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Received January 16, 1962. P.S.E.B.M., 1962, v109.

Folinic Acid Activity in Leucocytes.* (27353)

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Leukemic leucocytes contain a higher concentration of folinic acid active material than mature leucocytes(1,2). In these investigations it was noted that the increased levels of citrovorum factor activity (CF) correlated with degree of immaturity of the leukemic cell type, the highest elevations being present in acute leukemic cells. Furthermore, although some free CF activity was present, most of the activity in leucocytes was in a bound form. Ellison and Hutchinson(2) noted that CF activity of leucocytes was maximal only when the sample was protected from oxidation by including ascorbate in the assay procedure. Thus, the indications are that several folate forms exist in leucocytes which may be designated as free, bound, oxygen-stable and oxygen-labile CF. Other workers have demonstrated the occurrence of oxygen-labile bound CF in liver(3), red cells(4), and blood plasma(5). However, the relative amounts of each CF form in leucocytes have not been previously reported. Hence the present study was undertaken to establish values for free, bound, oxygen-stable and oxygen-labile CF in normal leucocytes. Preliminary observations in leukemic leucocytes are also included.

Method. Six healthy, well nourished medical students were studied to establish normal values. The leukemic patients (Table II) are classified according to cell morphology of peripheral blood and bone marrow. These patients had received no therapy for leukemia. All subjects were on unrestricted diets with no vitamin supplements, except for normal subject E.M. who was receiving 100 μg folic acid daily.

Venous blood was obtained from individuals in a fasting state for leucocyte separation. Approximately 300 cc of blood was necessary to obtain sufficient numbers of leucocytes (0.5-1.0 g wet weight of cells) for assay in subjects with leucocyte counts in the normal range. Leucocytes were separated by the phytohemagglutinin method(6). Microscopic examination of leucocyte morphology showed no alteration after separation, and erythrocyte contamination was negligible. However, platelets were included in the leucocyte extracts. When the buffy coat layer was aspirated directly in patients with high leucocyte counts CF assay results were not essentially different from the activity of those samples obtained by the agglutination method. The dry weight of the residue was determined by heating at 100°C for 30 minutes to correct for variations of water content in the samples. The results are expressed as mμg of CF activity/mg of leucoctyes-dry weight. The samples were assayed immediately or stored at –20° until use.

The leucocytes from each subject were divided into fractions and assayed after treatment (Table I). In Table I the column entitled *Incubation* indicates that the fraction was incubated at 37°C in 0.25 M phosphate buffer at pH 6.6-6.8** for 18-20

* Supported by U.S.P.H.S. grant and Post-Sophomore Research Fellowship to J.S.O., 1958-59. This work in part included as M.D. Thesis by J.S.O., Creighton Univ. School of Medicine, 1960.

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** Although it has been stated that optimal pH for release of folate conjugates is 4.5(1), we have found that yields are much greater at pH 6.8, similar to Chang's findings(3).

TABLE I.

Fraction	Incubation	AA	Autoclaved	Designation
A	—	—	+	Free, oxygen-stable CF
B	—	—	With ascorbate	" , oxygen-labile "
C	+	+	" "	Bound, oxygen-labile CF
D	+	+	—	" , " , heat-labile CF
E	+	—	—	" , oxygen-stable CF

TABLE II. Morphologic Type of Leukemia.

Patient	Age	Type of leukemia	WBC/mm³	Peripheral blood morphology
D.B.	70	Chronic lymphocytic	200,000	99% mature lymphocytes 1% polymorphonuclear leucocytes
H.B.	68	*Idem*	180,000	79% mature lymphocytes 21% polymorphonuclear leucocytes
M.H.	3	Acute myelocytic	300,000	12% myeloblasts 40% pro- and metamyelocytes 48% polymorphonuclear leucocytes
J.N.	9	Acute monocytic	85,000	80% monoblasts 15% monocytes 5% polymorphonuclear leucocytes
A.L.	3	Acute myelocytic	225,000	10% promyelocytes 15% metamyelocytes 75% polymorphonuclear leucocytes

hours at a tissue/buffer concentration ratio of 100 mg wet leucocytes/cc of buffer. These conditions are those defined by Chang(3) for optimal release of bound folates in liver tissue, except that our system has a 10-fold decrease in the tissue/buffer ratio due to the small volume of samples obtained. The column titled *AA* indicates that the fraction was incubated in presence of ascorbate in a concentration of 10 mg/cc. The next column indicates that the fraction was autoclaved for 10 minutes at 120°C (15 lb pressure) prior to assay. "With ascorbate" indicates that the fraction was autoclaved for 30 minutes at 120°C (15 lb pressure) in presence of 50 mg of ascorbate/cc prior to assay. The sample that was not autoclaved was added aseptically to the assay media.

The CF assay media of Sauberlich and Baumann(7) was used. Growth response of *Pediococcus cerevisiae* (*Leuconostoc citrovorum*—American Type Culture Collection [A.T.C.C.] No. 8081) was estimated turbidimetrically at the end of 20 to 24 hours' incubation at 37°, after adding one drop of the organism-suspension to each assay tube. Standards were run with each determination and unknowns were read from a standard curve. In a few instances, *Streptococcus faecalis* (A.T.C.C. No. 8043) was used as the assay organism. However, *S. faecalis* responded erratically in presence of ascorbate and accurate determination of folates by this procedure was not possible. Therefore, these results are not included. Calcium leucovorin§ was the standard for the *Leuconostoc* assay. CF activity was calculated using leucovorin as the reference standard, without correcting for presence of the microbiologically inactive isomer(8).

Results and discussion. The results of the CF assay in normal leucocytes are represented in Fig. 1.

All samples assayed showed minimal activity when autoclaved in water and assayed immediately (Fraction A). Approximately 2% of the total activity was obtained by this procedure, a result comparable to that found by Chang in liver tissue, in which only 2% of the CF activity was found in a freely extractable form(3). The activity of this sample could be enhanced 2- to 3-fold by autoclaving in ascorbate (Fraction B). This finding is

§ Generously supplied by Dr. H. P. Broquist, Lederle Laboratories, Pearl River, N. Y.

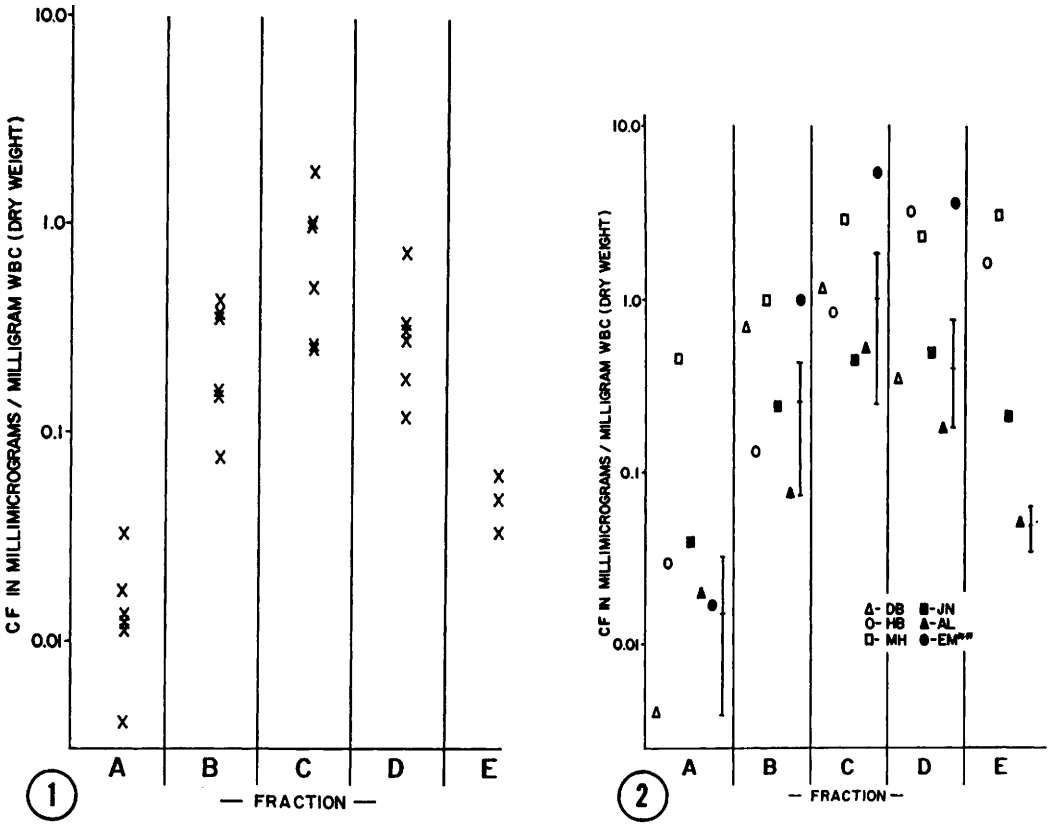

FIG. 1. CF activity of leucocytes in 6 normal adult humans. Fraction A represents free, oxygen-stable CF; Fraction B, free, oxygen-labile CF; Fraction C, bound, oxygen-labile CF; Fraction D, bound, oxygen-labile, heat-labile CF; Fraction E, bound, oxygen-stable CF.

FIG. 2. CF activity of leukemic leucocytes. For type of leukemia see Table I. Fractions as in Fig. 1. Subject E.M. is a normal control taking 100 μg of folic acid orally per diem. Vertical lines represent range and mean of 6 normals for each fraction.

perhaps best explained by the presence of oxygen-labile forms which are converted to stable CF by autoclaving in ascorbate. Indeed, it has been demonstrated that N-10-formyl-tetrahydrofolic acid and N-5, N-10-methenyl-THFA can be converted to CF by this procedure(9-11) and a similar conversion mechanism might account for the enhancement of CF activity found in this fraction.

After incubation, marked increases of CF activity were seen. Furthermore, the greatest yields were obtained when the fraction was incubated in presence of ascorbate and autoclaved in ascorbate (Fraction C). The activity was considerably reduced when ascorbate was lacking in either the incubation step or the autoclaving step.‖ The activity found when ascorbate was absent from *both* incubation and autoclaving steps was only approximately 10% of total activity (Fraction E). Although ascorbate may exert varying protective effects, the magnitude of the losses of activity suggests that oxidative-degradation of labile folates occurred in the absence of ascorbate. The finding that the greatest activity yields were obtained after autolysis indicates that the predominant intracellular folate forms in leucocytes are bound forms, similar to the findings in other tissues(3-5).

The wide variations in activity of sample D, the sample assayed aseptically, may be due to variations in pH of the assay media.

‖ In an effort to define where the greatest loss occurred, one sample was incubated in the absence of ascorbate, but autoclaved in ascorbate. It was found that approximately one-half of the activity was lost during the incubation procedure.

This procedure was devised to attempt the estimation of heat-labile forms such as THFA. It has been reported that THFA has approximately 50% of the activity of CF at pH 6.3, but this activity may vary from 15 to 75% depending on the pH of the media (12). Since the pH of the media was not rigidly controlled, this may account for the wide variations in CF activity of this fraction.

Despite variations in dietary intake of folic acid in the normal subjects, leucocyte CF activity in each fraction did not vary considerably from subject to subject.

Preliminary results in leukemic cells (Fig. 2) confirm the finding that, in general, CF activity of these cells is higher than normal. The highest elevations of CF activity were observed in cells from the patient with acute granulocytic leukemia (M.H.). This is in accord with the findings of Swenseid *et al.* and Hutchinson and Ellison(2) that acute leukemic leucocytes contained the greatest elevations of CF activity. However, patient J.N., who had acute monocytic leukemia, did not show the marked elevations found in patient M.H. Patients D.B. and H.B. both had chronic lymphatic leukemia with markedly similar morphologic cellular changes and almost identical clinical courses. Both patients died 10 years after the diagnosis of their disease which was untreated until just prior to death. However, the pattern of CF activity at the same approximate time in the course of their disease was dissimilar. Subject A.L. whose values fall within the normal range was later found to be taking cortisone at the time of assay. Of additional interest is a comparison of the results found in subject E.M. (daily oral dose of 100 μg of folic acid) with the leukemic patients. Despite similar quantitative increases in leucocytic CF activity in this subject and the leukemic patients, the fractional distribution of CF activity is dissimilar.

The pattern of CF activity of each fraction was extremely variable from patient to patient in the leukemic study. When sufficient quantities of leukemic leucocytes were available for duplicate assays, it was found that the values obtained checked closely, indicating that the method was not the source of the variability. Although there was an elevation of CF activity in at least one fraction in 4 of the 5 leukemic patients studied, no specific fraction appeared to be consistently elevated.

The foregoing results in leucocytes support findings in other studies that the *in vivo* folates exist predominantly as oxygen-labile bound forms(1-5). The chemical nature of these derivatives is not known. Recently, Rabinowitz has found that 80% of the folate activity in cells of *Clostridia* is tetrahydropteroyl-triglutamate(13). These observations indicate that tetrahydropteroyl-polyglutamates may be the predominant tissue folate forms and represent the oxygen-labile bound CF found in leucocytes by microbiological assay. However, the recent results of Silverman *et al.*(14) indicate that polyglutamates are not present in mouse leukemic cells. Thus, the chemical nature of the CF activity in leucocytes remains to be firmly established.

Summary. Intracellular CF activity in normal and leukemic leucocytes was studied. The concentration of CF which is easily extractable (free CF) as well as that which is microbiologically active for *Pediococcus cerevisiae* only after autolysis of the sample (bound CF) were investigated. Determinations were also made of that proportion of free and bound CF which loses activity after autoclaving in absence of ascorbate, as well as the amount of labile CF which can be converted to stable CF in presence of ascorbate. The bulk of the activity in normal and leukemic leucocytes is bound CF. Furthermore, approximately 90% of CF activity of free and bound CF is lost by autoclaving the sample in absence of ascorbate, indicating that oxygen-labile bound CF predominates in leucocytes. In preliminary studies of leukemic leucocytes, elevation of CF activity in at least one fraction was found in 4 out of 5 patients studied. However, qualitative differences in the pattern of CF activity were not observed.

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Received January 22, 1962. P.S.E.B.M., 1962, v109.

Differential Antagonism of Reserpine Eyelid Closure by Imipramine and Amphetamine. (27354)

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Stimulants such as amphetamine and antidepressants such as imipramine antagonize reserpine-induced eyelid closure in laboratory animals(1-5). Although amphetamine and imipramine seem to have similar effects in this respect, they belong to different classes of compounds with different pharmacological and clinical activities. The present paper will describe a differentiation of imipramine from amphetamine in the antireserpine eyelid closure test in mice.

Methods and materials. The tests were performed using Carworth Farms mice CF No. 1 weighing 16 to 24 g. Male and female animals were equally distributed throughout all groups. The animals were starved 4 hours prior to drug administration. Imipramine hydrochloride or d,l-amphetamine sulfate was administered orally at graded dose levels to 4 groups of 10 mice per dose. Control groups received the vehicle (water) only. In acute experiments, the animals were challenged with reserpine one hour after a single treatment with amphetamine or imipramine. In subacute experiments, mice were treated once daily with amphetamine or imipramine for 2 (in one experiment for 3) consecutive days, and challenged with reserpine 24 hours after the last drug treatment.

Reserpine U.S.P. (S.B. Penick), solubilized with a few drops of glacial acetic acid and diluted with water, was injected intraperitoneally at a dose of 2.5 mg/kg. At one-half, one and two hours after reserpine treatment, eyelid closure scores were taken according to the method of Rubin(6). The scores of the imipramine and amphetamine groups were averaged per group and expressed as percentages of the water control; the values of the 3 readings were averaged. The treatment with amphetamine or imipramine was performed while the mice were aggregated, 10 per cage. After the reserpine injection, each animal was placed under a 1000-ml glass beaker. The animals were not handled during the observation period of 2 hours.

Results. In acute studies, both imipramine (100 mg base/kg) and amphetamine (0.5 to 4.0 mg base/kg) showed marked antagonism to reserpine ptosis (imipramine, 45%; amphetamine, 18 to 63%). In contrast, after repeated administration, imipramine (50 to 200 mg base/kg/day) showed appreciable antagonism (7 to 72%), but amphetamine (4.0 to 64.0 mg base/kg/day) produced only slight antagonism (4 to 14%). The results of the 2-day experiments are shown in Fig. 1. It should be pointed out that an increase in the dose of amphetamine above 64 mg/kg resulted in death. Administration of imipramine for 3 days gave similar effects to the 2-day treatment. When amphetamine was administered for 3 days the reserpine eyelid closure was increased.