IN VITRO SYNTHESIS OF CITROVORUM FACTOR ACTIVITY BY RAT BLOOD

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THE recognition of a new factor in liver[1] and in fermentation liquors[2] which supports the growth of *Leuconostoc citrovorum* 8081 and its close relation to pteroyl glutamic acid (PGA)[3,4] has been followed by its synthesis (folinic acid,[5] leucovorin[6]) from PGA and isolation from liver in a crystalline form.[7] Excretion of this factor (CF) in urine is in proportion to the amount of PGA in the diet.[8] CF concentrates and leucovorin are more effective than PGA in reversing antagonists of PGA for micro-organisms and mice;[4] natural CF in this respect is nearly twice as active as the synthetic product.[7] Leucovorin is also much more active than PGA in inducing the disappearance of megaloblasts in cultures of bone marrow of untreated patients with pernicious anemia.[9] These findings along with observations on the enzyme systems present in rat liver[10] and in *Lactobacilli*[11] converting PGA to CF would indicate that CF or a derived product from it is the functional form of PGA.

The ability of blood cells to convert riboflavin to flavinadenine dinucleotide,[12] nicotinic acid to nicotinamide mononucleotide[13] and thiamine to co-carboxylase[14] suggested this search for the presence of a similar mechanism in blood for the conversion of PGA to CF activity.

Adult albino rats (inbred Wister strain) were maintained on the laboratory stock diet or in the case of folic acid-deficient rats, on the purified folic acid-free ration of Fatterpaker *et al.*,[15] with supplementation of 0·15 mg. per kg. of vitamin B_{12} (Merck) and 2 per cent. phthalylsulphathiazole. The blood, withdrawn under phenobarbitone anesthesia from the portal vein was collected into chilled, citrated Erlenmeyer flasks and quickly utilized for the experiments.

The procedure followed for the *in vitro* synthesis of CF consisted in incubating at 37° C. a mixture of 2 c.c. blood and 2 c.c. Krebs-Ringers phosphate buffer of pH 7·4, together with appropriate additions (Table I) and water to a total volume of 5 c.c. At the end of 3 hours, the contents in each vessel, after adjustment to neutral pH, were autoclaved for 15 minutes at 15 lb. pressure, cooled, homogenised and centrifuged. The supernatants which were often turbid were assayed titrimetrically[16] for CF activity using *L. citrovorum* 8081 as the test organism and leucovorin (Lederle) as the standard. The cells in duplicate sets of flasks were crushed well after incubation and pH brought to 7·6. The contents were next autolysed under toluene for 24 hours at 37° C. prior to assay after neutralization and autoclaving as above. Under these conditions there was maximum release of bound CF which accounted for most of the CF activity. Enzymic release of bound CF[23] was not resorted to on account of the high blanks.

From the results presented in Table I, it may be observed that the blood system could convert PGA to CF. This synthetic ability is destroyed if the blood were heated at 100° C. for 3 minutes. The values with the autolysed samples suggested that the enzymes for this conversion resided in the cells and was confirmed in other experiments with the formed

TABLE I

Synthesis of CF activity from PGA by normal and PGA-deficient rat blood

Supplements	Normal animals		PGA-deficient animals
	Before autolysis	After autolysis	After autolysis
	CF mμg. per c.c. blood		
Blood ..	0·20	4·92	<0·2
Blood+PGA (10 μg.) ..	1·72	8·90	9·0
Blood+PGA (10 μg.)+AA*(10 mg.) ..	3·20	13·72	13·0

* Freshly neutralised ascorbic acid (AA)

elements of the blood only. The effect of ascorbic acid in enhancing CF synthesis is appreciable as with liver slices.[10]

Maximum synthesis with intact cells occurred in 3 hours of incubation and, under comparable conditions crushed cells did not show much synthetic ability for CF. These observations excluded the possibility of any synthesis occurring during autolysis, the increased values for CF being therefore, attributable to liberation from bound forms only.

The results with blood from PGA-deficient animals, included in Table I, would show that though there is negligible CF activity present initially, incubation with PGA with or without AA produces nearly as much CF as with normal blood. However, there is evidence to suggest that in a folic acid deficiency induced by aminopterin there is almost complete impairment of this enzyme system.

Aminopterin addition to the incubating mixture (Table II) blocks the conversion of PGA to CF by the blood cells considerably (*cf.* 17); for these experiments, the standard curves were run in presence of aminopterin and it was ascertained that, in the dilutions employed, it made no difference from normal in the response to growth by the organism. Since aminopterin is also known to interfere with the utilization of CF in micro-organisms,[3,18] it would seem that this antagonist may have two or more important sites of action and interference with the metabolic alteration of PGA is only one of them.

TABLE II

Effect of aminopterin on in vitro *synthesis of CF from PGA by normal rat blood*

Supplements	CF mμg. per c.c. blood
Blood ..	5·0
Blood+FA (10 μg.) ..	9·4
Blood+FA (10 μg.)+Aminopterin (5 μg.) ..	4·8
Blood+FA (10 μg.)+AA (10 mg.) ..	14·2
Blood+FA (10 μg.)+AA (10 mg.) +aminopterin (5 μg.)	6·2

In an attempt to characterize the CF activity synthesized by the blood cells, a dose response curve was obtained for *L. citrovorum* against increasing concentrations of the blood filtrate and was found to differ from a similar curve with equivalent concentrations of Leucovorin (Fig. 1). Such a difference was also seen with the xanthine culture filtrate of *L. arabinosus* which synthesizes considerable CF activity from p-aminobenzoic acid[19] and has recently been

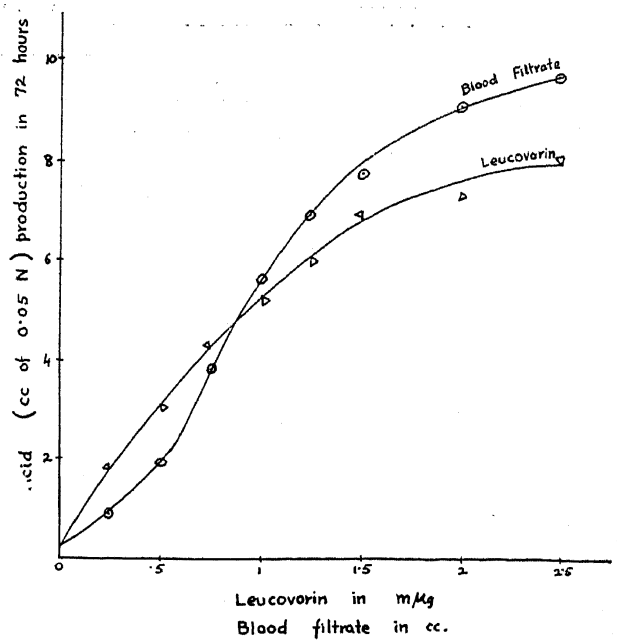

FIG. 1. Dose Response Curve

reported for Hemofolin,[20] a factor synthesized when leucocytes from bone narrow are incubated with PGA. While there are reports of probable new factors[21,22] and conjugates[23] related to CF, it is possible that presence of inhibitors or activators in blood filtrates and other crude extracts may account for the observed differential response. Bioautographic studies to characterize the CF activity are in progress.

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