The Influence of Vitamin B_{12} on the Content, Distribution and in Vivo Synthesis of Thiamine Pyrophosphate, Flavin Adenine Dinucleotide and Pyridine Nucleotides in Rat Liver[1]

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Apart from the well-known interrelationships among the B vitamins, there is reason to believe that folic acid and vitamin B_{12} may influence the functioning of other vitamins as cofactors. Thus, dietary folic acid has been known to determine rat liver stores of coenzyme A (CoA) and adenotriphosphate (ATP) (Popp and Totter, '52; Totter, '53); a decrease in liver DPN is also caused by aminopterin[2] (Strength et al., '54). The *in vivo* incorporation of nitcotinamide into pyridinonucleotides in rat liver is affected in a deficiency of vitamin B_{12} (Nadkarni et al., '57). Low blood level of citrovorum factor in the hyperthyroid, vitamin B_{12}-deficient rat is corrected by administration of vitamin B_{12} (Pfander et al., '52). In liver homogenates from vitamin B_{12}-deficient hens, the synthesis of citrovorum factor from added folic acid is less than in those from animals injected with the vitamin (Doctor et al., '54). The potentiating effect of vitamin B_{12} in the mobilization of folic acid has also been reported from this laboratory (Sreenivasan, '51; Fatterpaker et al., '55a). The general influence of vitamin B_{12} on carbohydrate and lipid metabolism has been linked to a primary relation to sulphydryl biosynthesis (Ling and Chow, '54; Register, '54; Kasbekar et al., '56, '59a). Distinguished from these apparently collateral findings is the reported elevation of CoA in livers of vitamin B_{12}-deficient rats and mice (Boxer et al., '53, '55; Wong and Schweigert, '56).

The present work relates to a study of the influence of vitamin B_{12} on the intracellular distribution of thiamine pyrophosphate (TPP), flavin adenine dinucleotide (FAD) and pyridine nucleotides (PN), and to their *in vivo* synthesis from the corresponding administered vitamins, in the rat liver. Data on the distribution of these cofactors in liver cells of the normal rat are available in the works of Goethart ('52) and Dianzani and Dianzani Mor ('57) on TPP, of Carruthers and Suntzeff ('54) and Dianzani ('55) on pyridine nucleotides (PN) and of Schneider and Hogeboom (Schneider, '56) on FAD.

EXPERIMENTAL

Young, male Wistar rats weighing approximately 100 gm each were used. The animals, housed individually in raised mesh-bottom cages, were initially depleted of their vitamin B_{12} reserves by maintenance on a purified, iodo-casein ration. This consisted of the following percentage composition: hot, alcohol-extracted casein, 18; iodinated casein,[3] 0.15; arachis oil, 6; shark liver oil, 2; sucrose, 9.85; maize starch 60; and salt mixture (U.S.P. XIV), 4; with vitamins to provide in milligrams per kilogram of diet: thiamine·HCl, 6; riboflavin, 10; nicotinic acid, 30; calcium pantothenate, 20; pyridoxine·HCl 6; biotin, 1; folic acid, 5; *p*-aminobenzoic acid, 100; choline·Cl, 500; inositol, 500; 2-methyl-1, 4-naphthoquinone, 10; and α-tocopherol,

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[2] Strength, D. R., and N. I. Mondy 1953 Choline dehydrogenase activity and DPN content of rat livers following aminopterin injection. Federation Proc., *12*: 276 (abstract).

[3] Protomone, obtained from Cerophyl Laboratories, Kansas City, Mo.

50. The vitamin additions provided in this basal diet were considered adequate for the hyperthyroid condition. At the end of 4 weeks, animals were divided into two groups, one of which continued to receive the basal diet modified by the omission of iodinated casein and *p*-aminobenzoic acid and substitution by 2% of succinyl sulphathiazole. The latter addition was compensated for by adjusting the percentage of starch. The second, control group, received the modified diet with a supplement of vitamin B_{12} (150 μg/kg of diet). After a further 5-week period, the animals in both groups were divided into 4 sub-groups of 7 to 8 rats each, one of which was killed immediately to establish the liver distribution pattern of the cofactors in the vitamin B_{12}-deficient and replete states. The remaining three sub-groups of each group were used to study the synthesis of each of the three cofactors from the respective, administered vitamins. The rats were injected, intraperitoneally, on 5 successive days, with 5 mg of thiamine·HCl, 5 mg of riboflavin, or 1 mg of nicotinamide per rat per day. All animals were killed on the 5th day, 8 hours following the final injection of the test-vitamin.

The normal intracellular distribution pattern of the cofactors was secured on liver of 10 adult male rats weighing approximately 200 gm, maintained on the laboratory stock diet consisting of (gm per 100 gm of diet): whole wheat flour, 75; whole milk powder, 2; casein, 12; dried yeast, 2; arachis oil, 3; shark liver oil, 2; sodium chloride, 2; and calcium carbonate, 2.

Animals were exsanguinated and livers were perfused with ice-cold 0.25 M sucrose, promptly excised, blotted and made into 10% homogenates with 0.25 M sucrose in a Potter-Elvehjem homogenizer. The homogenates were separated by the differential centrifugation procedure of Schneider and Hogeboom ('50) into nuclear, mitochondrial and microsomal plus supernatant fractions using an International (PR-2) refrigerated centrifuge. Total and free thiamine in portions of whole homogenates and fractions were determined by a modification of the fluorometric method of Hennessy ('41) before and after hydrolysis of TPP complexes with taka-diastase for 18 hours at 37°C, the TPP content being calculated by difference. The total and non-FAD (flavin mono nucleotide, FMN plus free riboflavin) riboflavin were determined by the fluorometric procedure of Bessey et al. ('49) and the FAD content derived from these values with use of proper conversion factor, as indicated by these authors. The determination of total PN in homogenates and in fractions, as well as the differential determination of their oxidized and reduced forms in whole homogenates, was carried out fluorometrically by the procedure outlined by Dianzani ('55).

For determinations of blood erythrocyte count and hemoglobin content and of plasma vitamin B_{12} concentration, the animals were bled from tail veins and adequate samples collected in heparinated vials. The erythrocyte count was made by the standard method. Hemoglobin was determined by acid-hematin method in a Klett-Summerson photoelectric colorimeter (Kolmer et al., '51). Plasma vitamin B_{12} was determined by the method of Ross ('52) using *Euglena gracilis* as the test organism.

Portions of liver homogenates were incubated at 37°C for 12 hours under toluene with papain (25 mg/gm of fresh liver) to liberate the bound vitamin B_{12}, which was assayed using *E. gracilis* according to the method of Hoff-Jorgenson ('54).

The results were analyzed for statistical significance by calculating the t value of Fisher (Fisher and Yates, '53). Only the differences with a t value corresponding to a probability $P < 0.05$ were accepted as significant.

RESULTS

Content and distribution of cofactors and free vitamins in normal liver cytoplasm. The data obtained with liver homogenates of normal, stock diet-fed animals are reported in table 1. About 30% of the TPP of the homogenate was found contained in mitochondria, while the rest was present almost exclusively in the supernatant. The distribution pattern compares with data reported by others (Goethart, '52; Dianzani and Dianzani Mor, '57) under similar conditions. Free thiamine, also maximally localized in the supernatant

TABLE 1

Content and distribution of cofactors and free vitamins in cytoplasm fractions of normal rat liver. Mean values from 10 independent determinations

Cytoplasm fraction	TPP		Free thiamine		FAD		FMN + free riboflavin		Total PN	
	μg/gm[1]	%	μg/gm	%	μg/gm	%	μg/gm	%	μg/gm	%
Homogenate	17.20 ± 1.31[2]		4.91 ± 0.34		24.80 ± 1.40		8.56 ± 0.61		845 ± 25	
Nuclear	0.69 ± 0.13	4.3 ± 0.9	1.01 ± 0.09	22.9 ± 2.1	4.26 ± 0.81	17.7 ± 2.5	1.80 ± 0.21	21.9 ± 2.3	184 ± 16	24.5 ± 2.1
Mitochondrial	5.01 ± 0.92	31.4 ± 4.9	0.51 ± 0.06	11.6 ± 1.4	13.94 ± 1.13	58.2 ± 4.7	1.80 ± 0.19	21.9 ± 2.3	121 ± 11	16.2 ± 1.2
Supernatant	10.26 ± 1.11	64.3 ± 5.6	2.89 ± 0.13	65.5 ± 4.3	5.75 ± 0.61	24.0 ± 3.1	4.63 ± 0.88	56.2 ± 4.3	442 ± 18	59.2 ± 2.9
Recovery, %		92.8 ± 2.6		89.8 ± 1.8		96.6 ± 3.4		96.3 ± 3.1		88.4 ± 3.4

[1] Gram weight of fresh tissue.
[2] Standard error of mean.

TABLE 2

Growth rate and blood and liver values in vitamin B_{12} deficiency[1]

Group	Body weight[2]		Erythrocytes	Hemoglobin	Vitamin B_{12}	
	Initial	Final			Plasma	Liver
	gm	gm	× 10^6/mm³	gm/100 ml	μμg/ml	mμg/gm
Vitamin B_{12}-deficient	147 ± 4[3]	188 ± 4	4.0 ± 0.3	11.7 ± 0.5	87 ± 41	29.3 ± 9.2
Vitamin B_{12}-fed	146 ± 3	256 ± 5	6.9 ± 0.3	14.8 ± 0.7	834 ± 34	102.5 ± 11.3
Normal[4]	—	—	7.8 ± 0.4	15.1 ± 0.4	688 ± 29	86.4 ± 6.5

[1] Results are average values obtained from at least 7 independent determinations in each series.
[2] Initial weight refers to weight at time of grouping after 4 weeks of iodo-casein feeding; final weight is 5 weeks after grouping and maintenance on succinyl sulphathiazole-containing diets.
[3] Mean value ± standard error of mean.
[4] Adult rats of approximately 200 gm maintained on laboratory stock diet.

TABLE 3

Effect of vitamin B_{12} on the content and distribution of TPP and free thiamine in liver cytoplasm and on the incorporation of thiamine administered into these components[1]

Cytoplasm fraction	Vitamin B_{12}-fed (8 rats)				Vitamin B_{12}-deficient (8 rats)				Thiamine-injected			
	TPP		Free thiamine		TPP		Free thiamine		Vitamin B_{12}-fed (7 rats)		Vitamin B_{12}-deficient (9 rats)	
									TPP	Free thiamine	TPP	Free thiamine
	µg/gm	*%*	*µg/gm*	*%*	*µg/gm*	*%*	*µg/gm*	*%*	*Increase over uninjected control*			
									%	*%*	*%*	*%*
Homogenate	13.20 ± 1.01[2]		4.28 ± 0.13		7.23 ± 0.83		3.97 ± 0.30		78.7 ± 4.1	90.8 ± 3.8	48.2 ± 2.1	94.2 ± 3.9
Nuclear	0.62 ± 0.02	5.2 ± 0.4	0.76 ± 0.07	19.2 ± 1.6	0.56 ± 0.04	8.8 ± 0.6	0.63 ± 0.02	17.9 ± 1.0	124.2 ± 5.3	64.5 ± 3.0	12.5 ± 0.8	106.3 ± 8.8
Mitochondrial	3.49 ± 0.11	29.3 ± 2.1	0.34 ± 0.01	8.6 ± 0.6	0.86 ± 0.03	13.4 ± 1.2	0.33 ± 0.03	9.3 ± 0.8	100.6 ± 2.9	70.6 ± 3.1	62.8 ± 2.1	112.1 ± 7.3
Supernatant	7.81 ± 0.81	65.5 ± 6.2	2.86 ± 0.03	72.1 ± 1.8	4.99 ± 0.29	77.8 ± 5.3	2.57 ± 0.11	72.8 ± 4.3	74.4 ± 1.8	109.8 ± 4.3	51.4 ± 3.3	86.8 ± 2.3
Recovery, %		90.3 ± 3.3		92.6 ± 2.7		88.7 ± 3.1		88.9 ± 2.0	93.4 ± 2.9	95.8 ± 1.1	89.6 ± 2.0	88.2 ± 3.0

[1] Animals injected intraperitoneally with 5 mg of thiamine·HCl/rat/day for 5 consecutive days and killed 8 hours following final injection on the 5th day.

[2] Standard error of mean.

TABLE 4

Effect of vitamin B_{12} on the content and distribution of FAD and FMN + free riboflavin in liver cytoplasm and on the incorporation of riboflavin[1] administered into these components

Cytoplasm fraction	Vitamin B_{12}-fed (8 rats)				Vitamin B_{12}-deficient (8 rats)				Riboflavin injected			
									Vitamin B_{12}-fed (7 rats)		Vitamin B_{12}-deficient (9 rats)	
	FAD		FMN + free riboflavin		FAD		FMN + free riboflavin		FAD	FMN + free riboflavin	FAD	FMN + free riboflavin
	μg/gm	*%*	*μg/gm*	*%*	*μg/gm*	*%*	*μg/gm*	*%*	*Increase over uninjected control*			
									%	*%*	*%*	*%*
Homogenate	24.50 ± 1.61[2]		10.13 ± 0.42		12.79 ± 1.83		12.48 ± 1.32		67.6 ± 2.6	75.1 ± 3.8	41.4 ± 2.9	68.9 ± 3.1
Nuclear	4.36 ± 0.33	19.5 ± 3.6	1.65 ± 0.01	16.9 ± 0.9	3.37 ± 0.03	29.4 ± 1.2	2.55 ± 0.04	23.3 ± 1.2	92.2 ± 3.7	38.8 ± 1.6	54.3 ± 2.2	27.8 ± 1.5
Mitochondrial	13.69 ± 0.58	61.3 ± 2.4	2.43 ± 0.02	24.8 ± 1.2	4.63 ± 0.05	40.4 ± 1.8	1.74 ± 0.04	15.9 ± 1.3	54.9 ± 1.4	53.1 ± 2.7	20.7 ± 2.1	70.7 ± 4.6
Supernatant	4.29 ± 0.17	19.2 ± 2.9	5.70 ± 0.32	58.3 ± 2.4	3.45 ± 0.05	30.1 ± 1.6	6.65 ± 0.21	60.8 ± 1.9	82.3 ± 2.9	75.6 ± 3.7	52.5 ± 3.9	84.2 ± 2.1
Recovery, %		91.2 ± 2.7		96.5 ± 1.3		89.5 ± 3.4		87.6 ± 3.1	91.1 ± 2.1	90.3 ± 3.9	88.8 ± 3.8	87.6 ± 3.9

[1] As for thiamine (table 3), with injections of 1 mg of riboflavin/rat/day.
[2] Standard error of mean.

TABLE 5

Effect of vitamin B_{12} on the content and distribution of total PN in liver cytoplasm and on its synthesis from administered nicotinamide[1]

Cytoplasm fraction	Vitamin B_{12}-fed (8 rats)		Vitamin B_{12}-deficient (8 rats)		Nicotinamide-injected	
					Vitamin B_{12}-fed (7 rats)	Vitamin B_{12}-deficient (9 rats)
	μg/gm	*%*	*μg/gm*	*%*	*increase over uninjected control*	
					%	*%*
Homogenate	641 ± 23[2]		392 ± 22		65.2 ± 3.9	43.8 ± 3.1
Nuclear	115 ± 11	20.1 ± 1.8	89 ± 5	26.2 ± 1.9	101.7 ± 4.2	13.5 ± 1.2
Mitochondrial	100 ± 8	17.4 ± 1.8	25 ± 4	7.6 ± 0.5	50.0 ± 3.8	60.0 ± 2.9
Supernatant	357 ± 11	62.5 ± 4.0	224 ± 13	66.2 ± 2.7	57.1 ± 3.1	56.2 ± 1.9
Recovery, %		89.2 ± 3.3		86.3 ± 1.8	89.0 ± 2.3	87.1 ± 2.1

[1] As for thiamine (table 3), with injections of 5 mg of nicotinamide/rat/day.
[2] Standard error of mean.

fraction was, however, present in appreciable amounts in the nuclear fraction, its concentration in this fraction even exceeding that of TPP ($P < 0.05$).

The distribution pattern of FAD differed from that of TPP, in its greater association with the mitochondria than with the supernatant fraction. The proportion of FAD found in association with the mitochondria was similar to that reported (about 65%) by Schneider and Hogeboom (Schneider, '56). Although about 60% of the non-FAD riboflavin was contained in the supernatant, the proportion associated with mitochondria was small as compared with the FAD content of this fraction.

A major fraction of the total liver PN was localized in the supernatant, in confirmation of earlier observations (Carruthers and Suntzeff, '54; Dianzani, '55). A significant proportion was, however, contained in the nuclear fraction as well.

Alterations in vitamin B_{12} deficiency. The data on blood erythrocyte count and hemoglobin concentration and especially on plasma and liver content of vitamin B_{12} (table 2), apart from observations on growth noted below, connote the severity of the deficiency attained. During the 5-week period for which the animals were maintained on the succinyl sulphathiazole- containing diets, the vitamin B_{12}-deficient ration promoted an average gain in body weight of 8 gm/week compared with an average of 22 gm using the control ration with vitamin B_{12} (table 2). Data on blood erythrocyte count and hemoglobin concentration and especially on plasma and liver content of vitamin B_{12} (table 2) also connote the severity of the deficiency attained. Table 6 records the values obtained for oxidized and reduced forms of PN. The effects of vitamin B_{12} deficiency are summarized as follows: (a) there was a marked reduction in the liver content of the cofactors studied, being about 44% for TPP, 48% for FAD and 64% for total PN; (b) the mitochondria exhibited the largest depletion of the cytoplasm fractions. The reductions were 75, 66 and 75%, respectively, for TPP, FAD and PN. Significant reductions in the concentrations of these cofactors were also apparent in the other two fractions, especially in the supernatant; (c) the content and distribution of free thiamine remained essentially unaltered. With non-FAD riboflavin, there was small but significant elevation in both nuclear ($P < 0.005$) and supernatant ($P < 0.05$) fractions, the total being not significantly altered; and (d) the observed decrease in the proportion of oxidized to reduced forms of pyridine nucleotides (PN/PNH ratio) in livers of vitamin B_{12}-deficient animals (table 6) is in accordance with the findings of Nadkarni et al., '57). It is of interest to note that the changes in PNH content were much less pronounced in vitamin B_{12} deficiency.

Effect of vitamin B_{12} on incorporation of administered vitamins into liver cofactors. Tables 3 to 5 also include the results of experiments on the conversion by liver enzymes *in vivo* of the administered vitamins into their respective cofactors. Incorporation of thiamine, riboflavin and nicotinamide into their coenzyme forms is considerably less in the vitamin B_{12}-defi-

TABLE 6

Effect of vitamin B_{12} on oxidized and reduced PN in liver homogenates

Group	No. of rats	Oxidized pyridine nucleotides (PN)	Reduced pyridine nucleotides (PNH)	PN/PNH
		μg/gm	*μg/gm*	
Normal	10	639 ± 22[1]	206 ± 9	3.10 ± 0.09
Vitamin B_{12}-deficient	8	237 ± 18	155 ± 5	1.53 ± 0.11
Vitamin B_{12}-fed	8	476 ± 23	165 ± 6	2.88 ± 0.13
Vitamin B_{12}-deficient—nicotinamide injected	7	343 ± 26	221 ± 7	1.55 ± 0.14
Vitamin B_{12}-fed nicontinamide injected	8	809 ± 19	250 ± 11	3.24 ± 0.19

[1] Standard error of mean.

cient animals, although the extent of incorporation is appreciable even in this group. A relatively greater proportion of free thiamine and of non-FAD riboflavin was observed in the deficient group. The PN/PNH ratio was not significantly altered as a result of nicotinamide administration, the difference between the deficient and control groups being maintained (table 6).

The decreased ability in vitamin B_{12} deficiency of the liver enzymes for conversion of the administered vitamins into their respective cofactors is reflected in all of the cytoplasmic fractions and is largely seen in the nuclear fraction for TPP and PN and in the mitochondrial fraction for FAD. The deficient animals showed higher gains of free thiamine in the nuclear and mitochondrial fractions, whereas the differences with respect to non-FAD riboflavin were confined to the supernatant fraction.

DISCUSSION

The present observations demonstrate an impairment in the retention of TPP, FAD and PN in the vitamin B_{12}-deficient rat liver, as well as in their biosynthesis from the corresponding vitamins administered. In general, the observed effects point to greater depletion of cofactors from mitochondria than from other fractions. If these changes are not always reflected in liver levels of the free vitamins, it may be because of some losses through excretion; this may also imply a decreased use of the free vitamins for synthesis of coenzymes. Dianzani ('55) and Dianzani and Dianzani Mor ('57) have reported similar modifications in the distribution of TPP and PN in mitochondria from fatty livers caused by choline deficiency or CCl_4 poisoning. As discussed by these authors, such changes could result from decreased rates of synthesis of the cofactors or their increased degradation or from both causes. Thus it is possible that the synthesis of TPP through phosphorylation of thiamine and of DPN through the Kornberg reaction is diminished *in vivo* as a consequence of the reduced concentration of ATP in fatty livers; increased decomposition of TPP and DPN may be favored by increased acid phosphatase activity and through pyrophosphorolytic cleavage, respectively. Increased degradation of PN may also occur through partial displacement from the mitochondrial into the supernatant fraction, where DPNase is very active. Since both vitamin B_{12} and choline could exert similar lipotropic effects, it is probable that the same types of causes operate in either deficiency.

A rapid depletion of rat liver and its mitochondrial vitamin B_{12} could result from hyperthyroidism (Kasbekar et al., '59a) and acute carbon tetrachloride poisoning (Kasbekar et al., '59b). These conditions are known to cause morphological damage to mitochondrial structure with consequent displacement of intramitochondrial constituents into the surrounding medium (Dianzani, '54, '55; Dianzani and Dianzani Mor, '57; Maley and Lardy, '55; Kasbekar and Sreenivasan, '56). The protection afforded by prior administration of vitamin B_{12} under these conditions of stress (Fatterpaker et al., '55b; Kasbekar et al., '59a, '59b) could arise from its general lipotropic effect and from its known influence on sulphydryl conservation which, in turn, is essential for maintenance of mitochondrial integrity (Tapley, '56; Hunter et al., '56). It has also been suggested that vitamin B_{12} may be necessary for the synthesis of porphyrin-containing proteins of the cell (O'Dell et al., '55) which, apart from their function as respiratory carriers, are apparently also of importance in determining mitochondrial morphology (Gamble, '57). The observed alterations in mitochondrial cofactors in vitamin B_{12} deficiency may thus have an important though indirect, bearing on this function of the vitamin in the maintenance of mitochondrial organization in so far as the nucleotidation and phosphorylation reactions involved in the synthesis of cofactors are ATP-dependent and any structural damage to mitochondria renders the synthesis of ATP inoperative (Kielley and Kielley, '51; Dianzani, '54).

The effect of vitamin B_{12} also has to be assessed in terms of its known relationship to nucleotide biosynthesis. Although the nature of this relationship is obscure, evidence exists to suggest its involvement in the biosynthesis of both ribose[4] and de-

[4] Ling, C. T., and B. F. Chow 1954 Effect of vitamin B_{12} on ribose formation in erythrocytes. Federation Proc., *13*: 253 (abstract).

oxyribose (Downing and Schweigert, '56; Wong and Schweigert, '57) moieties of nucleic acids.

It is interesting to note that the injection of a vitamin could enhance the liver content of the corresponding cofactor despite dietary adequacy of the vitamin concerned. Kaplan and coworkers ('56) had observed a 10-fold increase in liver PN following administration of nicotinamide into normal mice.

According to Hogeboom and Schneider ('52), the DPN synthesis is localized in the liver cell nucleus. In the vitamin B_{12}-deficient rats, the extent of impairment in PN synthesis from administered nicontinamide is more pronounced in the nuclear fraction (table 5). A similar impairment in TPP synthesis from administered thiamine is again better reflected in the nuclear fraction than in the mitochondria and least in the supernatant fraction (table 3); this nuclear impairment is accompanied by an appreciable rise in free thiamine in this fraction, suggesting that TPP, like DPN, may be synthesized in the liver nucleus from thiamine, a process susceptible to vitamin B_{12}-deficiency.

On the other hand, the impairment in FAD synthesis from riboflavin administered to the vitamin B_{12}-deficient rat (table 4) is better reflected in the mitochondrial fraction. The increase in the proportion of non-FAD riboflavin in the vitamin B_{12}-deficient animals, with or without riboflavin administration, is, however, confined mainly to the supernatant fraction. The synthesis of FAD from FMN and ATP is also localized in the supernatant fraction (Schneider, '56).

An increase in the proportion of reduced PN in fatty livers (Dianzani, '55) and in those from vitamin B_{12}-deficient rats (Nadkarni et al., '57) has been reported. Such a condition may provoke predomination of fatty acid synthesis as compared with breakdown (Lynen, '54). The decreased content (table 6) of oxidized PN in vitamin B_{12} deficiency (Nadkarni et al., '57) may point to its selective destruction by the DPNase (McIlwain and Rodnight, '49; Zatman et al., '53) contained in mitochondrial and supernatant fractions, as well as to decreased synthesis.

SUMMARY

Rats depleted of their vitamin B_{12} reserves were used to study the effects of dietary vitamin B_{12} on (1) the content and distribution in liver cytoplasm of thiamine pyrophosphate (TPP) and free thiamine, flavin adenine dinucleotide (FAD) and non-FAD riboflavin (FMN + free riboflavin) and total pyridine nucleotides (PN), and (2) the *in vivo* synthesis of these cofactors from the respective vitamins.

In the normal, stock diet-fed animals, about 30 % of total liver TPP was localized in the mitochondria while the rest was found almost exclusively in the supernatant fraction (including microsomes). Free thiamine was distributed between the supernatant (65%) and the nuclear fraction (23%). About 60% of total liver FAD content was associated with mitochondria while about 55% of the total non-FAD content was contained in the supernatant fraction, the balance in either case being almost equally distributed between the remaining two fractions. A major portion (60%) of the total liver PN was localized in the supernatant; the nuclear fraction also contained appreciable amounts (25%).

The liver content of the cofactors was markedly affected in vitamin B_{12} deficiency with average reductions of 45, 48 and 64%, respectively, in TPP, FAD and PN. The effects were largely confined to the mitochondrial fraction and were not accompanied by corresponding changes in the content of the free vitamins. A decrease in oxidized pyridine nucleotides in liver homogenates with proportional lowering of the ratio of oxidized to reduced pyridine nucleotides (PN/PNH) was observed.

The incorporation of injected thiamine, riboflavin and nicotinamide into the liver coenzymes was impaired in vitamin B_{12} deficiency. The impairment in TPP and PN synthesis was reflected to a greater extent in the nuclear fraction than in other fractions, while that in FAD synthesis was seen to an almost equal extent in all fractions.

In the vitamin B_{12}-deficient animal there was also appreciable incorporation of administered vitamins into their cofactors;

the concentration of the free forms of the vitamins was, however, relatively greater.

The results, suggesting an impairment in the biosynthesis of these cofactors in vitamin B_{12} deficiency, are discussed. Conditions arising out of possible damage to mitochondrial integrity, as well as the effects of the vitamin in relation to the mode and site of synthesis of the cofactors, are discussed.

LITERATURE CITED

Bessey, O. A., O. H. Lowry and R. H. Love 1949 The fluorometric measurement of the nucleotides of riboflavin and their concentration in tissues. J. Biol. Chem., *180:* 755.

Boxer, G. E., W. H. Ott and C. E. Shonk 1953 Influence of vitamin B_{12} on the coenzyme A content of the liver of chicks. Arch. Biochem. Biophys., *47:* 474.

Boxer, G. E., C. E. Shonk, E. W. Gilfillan, G. A. Emerson and E. L. Oginsky 1955 Changes in coenzyme A concentration during vitamin B_{12} deficiency. Ibid., *59:* 24.

Carruthers, C., and V. Suntzeff 1954 The distribution of pyridine nucleotides in cellular fractions of some normal and malignant tissues. Cancer Res., *14:* 29.

Dianzani, M. U. 1954 Uncoupling of oxidative phosphorylation in mitochondria from fatty livers. Biochim. Biophys. Acta, *14:* 514.

——— 1955 Content and distribution of pyridine nucleotides in fatty livers. Ibid., *17:* 391.

Dianzani, M. U., and M. A. Dianzani Mor 1957 Displacement of thiamine pyrophosphate from swollen mitochondria. Ibid., *24:* 564.

Doctor, V. M., J. F. Elam, P. Sparks, C. M. Lyman and J. R. Couch 1954 Studies on the conversion of folic acid to citrovorum factor by avian liver homogenate. Arch. Biochem. Biophys., *48:* 249.

Downing, M., and B. S. Schweigert 1956 Role of vitamin B_{12} in nucleic acid metabolism. IV. Metabolism of C^{14}-labeled thymidine by *Lactobacillus leichmannii.* J. Biol. Chem., *220:* 521.

Fatterpaker, P., U. Marfatia and A. Sreenivasan 1955a Role of folic acid and vitamin B_{12} in transmethylations. I. Formation of creatine *in vitro* and *in vivo.* Ind. J. Med. Res., *43:* 43.

——— 1955b Protective action of vitamin B_{12} in the hyperthyroid rat. Nature, *176:* 165.

Fisher, R. A., and F. Yates 1953 Statistical Tables for Agricultural, Biological and Medical Research. Oliver and Boyd, Edinburgh.

Gamble, Jr., J. L. 1957 Aggregation of mitochondria, mitochondrial fragments, and microsomes by cytochrome C. Biochim. Biophys. Acta, *23:* 306.

Goethart, G. 1952 Thiamine pyrophosphate in fractions from rat-liver homogenates—the influence of thiamine deficient diet. Ibid., *8:* 479.

Hennessy, D. J. 1941 Chemical methods for the determination of vitamin B_1. Ind. Eng. Chem., (Anal. ed.), *13:* 216.

Hoff-Jorgensen, E. 1954 Microbiological assay of vitamin B_{12}. In Methods of Biochemical Analysis, vol. 1. Interscience Publishers Inc., New York, p. 81.

Hogeboom, G. H., and W. C. Schneider 1952 Cytochemical Studies. VI. The synthesis of diphosphopyridine nucleotide by liver cell nuclei. J. Biol. Chem., *197:* 611.

Hunter, Jr., F. E., J. Davis and L. Carlat 1956 The stability of oxidative and phosphorylative systems in mitochondria under anaerobic conditions. Biochim. Biophys. Acta, *20:* 237.

Kaplan, N. O., A. Goldin, S. R. Humphreys, M. M. Ciotti and F. E. Stolzenbach 1956 Pyridine nucleotide synthesis in the mouse. J. Biol. Chem., *219:* 287.

Kasbekar, D. K., and A. Sreenivasan 1956 Lability of intramitochondrial components in experimental liver injury. Nature, *178:* 989.

Kasbekar, D. K., D. V. Rege and A. Sreenivasan 1956 Protective action of vitamin B_{12} in experimental liver injury. Ibid., *178:* 989.

Kasbekar, D. K., W. V. Lavate, D. V. Rege and A. Sreenivasan 1959a A study of vitamin B_{12} protection in experimntal thyrotoxicosis in the rat. Biochem. J., *72:* 374.

——— 1959b A study of vitamin B_{12} protection in experimental liver injury to the rat by carbon tetrachloride. Ibid., *72:* 384.

Kielley, W. W., and R. K. Kielley 1951 Myokinase and adenosinetriphosphatase in oxidative phosphorylation. J. Biol. Chem., *191:* 485.

Kolmer, J. A., E. H. Spaulding and H. W. Robinson 1951 Approved Laboratory Technic, ed. 5, Appleton-Century-Crofts, New York.

Ling, C. T., and B. F. Chow 1954 The influence of vitamin B_{12} on carbohydrate and lipide metabolism. J. Biol. Chem., *206:* 797.

Lynen, F. 1954 Participation of coenzyme A in the oxidation of fat. Nature, *174:* 962.

McIlwain, H., and R. Rodnight 1949 Breakdown of oxidized forms of coenzymes I and II by an enzyme from the central nervous system. Biochem. J., *45:* 337.

Maley, G. F., and H. A. Lardy 1955 Efficiency of phosphorylation in selected oxidations by mitochondria from normal and thyrotoxic rat livers. J. Biol. Chem., *215:* 377.

Nadkarni, G. B., D. S. Wagle and A. Sreenivasan 1957 Vitamin B_{12} and the metabolism of pyridine nucleotides. Nature, *180:* 659.

O'Dell, B. L., J. S. Gordon, J. H. Bruemmer and A. G. Hogan 1955 Effect of a vitamin B_{12} deficiency and of fasting on oxidative enzymes in the rat. J. Biol. Chem., *217:* 1955.

Pfander, W. H., L. S. Dietrich, W. J. Monson, A. E. Harper and C. A. Elvehjem 1952 Citrovorum factor, vitamin B_{12}, and folic acid activity of whole blood of several species. Proc. Soc. Exp. Biol. Med., *79:* 219.

Popp, E. M., and J. R. Totter 1952 Studies on the relationship between folic acid and coenzyme A. J. Biol. Chem., *199:* 547.

Register, U. D. 1954 Effect of vitamin B_{12} on liver and blood non-protein sulphydryl compounds. Ibid., *206:* 705.

Ross, G. I. M. 1952 Vitamin B_{12} assay in body fluids using *Euglena gracilis.* J. Clin. Pathol., *5:* 250.

Schneider, W. C. 1956 Structural factors in metabolic regulations. Proc. 3rd Int. Congr. Biochem., Brussels, 1955. Academic Press, New York, p. 305.

Schneider, W. C., and G. H. Hogeboom 1950 Intracellular distribution of enzymes. V. Further studies on the distribution of cytochrome C in rat liver homogenates. J. Biol. Chem., *183:* 123.

Sreenivasan, A. 1951 Metabolic inter-relationships between folic acid and vitamin B_{12}. Current Sci. (India), *20:* 319.

Strength, D. R., N. I. Mondy and L. J. Daniel 1954 The effect of aminopterin on diphosphopyridine nucleotide. Arch. Biochem. Biophys., *48:* 141.

Tapley, D. F. 1956 The effect of thyroxine and other substances on the swelling of isolated rat liver mitochondria. J. Biol. Chem., *222:* 325.

Totter, J. R. 1953 The biological function of pteridine derivatives. J. Cell. and Comp. Physiol., Suppl. 1, *41:* 241.

Wong, W. T., and B. S. Schweigert 1956 Role of vitamin B_{12} in nucleic acid metabolism. II. Liver coenzyme A levels in the rat. Arch. Biochem. Biophys., *60:* 126.

——— 1957 Role of vitamin B_{12} in nucleic acid metabolism. V. Liver deoxyriboaldolase activity. Proc. Soc. Exp. Biol. Med., *94:* 455.

Zatman, L. J., N. O. Kaplan and S. P. Colowick 1953 Inhibition of spleen diphosphopyridine nucleotidase by nicontinamide—an exchange reaction. J. Biol. Chem., *200:* 197.