Determination of Folic Acid Metabolites in Normal Subjects and in Patients with Nutritional Megaloblastic Anemia.* (27332)

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Considerable advances in development of methods for detection of vit. B_{12} and folic acid deficiencies have been made during the last decade. Vit. B_{12} deficiency may be established by measuring serum concentrations of the vitamin by microbiological assays (1-4). Direct methods for determination of folic acid deficiency have been less successful due to the multiplicity of folic acid components found in blood and their uneven distribution between red cells and serum(5).

Until very recently *S. fecalis* has been used for determination of folic acid (F.A.) activity. This test organism, however, does not estimate the folic acid polyglutamates (F.A. conjugates) which constitute about 90% of all F.A. activity in the blood. Moreover, the *S. fecalis* assay has been carried out only on serum, which contains less than 10% of the F.A. found in whole blood. Therefore, only a small fraction (about 1%) of the blood F.A. activity was measured by this method. Thus, it is not surprising that no significant deviations in F.A. levels were found in patients clinically suspected of F.A. deficiency.

For this reason, indirect methods were introduced employing oral or intravenous loads of synthetic F.A. (pteroylglutamic acid) and estimating rate of disappearance from the serum or amount excreted in the urine. However, results obtained by these indirect methods may measure F.A. utilization or storage rather than deficiency.

Microbiological methods for measuring the level of F.A. in serum with the aid of *L. casei* have been described by Baker, Herbert and coworkers(6,7) and at about the same time we introduced determinations of F.A. metabolites in whole blood and serum using 3 different organisms: *L. casei, S. fecalis* and *P. cerevisiae* (*L. citrovorum*)(8,9).

The present paper describes the merits of the 3 different bioassays used in detection of folic acid deficiency.

Materials. Twenty-nine women with severe nutritional anemia admitted to the Rangoon General Hospital (Rangoon, Burma) were examined; their hemoglobin values ranged from 7.0 g % down to 0.9 g %, with a mean of 2.8 g %. The bone-marrow of all patients showed extreme megaloblastic transformation. For comparison blood from 5 non-anemic Burmese individuals was assayed for F.A. activity. The blood samples from Burma were flown to Jerusalem, where they were received within 3-4 days. Normal values of the various F.A. metabolites were established in 43 healthy non-anemic Israeli subjects.

Methods. Microbiological assays used are those reported earlier(9). The procedures are modifications of the method described by Toennies *et al.*(10).

Lactobacillus casei ATCC No. 7469 was used for determination of "total folic acid" activity, as it responds to both free and conjugated forms of F.A. This designation is given despite the further increase in values of the *L. casei* assays after incubation of human blood with chicken pancreas conjugase, presumably due to deconjugation of hepta- and other polyglutamates.

Streptococcus fecalis No. 9 (more sensitive than strain ATCC No. 8043) was employed for determination of the free forms of F.A. and folinic acid.

Pediococcus cerevisiae (*Leuconostoc citrovorum*) ATCC 8081 was used for determination of free and conjugated folinic acid (citrovorum factor) and related forms.

As some of the F.A. metabolites are unstable and activity may have decreased dur-

* Aided by grants from B. de Rothschild Foundation for Advancement of Science in Israel, Inc., and Rebecca Rose Memorial Fund, New York.

TABLE I. Folic Acid Metabolites and Vitamin B_{12} in Blood of Healthy Subjects and in Patients with Megaloblastic Anemia.

No. of subjects			Hemoglobin, g %	Folic acid, mμg/ml *L. casei* Whole blood	*L. casei* Serum	*S. fecalis* Whole blood	*S. fecalis* Serum	*P. cerevisiae* Whole blood	*P. cerevisiae* Serum	Vit. B_{12}, μμg/ml serum
43	Healthy (Jerusalem)	Range	13.5–15.5	47–149	3.2–15	3–20	.25–1.7	1.5–25	.25–1.3	200–500
		Mean		89	8.26	12	.75	6.35	.3	350
5	Non-anemic (Rangoon)	Range		15–59		3.7–10.2		1.1–5.6		170–680
		Avg		37		8.1		2.0		415
29	Anemic (Rangoon)	Range	.9–7.0	2–17.4	1.2–5*	.8–6.8	1–3.9*	.1–1.4		150–1900
		Mean	2.8	4.4	3.1	2.3	1.9	.4		645

* Only 16 of these 29 sera were examined.

ing transport, experiments were set up to test this possibility. Citrated blood samples from 4 individuals were incubated at 30°C for 4 and 7 days and F.A. activity of the blood was determined with the 3 bioassays.

Vit. B_{12} was determined in the serum with the *Escherichia coli* mutant as described previously (3,4).

Results. The whole blood "total folic acid" activity (as measured with *L. casei*) in 43 normal Israeli subjects ranged from 47 to 149 mμg/ml with a mean value of 89.0 mμg/ml (Table I). "Total folic acid" activity of sera of the same group of individuals ranged from 3.2 to 15.0 mμg/ml with a mean of 8.26 mμg/ml. Ratio of whole blood to serum "total folic acid" values is about 10:1. Free F.A. determinations on the normal subjects carried out with *S. fecalis* gave values as low as 10% of those obtained with *L. casei*. Values for whole blood with *S. fecalis* ranged from 3.0 to 20.0 mμg/ml with a mean of 12.0 mμg/ml, while those of the serum from 0.25 to 1.7 mμg/ml with a mean of 0.75 mμg/ml. Determination of folinic acid and related forms (*Pediococcus cerevisiae assays*) in normals were some 50% lower than those obtained with *S. fecalis*. In healthy subjects the ratio of *L. casei* : *S. fecalis* : *P. cerevisiae* values amounted to 20:2:1, both in whole blood and serum.

In the group of 29 Burmese patients with severe megaloblastic anemia, F.A. values were considerably lower than in the Israeli normals (Table I). Very striking decreases were found in "total folic acid" activity of whole blood which amounted to approximately 5% of the normal mean (4.4 mμg/ml as compared to 89.0 mμg/ml in normals). The "total folic acid" was also markedly reduced in the serum. Since serum values were similar to those found in whole blood it follows that the cellular elements were practically depleted of their F.A. content.

The *S. fecalis* assay (which measures free forms of F.A. as well as folinic acid) also revealed a decrease in F.A. metabolites of whole blood (from 12.0 mμg/ml to 2.3 in mμg/ml). The serum values obtained with this assay were in the same range as in whole blood but were somewhat higher than normal mean serum value.

Folinic acid activity (*P. cerevisiae* assay) was determined in whole blood only and was found to be extremely low; the mean value was 0.4 mμg/ml as compared to 6.3 mμg/ml in normals.

Serum vit. B_{12} values were normal or even increased in most instances.

F.A. activity of blood samples from 5 non-anemic Burmese subjects was also lower than that of the Israeli normals, particularly with the *L. casei* and *P. cerevisiae* assays. Nevertheless, the values found in these Burmese subjects were very much higher than those found in the anemic patients (Table I). Clinical material and laboratory findings will be detailed elsewhere.

Effect of storage on F.A. activity in whole blood. Incubation at 30°C of untreated blood samples for 4 and 7 days respectively had no detrimental effect on F.A. activity tested with *L. casei;* some samples even showed increased values. F.A. activity as-

TABLE II. Effect of Storage (at 30°C) on Folic Acid Activity (in mμg/ml) in Blood.

Incubation time at 30°C (days)	Assay carried out with											
	L. casei				*S. fecalis*				*P. cerevisiae*			
Sample No.	1	2	3	4	1	2	3	4	1	2	3	4
0	160	97	34.8	56	20	16	2.0	6.3	13.2	12	1.75	3.6
4	162	144	21.5	—	17	3.7	1.7	5.1	7.2	2.9	1.65	3.95
7	200	132	29	72	7	4.7	2.0	5.3	3.6	3.4	1.10	3.62

sayed with *S. fecalis* and *P. cerevisiae* decreased in some samples, but not in others (Table II).

Discussion. The material presented here clearly shows that F.A. deficiency can be revealed by direct measurement of F.A. metabolites in the blood. The most reliable information can be obtained by examining whole blood rather than serum. In the material presented here the most significant decrease in blood F.A. values was found with *L. casei* and *P. cerevisiae*, the sensitivity of both assays being approximately equal. *P. cerevisiae* assay has the advantage of measuring a distinct group of metabolites, *i.e.*, the folinic acid group. However, the concentration of the latter in the blood is small (about 8% of the total F.A.). Moreover, the folinic acid compounds seem to be the least stable F.A. metabolites of the blood (Table II). *L. casei*, on the other hand, measures a heterogenous group of F.A. metabolites (free forms and polyglutamates), so that much higher values are found in the blood and a greater deviation from the norm can be expected in deficiency states. Another advantage of the *L. casei* assay is a greater stability of F.A. metabolites measured by this organism, as evidenced by values obtained following storage (Table II). Our previous experience has shown that in nutritional anemia of only moderate degree *L. casei* assays revealed a significant decrease as compared to normals, whereas *S. fecalis* and *P. cerevisiae* assays showed values within the normal range both in whole blood and serum(11).

The most striking finding in the present series of patients with severe megaloblastic anemia is the complete disappearance of the measured F.A. metabolites from the blood cells. Whereas, in normal Israeli subjects 90% of the F.A. activity is found in whole blood and only 10% in the serum, in the patients described here the values in whole blood and serum were about the same, hence the cellular elements were depleted of their F.A. content. The high concentration of F.A. metabolites in normal blood cells and its extreme depletion in a severe deficiency state focuses attention on the importance of F.A. in the metabolism of the cellular elements of the blood and the red cells in particular. This problem calls for further investigation. The vit. B_{12} concentrations were normal or above normal in most cases. It is thus evident that the megaloblastic transformation of the bone-marrow was due to F.A. deficiency only, and the available vit. B_{12} was not utilized.

Summary. Three microbiological assays were employed simultaneously (*L. casei, S. fecalis*, and *P. cerevisiae*) for determination of folic acid (F.A.) metabolites in whole blood and serum. Normal values were established with all these bioassays in healthy subjects. In a group of patients with severe megaloblastic anemia concentrations of all the F.A. metabolites in the whole blood were found to be remarkably reduced. The most

striking decrease was found with the *L. casei* and *P. cerevisiae* assays. The results indicate an almost complete depletion of F.A. content of the red cells.

The able assistance of Mrs. M. Jablonska is gratefully acknowledged.

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Received July 7, 1961. P.S.E.B.M., 1962, v109.

Cardiac Tissue Response to Endotoxin.* (27333)

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Myocardial injury is considered to play a primary role in the development of irreversibility to transfusion for hemorrhagic shock (1). The evidence for such injury is biochemical and functional data that allow various interpretations. There is no morphologic evidence for such injury, in spite of the fact that the catechol amines and bacterial toxins, both of which are present in excessive amounts in the blood of the animal in hemorrhagic shock, produce morphologic injury in the myocardium and other tissues of normal animals(2,7). The absence of such morphologic evidence in the shocked animal might be because it cannot react to injury. To test this hypothesis experiments were performed to see if the morphologic pathology caused by these substances in the normal animal can also develop in the shocked animal that is allowed to recover and survive long enough to allow the reaction to injury to become manifest.

* Aided by a grant from Nat. Inst. Health, Bethesda, Md., and by a Contract with Office of the Surgeon General, U. S. Army.

Materials and methods. Hybrid albino adult rabbits were used. Sublethal doses of *E. coli* endotoxin (0.5 mg/kg) or 0.1 mg/kg of norepinephrine or epinephrine were given intravenously singly or together. The experiments were repeated in rabbits that had received dibenamine (25 mg/kg) intravenously 3 hours in advance. Eleven rabbits were bled to produce reversible hemorrhagic shock of 2 hours duration, and were given 100 μg of endotoxin just before transfusion. These rabbits did not receive catechol amines because the blood concentration of these substances is already more than adequate in shock(7,8).

All rabbits which were in apparently good condition 48-72 hours later were killed. In addition to routine postmortem examination, tissue sections of lung, liver, spleen, kidney, and heart, fixed in 10% neutral formalin, were prepared and stained with hematoxylin and eosin or with the von Kossa stain, and examined microscopically.

Results. (Table I). A. Normal rabbits. Six animals which received neither endotoxin