of mitochondrial morphology and levels of liver sulphydryl compounds.

EXPERIMENTAL

Induction of thyrotoxicosis in rats. Weanling rats (Wistar strain) 3–4 weeks old and weighing about 40 g. were used. They were fed on a purified diet of the following composition (per cent, by wt.): ethanol-extracted casein 10, starch

68, sucrose (vitaminized) 10, salt mixture (U.S. Pharmacopoeia IV) 4, sesame oil 6 and shark-liver oil 2. Commercial casein was extracted with ethanol first by refluxing for 12 hr. and subsequently in a Soxhlet-type extractor for 48 hr. The extracted casein usually contained less than 10 μmg. of vitamin B_{12}/g. Vitamins were added at the following concentrations (mg./kg. of diet): thiamine hydrochloride 6, riboflavin 10, niacin 30, calcium pantothenate 20, pyridoxine 6, biotin 1, inositol 500, *p*-aminobenzoic acid 100, choline chloride 500, menadione (2-methyl-1:4-naphthaquinone) 10, vitamin E 50 and folic acid 5. Vitamin B_{12} was omitted. Iodinated casein (Protamone, Cerophyl Laboratories, Kansas City, Mo., U.S.A.) was incorporated (0·3%) into this diet for an initial period of 7 days, after which the animals were divided into four groups of four to six animals each, which were fed on the following diets: I, basal diet; II, basal diet + vitamin B_{12}; III, basal diet + iodinated casein; IV, basal diet + iodinated casein + vitamin B_{12}.

Each rat of groups II and IV was given 10 μg. of vitamin B_{12} (Cobione, Merck and Co., Inc.) intraperitoneally every other day. The animals were weighed thrice a week. After the fourth week from grouping, when the growth rate of animals in group III had declined considerably compared with that of the basal group and distinct symptoms of hyperthyroidism including altered haematological findings were present, the animals were used for the various experiments.

The laboratory stock ration used in some cases was (g./100 g. of diet): whole-wheat flour 75, casein 12, whole-milk powder 2, dried yeast 2, arachis oil 5, sodium chloride 2 and calcium carbonate 2.

Preparation of tissues. The rats were kept without food overnight and were then killed by decapitation and allowed to bleed; the livers were quickly excised and chilled in cold 0·25 M-sucrose solution. After 5 min. the livers were dried rapidly by pressing between filter papers, the connective tissue was removed and weighed pieces were homogenized to a 10% suspension in a previously chilled Potter–Elvehjem glass homogenizer. The sucrose solution contained 1·8 mM-$CaCl_2$ when homogenates were used for centrifugal separation of cell fractions, in order to avoid agglutination of mitochondria and consequent contamination of nuclear fractions (Hogeboom & Schneider, 1952). Although Ca^{2+} ions are known (Ernster & Low, 1955; Slater & Cleland, 1952) to affect mitochondrial behaviour to some extent, the effect may be uniform with all the groups.

When blood was required, ether anaesthesia was used and blood was withdrawn by venepuncture and immediately citrated, and portions were centrifuged for separate estimations in plasma and cells.

Centrifugal separation of cell fractions. The homogenates were strained through cloth and portions were fractionated in an International Refrigerated Centrifuge (PR-2), essentially by the procedure of Schneider & Hogeboom (1950). Nuclei were separated by centrifuging at 700 *g* for 10 min. and mitochondria at 5000 *g* for 10 min. Each time the sediment was washed once with 0·25 M-sucrose solution and the washings were added to the supernatant. The unsedimented translucent part of the homogenate from which most of the large-sized granules were removed was taken as the supernatant fraction. Succinic dehydrogenase activity, which is known to reside entirely in mitochondria (Schneider, Claude & Hogeboom, 1948), was used as a criterion for judging the purity of cell fractions. The enzyme activity was followed manometrically with 1 ml. of 0·1 M-phosphate buffer, pH 7·4, 0·2 ml. of 0·01 M-methylene blue, 1 ml. of liver homogenate (1:50), or its equivalent liver fraction, and 0·2 ml. of 0·01 M-sodium succinate in a final volume of 3 ml. It was found that in spite of using sucrose solution containing $CaCl_2$, some sedimentation of mitochondria with nuclei could not be avoided, and only 87% of the total succinic dehydrogenase activity was recoverable in the mitochondrial fraction; the nuclear fraction contained about 14% of the activity. Observations with the nuclear and mitochondrial fractions are therefore subject to these limitations.

Determination of vitamin B_{12}. The vitamin was liberated by incubation overnight under toluene with papain (British Drug Houses Ltd.) with 25 mg./g. of liver or fraction, or 25 mg./ml. of blood or plasma, in acetate buffer (pH 4·6). The samples were then autoclaved at 15 lb./in.^{2} for 5 min., homogenized, neutralized and made to volume. Necessary blanks were run alongside. Assays were carried out with *Lactobacillus leichmannii* (ATCC 7830) by a turbidimetric adaptation of the U.S. Pharmacopoeia method.

Assay of soluble sulphydryl compounds. The soluble sulphydryl content of liver and fractions and of blood was determined according to the method of Grunert & Phillips (1951). Results are expressed in terms of glutathione as standard.

Determination of glutathione. The alloxan 305 method of Patterson & Lazarow (1955) for determination of blood glutathione was used with the following modifications.

The metaphosphoric acid extract of liver or fractions, or of haemolysed blood, was saturated with NaCl and allowed to stand for 15–30 min. in a deep freeze to allow for complete extraction and precipitation of proteins. To 1 ml. of the protein-free filtrate, containing 5% of metaphosphoric acid, was added 1 ml. of 0·1 M-alloxan solution and the mixture was brought to pH 7·5 by successive and rapid additions of portions of 0·5 N-NaOH calculated previously to neutralize exactly the metaphosphoric acid and alloxan in the samples. At this pH, glutathione reacts maximally with alloxan in 6 min., after which the reaction product is stabilized by a further addition of 1 ml. of N-NaOH. The extinction of the resulting 'glutathione–alloxan' complex was read at 305 mμ in a Beckman DU spectrophotometer. The non-specific absorption in the sample was eliminated by reading the sample against a blank containing all the constituents except alloxan, which was replaced by water. The amount of glutathione in the sample was read from a standard curve obtained with graded amounts of glutathione treated similarly and which was corrected for absorption due to alloxan-decomposition products. The values reported are, however, subject to the limitations described by the original authors.

With 50–100 mg. amounts of liver as homogenate, and with 50 μg. additions of glutathione, recoveries ranging from 97·5 to 103·0% of added glutathione were obtained by this procedure.

Incorporation of cysteine into glutathione in vivo. In experiments on the incorporation of administered cysteine into liver sulphydryl compounds *in vivo*, the following procedure was adopted. L-Cysteine hydrochloride (30 mg.) in neutral solution (1 ml.) was given intraperitoneally to adult young rats (100 g.) and the animals were killed at

stated intervals. Vitamin B_{12} (10 μg./rat) was injected intraperitoneally 3 hr. before the cysteine. Total liver sulphydryl compounds and glutathione were determined as above.

Enzyme activities. The enzyme activities of the homogenates and cell fractions were determined immediately after fractionation. All incubations were at 37°. Adenosine triphosphatase was determined according to Schneider (1946) with the inclusion of Mg^{2+} ions to study the Mg^{2+} ion-activated adenosine triphosphatase reported present in nuclei (Novikoff, Hecht, Podber & Ryan, 1952). Pyrophosphatase activity was estimated by the procedure of McElroy, Coulombre & Hays (1951). For the determination of acid phosphatase activity the method of Palade (1951) and for alkaline phosphatase that of Dounce (1943) were followed.

Inorganic phosphate was estimated by the method of Tausky & Shorr (1953). All results are expressed as mg. of inorganic phosphate liberated/hr./g. of fresh liver.

Determination of lipids. The total lipid content was taken as the total ether-extractable material after drying at 90°. The weights were checked with those of the extracted residues.

Determination of phospholipids. Portions of homogenates or fractions were treated thrice with cold 10% trichloroacetic acid to extract acid-soluble phosphorus (Schneider, 1945). The residue was resuspended in water and washed twice with 95% ethanol to remove trichloroacetic acid. Phospholipids were then repeatedly extracted by boiling the residue with ether–ethanol (1:3) (Kennedy, 1953), and the extracts were combined and the solvents evaporated. The residual fatty material which contained the phospholipids was then digested with perchloric acid and phosphorus was determined in the digest by the method of Tausky & Shorr (1953). The phospholipids were calculated by using 22·7 as conversion factor.

Determination of total choline. The procedure adopted for the liberation of choline from tissue samples was a modification of the method of Luecke & Pearson (1944). To portions of liver homogenate or fractions corresponding to 1 g. of fresh liver was added 5 ml. of 3% H_2SO_4 and the mixture was autoclaved for 2 hr. at 15 lb./in.^{2} (Horowitz & Beadle, 1943). The hydrolysate was cooled, neutralized with $Ba(OH)_2$ to pH 5·0–5·5 and made to a convenient volume and filtered. The filtrate was used for the determination of choline: 5–10 ml. was passed through activated (Hennessey, 1941) Decalso (The Permutit Co. Ltd., London, W. 4) columns. After washing the column with 0·3% NaCl solution the choline was eluted with 5% NaCl solution. A portion of the eluate containing about 10–20 μg. of choline was used for precipitation as periodide by the method of Appleton, La Du, Levy, Steele & Brodie (1953). The precipitate was centrifuged in specially tapered centrifuge tubes and dissolved in redistilled and moisture-free ethylene dichloride. The light absorption was measured on the Beckman DU spectrophotometer at 365 mμ and results were read from a standard curve for choline in the range 0–50 μg.

Determination of non-protein nitrogen and free amino acids. Samples of plasma or liver homogenates were deproteinized with 10% trichloroacetic acid and the non-protein nitrogen was determined in the filtrates by digestion and direct nesslerization by the method described by Umbreit, Burris & Stauffer (1946).

Table 1. *Growth and haematological results*

Weanling rats were fed a purified vitamin B_{12}-free, 10% casein diet containing 0·3% of iodinated casein. After 7 days, iodinated casein was withdrawn from the basal diet and the animals (initial weight 47 ± 2 g.) were grouped as follows. Animals were killed 4 weeks after grouping. For details see text. Results are averages of five to six animals in each group.

Diet	Final body wt. (g.)	Gain in wt. (%)	Liver wt. (g.)	Liver wt. (g./100 g. body wt.)	10^6 × Erythrocytes/mm.^{3}	10^3 × Leucocytes/mm.^{3}	Haemoglobin (%)	Packed cell volume (%)	Vitamin B_{12} in blood (μmg./ml.)	Blood vitamin B_{12} in plasma (%)
Basal	60 ± 4	28	2·45 ± 0·25	4·00 ± 0·31	9·0	7·0	14·1	35·1	1·53 ± 0·43	43
Basal + vitamin B_{12}	71 ± 3	51	2·90 ± 0·30	3·81 ± 0·24	10·8	8·0	15·0	36·4	1·80 ± 0·30	45
Basal + iodinated casein	43 ± 3	−8	2·32 ± 0·23	4·51 ± 0·17	4·9	6·6	12·8	32·2	0·90 ± 0·55	61
Basal + iodinated casein + vitamin B_{12}	50 ± 2	+6	2·05 ± 0·28	3·94 ± 0·21	7·4	7·8	15·9	33·8	2·01 ± 0·25	54

Protein-free plasma and liver filtrates were assayed for free amino acids microbiologically, according to the procedures of Hier & Bergeim (1945) and of Solomen, Johnson, Sheffner & Bergeim (1951) respectively, in the uniform assay media of Henderson & Snell (1948).

RESULTS

All values reported represent averages in each case of not less than five independent samples.

The growth-rate and haematological results for rats in the different groups over a period of 5 weeks are given in Table 1. The growth rate of animals even in the basal group was suboptimum, presumably because of the low amount of protein (10%) in the diet.

Vitamin B_{12}

A marked fall in the concentration of vitamin B_{12} in the circulating blood was observable in the hyperthyroid rats. This was prevented by the administration of vitamin B_{12}. A similar fall in liver vitamin B_{12} of thyrotoxic animals was also corrected by the vitamin (Table 2). In the last case, most of the additional vitamin was present in the nuclear and supernatant fractions. It has also been found that the low levels of vitamin B_{12} in thyrotoxic animals deficient in vitamin B_{12} return to normal when iodinated casein is withdrawn from the diet.

Iodinated casein has been reported to obstruct vitamin B_{12} absorption in chicks (Kano, Anderson, Hougham & Charkey, 1954). However, no interference in the absorption of ingested vitamin B_{12} from the gut was observed in rats made hyperthyroid by feeding with iodinated casein diets. Determination of vitamin B_{12} levels in the livers at intervals of 6 and 24 hr. after 10 μg. of the vitamin (in 1 ml.) was given to the rat (Haffkine strain) by stomach tube under mild ether anaesthesia showed that within 6 hr. the vitamin content of hyperthyroid-animal livers rose to normal and then declined to original levels during 24 hr. (Table 3). The absorption of the vitamin therefore appeared to be determined solely by the extent of vitamin B_{12} deficiency in the animal.

Metabolism of sulphydryl compounds

In experiments with adult young rats on the laboratory stock diet, it was observed that maximal values for sulphydryl compounds in liver were reached within 3 hr. of cysteine administration (Table 4). Although a major portion of sulphydryl compounds in liver is normally accounted for as glutathione, only about 50% of the total sulphydryl compound reacted as glutathione at this time after administration of cysteine. Normal amounts of total sulphydryl compounds were reached about 24 hr. later. The glutathione concentration, however, steadily increased up to 12 hr., again reaching normal levels after 24 hr. Thus between 3 and 12 hr. there was a steady increase in the proportion of glutathione to total sulphydryl. Previous administration of 10 μg. of vitamin B_{12} to animals on stock diet did not make any difference to this pattern (Table 4). The absence of an effect due to vitamin B_{12} on the incorporation of cysteine into glutathione might be because these stock animals had sufficient liver stores of vitamin B_{12}. In hyperthyroid animals, vitamin B_{12} enhanced glutathione synthesis (Table 5).

From the results reported in Tables 6 and 7 it may be seen that the pronounced lowering of tissue and blood vitamin B_{12} in the hyperthyroid rat is accompanied by a major depletion of reserves of liver sulphydryl compounds, a greater portion of the depletion being limited to the soluble fraction of the liver cells only (Edwards & Westerfeld, 1952). The mitochondrial fraction was very low in content of sulphydryl compounds. The small amount present in the nuclei is relatively less labile. Vitamin B_{12} counteracts these conditions appreciably.

The incorporation of administered cysteine into liver and blood sylphydryl compounds *in vivo* is also found to be decreased in the hyperthyroid animal. Sulphydryl compound levels were determined 3 hr. after cysteine administration. Although the major increase occurred in the soluble fraction of liver, the nuclear sulphydryl compounds also rose appreciably. These studies are included in Tables 6 and 7.

Table 2. *Distribution of vitamin B_{12} in liver*

For details see Table 1 and text.

Diet	Vitamin B_{12} (μmg./g. of fresh liver or equivalent)			
	Whole liver	Nuclei	Mitochondria	Supernatant
Basal	45·3±5·2	14·2±1·0	12·9±0·7	16·8±2·7
Basal + vitamin B_{12}	49·2±3·7	20·8±1·8	13·2±1·2	12·9±0·9
Basal + iodinated casein	37·9±2·5	13·7±0·7	8·1±0·3	14·7±1·3
Basal + iodinated casein + vitamin B_{12}	96·3±4·5	41·3±3·0	18·4±0·4	31·9±3·1

An observation emerging from this study is the increase in blood sulphydryl compounds during thyrotoxicosis and its reversal by vitamin B_{12} (Table 6). Such an observation has also been reported earlier from this laboratory (Fatterpaker *et al.* 1955). However, a differential assay of blood sulphydryl compounds indicated that this rise is not due to glutathione, which is actually slightly depleted (Table 8).

Lipids and total choline

The pyridinonucleotide content of hyperthyroid rat-liver mitochondria has been shown to be lower than that of normal ones (Maley & Lardy, 1955), and since mitochondrial behaviour appears to be altered in thyrotoxicosis, as is evident from the altered vitamin B_{12} distribution and also from other studies (Kasbekar & Sreenivasan, 1959), it was of interest to study the lipid content of hyperthyroid animal livers. Studies on fat distribution of livers are included in Table 9.

The increase in neutral liver fat in hyperthyroid animals occurs both in the particulate and soluble fractions, the major variation occurring in the latter, wherein a greater portion of the neutral fat separates out on homogenization.

Phospholipids are major structural constituents of mitochondrial membrane. Depletion of these phospholipids could therefore accompany damage to mitochondria. It has been suggested that the lipotropic action of choline might arise as a result of its presence in the structural phospholipids of mitochondria and that fatty infiltration would follow a depletion of choline-containing phospholipids and hence an impairment of mitochondrial integrity. No major alteration in phospholipids of mitochondrial fraction was, however, observable in hyperthyroid animals, this being limited to the soluble fraction. The values for total choline indicate a 20–30 % decrease in all fractions (Tables 10 and 11).

Enzyme activities

Results of a study of the activities of certain phosphatases, particularly that of adenosine triphosphatase, which can be representative of the extent of damage to mitochondrial structure (Kielley & Kielley, 1951), are included in Tables 12–14.

The adenosine triphosphatase activity of hyperthyroid-animal livers was significantly increased, the major increase being limited to the mitochondrial fraction. There was little redistribution of the enzyme in the cell particles. Pyrophosphatase and acid phosphatase showed increased activities in iodinated casein-fed groups, whereas alkaline phosphatase activity was slightly reduced. A transfer of these enzymes from particulate to

Table 3. *Intestinal absorption of vitamin* B_{12}

Food cups were withdrawn for half-hour periods before and after vitamin B_{12} (10 μg.) was administered by stomach tube. Animals were killed for assay for liver and blood vitamin B_{12} at 6 and 24 hr. intervals.

	Liver					Blood		
Time (hr.) ...	0	6		24		0	6	24
Diet	(μmg./g.)	(μmg./g.)	% increase	(μmg./g.)	% increase	(μmg./ml.)	(μmg./ml.)	(μmg./ml.)
Basal + vitamin B_{12}	75·0 ± 5·0	102·5 ± 6·5	36·7	82·5 ± 4·0	11·0	1·05 ± 0·25	2·10 ± 0·30	1·50 ± 0·25
Basal + iodinated casein	60·0 ± 3·5	122·5 ± 8·0	104·1	72·5 ± 6·5	20·8	1·00 ± 0·20	1·00 ± 0·25	1·00 ± 0·25
Basal + iodinated casein + vitamin B_{12}	115·0 ± 4·5	157·5 ± 9·5	37·0	115·0 ± 8·5	0·00	1·55 ± 0·3	1·50 ± 0·25	1·50 ± 0·20

Table 4. *Glutathione synthesis from injected cysteine by normal rat liver* in vivo

Adult young rats (100 g.) on the laboratory stock diet were injected intraperitoneally with 30 mg. of cysteine hydrochloride in 1 ml. of neutral solution and the animals were killed at the stated intervals for removal of liver. Determinations of total sulphydryl compounds and glutathione were made according to methods detailed in the text. Vitamin B_{12}, where given, was also injected intraperitoneally (10 μg./rat) 3 hr. before the intraperitoneal injection of cysteine.

	Total sulphydryl (mg./100 g. of fresh liver)		Glutathione (mg./100 g. of fresh liver)	
	Without vitamin B_{12}	With vitamin B_{12}	Without vitamin B_{12}	With vitamin B_{12}
Normal	139·8±10·4	—	131·2±7·8	—
Time after cysteine (hr.)				
3	273·1±14·6	270·6±17·4	141·3±9·6	138·9±11·2
5	243·4±11·8	249·0±11·1	159·6±6·9	167·1±5·4
7	216·8±12·9	223·7± 9·8	176·0±5·8	179·2±7·3
12	194·0±12·1	210·1± 8·7	177·7±6·7	186·0±4·6
14	190·1±10·4	186·5±11·0	179·9±8·0	174·1±8·9
24	120·9±11·3	130·2±13·4	109·1±4·4	114·2±5·7

Table 5. *Incorporation of cysteine into liver glutathione*

Animals were fed and grouped as detailed in Table 1 and in the text. Cysteine administration was at 30 mg./100 g. body wt. Other details were as described in the text.

Time after cysteine (hr.)	Glutathione (mg./100 g. of fresh liver)		
	Basal diet + vitamin B_{12}	Basal diet + iodinated casein	Basal diet + iodinated casein + vitamin B_{12}
0	85·5±5·4	37·1±3·8	59·2±4·1
3	98·9±6·1	45·0±2·9	70·3±2·8
7	120·1±5·8	55·4±4·0	95·5±5·6
14	135·0±4·9	61·2±3·5	105·5±3·7
24	89·7±6·0	35·4±5·1	66·0±3·9

Table 6. *Incorporation of cysteine into sulphydryl compounds of liver and blood*

For details see text and Tables 1 and 5. In the groups injected with cysteine intraperitoneally, the animals were killed 3 hr. after its administration.

Animals and diet	Sulphydryl compounds (mg./100 g. fresh wt.)			
	Without cysteine		With cysteine	
	Liver	Blood	Liver	Blood
Basal diet				
Vitamin B_{12}-deficient	75·2±7·1	10·9±1·8	213·0±16·1	23·9±2·9
Vitamin B_{12}-fed	83·0±6·5	11·5±1·5	259·2±14·2	23·2±3·1
Hyperthyroid diet				
Vitamin B_{12}-deficient	27·2±4·2	25·6±2·6	96·4±11·3	39·2±2·6
Vitamin B_{12}-fed	57·3±8·9	18·7±1·4	209·1± 7·5	27·6±3·2

Table 7. *Distribution of sulphydryl compounds in liver fractions*

For details, see Table 6 and text.

Animals and diet	Sulphydryl compounds (mg./100 g. equivalent of liver)			
	Without cysteine		With cysteine	
	Nuclei	Supernatant	Nuclei	Supernatant
Basal diet				
Vitamin B_{12}-deficient	11·6±1·1	62·3±7·8	16·4±2·6	184·5±10·1
Vitamin B_{12}-fed	12·0±0·7	67·1±6·5	18·2±2·9	227·2±11·8
Hyperthyroid diet				
Vitamin B_{12}-deficient	9·0±0·9	17·8±5·9	12·6±1·8	82·8±7·6
Vitamin B_{12}-fed	10·5±1·0	45·6±7·2	14·1±2·4	185·0±14·5

Table 8. *Incorporation of cysteine into blood glutathione*

The glutathione values reported are for samples of blood whose total sulphydryl values are given in Table 6.

Diet	Glutathione (mg./100 ml.)	
	Without cysteine	With cysteine
Basal	8·94±1·04	13·11±1·82
Basal + vitamin B_{12}	10·12±0·81	16·24±1·41
Basal + iodinated casein	5·42±1·27	7·61±1·16
Basal + iodinated casein + vitamin B_{12}	7·96±0·68	10·16±1·02

Table 9. *Distribution of liver lipids*

For details of diets and grouping see Table 1 and text.

Group	Lipids (mg./g. of fresh liver)			
	Whole homogenate	Nuclei	Mitochondria	Supernatant
Control				
Vitamin B_{12}-deficient	24·7±1·3	4·7±0·3	5·5±0·4	14·4±0·8
Vitamin B_{12}-protected	19·9±2·1	4·0±0·1	4·9±0·1	10·8±1·1
Hyperthyroid				
Vitamin B_{12}-deficient	32·8±1·9	6·2±0·3	7·2±0·1	19·7±1·3
Vitamin B_{12}-protected	23·6±2·4	4·6±0·2	5·9±0·3	13·2±1·0

Table 10. *Distribution of liver phospholipids*

For details of diets and grouping see Table 1 and text.

Diet	Phospholipids (mg./g. fresh wt. equivalent of liver)			
	Whole homogenate	Nuclei	Mitochondria	Supernatant
Basal	12·68±0·36	2·15±0·07	4·31±0·10	6·12±0·16
Basal + vitamin B_{12}	12·81±0·40	2·21±0·12	4·43±0·14	6·14±0·21
Basal + iodinated casein	10·93±0·24	2·04±0·05	3·91±0·08	4·92±0·15
Basal + iodinated casein + vitamin B_{12}	11·64±0·32	2·20±0·10	4·13±0·06	5·45±0·19

Table 11. *Distribution of total liver choline*

For details of diets and grouping see text.

Diet	Choline (mg./g. fresh wt. equivalent of liver)			
	Whole homogenate	Nuclei	Mitochondria	Supernatant
Basal	4·81±0·24	0·92±0·08	1·50±0·12	2·52±0·14
Basal + vitamin B_{12}	5·01±0·19	1·08±0·09	1·46±0·15	2·63±0·07
Basal + iodinated casein	3·82±0·07	0·64±0·05	1·14±0·11	1·96±0·11
Basal + iodinated casein + vitamin B_{12}	4·51±0·17	0·99±0·11	1·36±0·07	2·99±0·16

Table 12. *Liver adenosine triphosphatase activity*

For details of diets, grouping and methods see Table 1 and text.

Animals and diet	P liberated (mg./hr./g. of fresh liver)			
	Whole homogenate	Nuclei	Mitochondria	Supernatant
Basal diet				
Vitamin B_{12}-deficient	24·0±2·4	7·9±0·4	6·5±1·4	10·0±0·9
Vitamin B_{12}-fed	17·2±1·8	6·1±0·2	2·4±0·6	8·8±1·1
Hyperthyroid diet				
Vitamin B_{12}-deficient	27·6±2·1	9·7±0·2	8·0±1·7	11·3±0·7
Vitamin B_{12}-fed	20·7±3·2	7·7±0·3	4·4±1·5	9·1±0·8

Table 13. *Activities of liver pyrophosphatase, acid phosphatase and alkaline phosphatase*

For details of diets, groupings, etc. see Table 1 and text.

Animals and diet	P liberated (mg./hr./g. of fresh tissue)		
	Pyrophosphatase	Acid phosphatase	Alkaline phosphatase
Basal diet			
Vitamin B_{12}-deficient	27·4±2·4	3·84±0·18	0·99±0·03
Vitamin B_{12}-fed	21·3±2·0	3·26±0·12	1·13±0·07
Hyperthyroid diet			
Vitamin B_{12}-deficient	30·8±3·1	3·92±0·21	0·85±0·08
Vitamin B_{12}-fed	24·3±1·8	3·80±0·17	1·14±0·05

Table 14. *Distribution of phosphatases in liver-cell fractions*

For details of diets, groupings, etc. see Table 1 and text. N, Nuclei; M, mitochondria; S, supernatant.

Diet	Percentage distribution of enzyme activity								
	Pyrophosphatase			Acid phosphatase			Alkaline phosphatase		
	N	M	S	N	M	S	N	M	S
Basal	16·7	14·9	65·3	12·3	43·2	41·4	68·9	0	25·8
Basal + vitamin B_{12}	23·5	19·7	58·2	14·7	51·8	31·9	83·0	0	14·9
Basal + iodinated casein	13·9	11·4	74·0	11·8	40·0	50·0	65·0	0	32·6
Basal + iodinated casein + vitamin B_{12}	18·1	15·6	65·9	12·9	41·8	40·8	72·9	0	18·4

Table 15. *Concentrations of non-protein nitrogen and free amino acid in liver*

For details of diets, groupings, etc. see Table 1 and text. Values for amino acids are those for the amino acids given in column 1, which gave the same assay.

	Control (mg./100 g. of fresh liver)		Hyperthyroid (mg./100 g. of fresh liver)	
	Vitamin B_{12}-deficient	Vitamin B_{12}-fed	Vitamin B_{12}-deficient	Vitamin B_{12}-fed
Non-protein N	310±14	290±8	420±12	340±19
L-Arginine	3·36±0·12	1·72±0·08	4·66±0·12	3·68±0·16
L-Histidine	12·14±0·78	10·01±1·10	15·40±0·96	12·14±0·84
DL-Isoleucine	3·68±0·12	2·56±0·06	3·71±0·09	2·91±0·13
DL-Leucine	9·98±0·30	7·74±0·07	10·11±0·10	8·85±0·25
L-Lysine	7·79±1·81	4·91±1·12	9·26±1·51	7·72±0·68
DL-Methionine	12·41±0·86	8·62±0·40	16·12±0·75	10·18±0·94
DL-β-Phenylalanine	11·55±0·32	5·18±0·06	15·48±0·12	8·65±0·11
DL-Threonine	54·16±1·56	19·78±1·18	64·98±2·48	32·32±0·99
DL-Tryptophan	1·42±0·06	0·98±0·01	2·07±0·04	1·17±0·02
DL-Valine	9·29±0·79	7·04±0·69	9·61±0·46	8·65±0·76

Table 16. *Concentrations of non-protein nitrogen and free amino acids in plasma*

For details of diets, groupings, etc. see Tables 1 and 15 and text.

	Control (mg./100 ml. of plasma)		Hyperthyroid (mg./100 ml. of plasma)	
	Vitamin B_{12}-deficient	Vitamin B_{12}-fed	Vitamin B_{12}-deficient	Vitamin B_{12}-fed
Non-protein N	100·4±64·0	51·20±38·3	121·6±89·0	89·7±10·9
L-Arginine	18·67±0·26	4·62±0·15	20·94±0·21	8·14±0·19
L-Histidine	6·00±0·14	2·28±0·09	6·00±0·12	2·70±0·04
DL-Isoleucine	3·39±0·15	2·64±0·06	4·78±0·10	3·16±0·11
DL-Leucine	6·15±0·22	2·25±0·31	6·75±0·25	2·54±0·19
DL-Lysine	7·45±0·61	5·02±0·52	11·23±0·41	7·21±0·33
DL-Methionine	5·94±0·10	0·84±0·04	6·24±0·04	1·32±0·06
DL-β-Phenylalanine	7·20±0·09	2·01±0·02	7·24±0·05	2·92±0·02
DL-Threonine	4·88±0·26	4·16±0·46	5·70±0·31	4·62±0·29
DL-Tryptophan	3·24±0·16	2·34±0·25	4·80±0·18	2·94±0·14
DL-Valine	6·60±0·12	3·00±0·08	6·80±0·11	3·70±0·18

the soluble fraction is evident from distribution studies with cell fractions of normal and hyperthyroid-animal livers, and would point to damage to the particulate fractions *in vivo*. Vitamin B_{12} counteracts these changes to a considerable extent.

Free amino acids in tissues of thyrotoxic animals and the effect of vitamin B_{12}

In chicks, an increased urinary nitrogen excretion in thyrotoxicosis has been attributed to a catabolism of tissue proteins (Rupp, Paschkis & Cantarow, 1951). A quantitative study of non-protein nitrogen and free amino acid levels of plasma and liver was therefore undertaken. It is evident from the results (Tables 15 and 16) that there is a considerable rise in non-protein nitrogen and in free amino acids in both plasma and liver, the increase being more marked in plasma. Vitamin B_{12} administration exerts a moderating effect on these levels, indicating that the vitamin checks the drain on tissue protein. Charkey, Kano & Anderson (1954) observed a similar moderating effect of the vitamin on free amino acid levels in chick blood during fasting.

DISCUSSION

The foregoing results indicate that the deficiency of vitamin B_{12} in thyrotoxicosis does not arise as a result of an interference with the intestinal absorption of the vitamin, which depends on the vitamin B_{12} status of the animal, but is due apparently to the decreased retention of vitamin B_{12} or to its increased metabolic requirement or to both. There is a pronounced decrease of the vitamin in liver and its mitochondrial fraction. A similar decrease in blood vitamin B_{12} is accompanied by a loss of the erythrocyte vitamin to the plasma. These observations therefore suggest damage to cells and cellular particles.

Damage to cells and cellular particles on ageing or on subjecting them to adverse environmental conditions is known to lead, among other things, to changes in enzyme activities and distribution (Kielley & Kielley, 1951). Similar changes have been described in mitochondrial fractions from fatty livers (Dianzani, 1954). Decreased levels of pyridinonucleotides and uncoupling of oxidative phosphorylation in mitochondrial preparations from thyrotoxic-rat livers have also been reported (Maley & Lardy, 1955). The increased mitochondrial adenosine triphosphatase activity and altered distribution of phosphatases in vitamin B_{12} deficiency suggest analogous changes in mitochondrial properties.

In the hyperthyroid animals no appreciable change in phospholipids of liver mitochondria was observed. It is possible that an alteration in the nature of mitochondrial phospholipids underlies damage to the mitochondrial membrane. Witter & Cottone (1956) suggested that lecithin in mitochondria might be converted into lysolecithin during mitochondrial damage. Vitamin B_{12} may aid in the maintenance of mitochondrial integrity.

The lowering of blood and liver vitamin B_{12} in the hyperthyroid animals is closely paralleled by a depletion of glutathione reserves of these tissues. It has been demonstrated by Goldzieher, Rawls & Goldzieher (1953) that administration of cysteine results in an increase in the non-protein sulphydryl compounds of rat liver. The incorporation of administered cysteine into liver and blood glutathione also suffers in the hyperthyroid animals. These results therefore suggest the participation of vitamin B_{12} in the metabolism of sulphydryl compounds. A depletion of blood glutathione in pernicious-anaemia patients and in vitamin B_{12}-deficient rats has been reported to be restored to normal by administration of vitamin B_{12} (Ling & Chow, 1953, 1954; Register, 1954). During thyrotoxicosis the depletion of blood glutathione is accompanied by an increase in the content of total sulphydryl compounds.

The mechanism by which vitamin B_{12} could exert protection against damage to cellular structures is not clear. It is possible that this protection might arise from the effect of the vitamin on glutathione reserves of the cell because of the protection of mitochondrial integrity by sulphydryl groups. Thus Tapley & Cooper (1956) found that sulphydryl-binding compounds produced a rapid and pronounced mitochondrial swelling, suggesting that sulphydryl groups might play an important part in determining the mitochondrial structure or permeability or both. The suggestion of Hunter, Davis & Carlat (1956) that oxidation of sulphydryl groups might precede damage to mitochondrial structure supports this hypothesis. Witter & Cottone (1956) observed that lysolecithinase of snake venom was capable of producing damage to mitochondrial structure as a result of conversion of lecithin into lysolecithin. If the damage to mitochondria arises from an activation of lysolecithinase of mitochondria, Labarre's observation (1945) that the haemolytic effect of cobra venom in rabbits can be abolished by a simultaneous or prior administration of glutathione would again suggest a role for this metabolite in protecting cellular material from damage. Factors producing vitamin B_{12} deficiency and leading to a depletion of glutathione might therefore be expected to lead to an impairment of mitochondrial structure, of oxidative phosphorylation and eventually to a general metabolic derangement. These would be corrected by vitamin B_{12} probably through its influence on glutathione reserves.

SUMMARY

1. Effects of experimentally induced hyperthyroidism in rats and protection by vitamin B_{12} have been investigated.

2. It was observed that thyrotoxicosis results in a vitamin B_{12} deficiency in blood and in liver and its mitochondrial fraction.

3. In thyrotoxicosis there is no interference in the intestinal vitamin B_{12} absorption, which appears to depend mainly on the vitamin B_{12} status of the animal.

4. The depletion of liver and blood glutathione closely follows the fall in tissue vitamin B_{12} levels.

5. The incorporation of administered cysteine into liver glutathione is also decreased in thyrotoxicosis.

6. There is an increased accumulation of fat in liver and its centrifugally separated fractions, particularly the supernatant. A slight drop in total choline content is seen in all fractions; however, phospholipid content does not undergo significant alterations, those observed being limited to the soluble fraction.

7. An increased mitochondrial adenosine triphosphatase and an altered distribution of other soluble phosphatases in liver also suggest damage to the cellular particles in the thyrotoxic animal.

8. A considerable increase in non-protein nitrogen and free amino acids occurs in the liver and plasma of hyperthyroid animals.

9. The protective action of vitamin B_{12} is reflected in a correction of the various metabolic derangements observed in thyrotoxicosis.

10. The observations are discussed with special reference to the significance of sulphydryl compounds in the maintenance of normal mitochondrial properties.

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