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ENZYMIC CLEAVAGE OF FOLIC ACID BY EXTRACTS FROM HUMAN BLOOD CELLS

I. PREPARATION AND CO-FACTOR REQUIREMENTS OF THE ENZYME SYSTEM

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Several reports have described the inactivation of folic acid encountered during the assay of this vitamin in animal tissues[1]. SILVERMAN *et al.*[2] have demonstrated that folic acid on incubation with rat liver extracts suffers a loss in the growth-promoting properties for *Streptococcus faecalis* R. Comparatively less is known, however, about the enzymes or the pathways through which folic acid is inactivated in animal tissues. During the course of this work two abstracts have appeared which report that rat liver slices[3] and pigeon liver extracts[4] can degrade folic acid with the liberation of a diazo-

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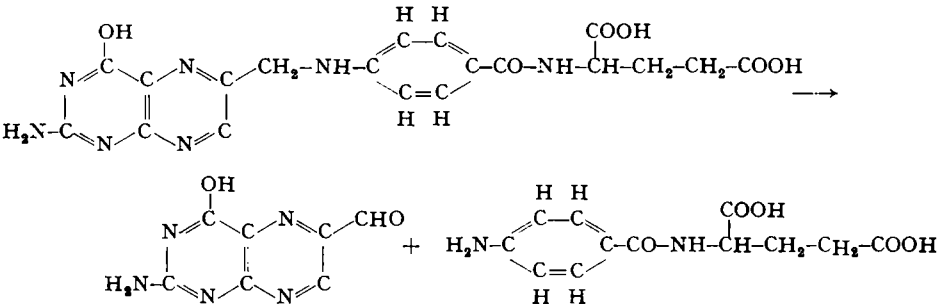
tizable amine. In this communication we present results to show that a preparation from normal human blood cells can enzymically degrade folic acid under physiological pH conditions at the C_9–N_{10} linkage with the liberation of *p*-aminobenzoylglutamic acid, and 2-amino-4-hydroxy-6-formyl pteridine.

The overall reaction may be formulated as follows:



Preparation, the nature of the products formed, as well as the co-factor requirements for this enzyme system, are described. Evidence is presented to indicate that normal human blood contains inhibitors for the enzyme which cleaves folic acid.

TECHNIQUES

Enzymic degradation of folic acid

Routine determinations of enzyme activity were carried out by following the liberation of the diazotisable amine from folic acid by the BRATTON AND MARSHALL test [5]. In view of our previous observations[6] that acid extracts of blood can actively degrade folic acid, it was necessary, especially when working with untreated haemolysed extracts of blood cells, to modify the order in which the reagents were added. The following procedure was found to produce the minimum of interference. The reaction mixture was treated with 1 ml of 0.1% sodium nitrite and then with 1 ml of 30% trichloroacetic acid. The precipitated protein was removed by centrifugation, and to a suitable aliquot were added 1 ml of 0.5% ammonium sulphamate and then 1 ml of 0.1% *N*-naphthylethylenediamine hydrochloride in the usual way. The intensity of colour was measured in a Klett-Summerson Colorimeter with a 540 mμ filter. It may be noted that when extracts of cells prepared as described in the present studies were employed, no modification of the BRATTON AND MARSHALL test was necessary.

Standards employed

A standard curve was plotted by employing pure *p*-aminobenzoic acid as the standard. On the basis that *p*-aminobenzoic acid and *p*-aminobenzoylglutamic acid, which may be formed from folic acid, give the same molal colour[7], the experimental values (μg of folic acid degraded) were calculated from this curve.

Protein content

Protein was estimated by the optical density method suggested by KALCKAR *et al.*[8], using the following formulation: Protein (mg per ml) = 1.45 E_{280} — 0.74 E_{260}, where E_{280} and E_{260} are the extinctions of the solution at wavelengths 280 and 260 mμ.

Phosphate buffers

Prepared by mixing KH_2PO_4 and Na_2HPO_4.

Materials employed

p-Aminobenzoic acid – Nutritional Biochemicals Corporation; L(+) *p*-Aminobenzoyl-glutamic acid – S.A.F. Hoffman-La Roche & Co. Ltd.; Folic acid (crystalline) – Nutritional Biochemicals Corporation; Adenosine triphosphate disodium salt (ATP) 95% pure – Nutritional Biochemicals Corporation; Glutathione – Nutritional Biochemicals Corporation; Tris(hydroxymethyl)aminomethane – L. Light & Co., Ltd.; L-Cysteine monohydrochloride monohydrate – H.M. Chemical Co.,

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Ltd.; Thioglycollic acid – B.D.H. Spot Test reagent; Coenzyme A, approx. 75% pure Nutritional Biochemicals Corporation; Diphosphopyridine nucleotide (DPN) Approx. $4H_2O$ – Sigma Chemical Co.

Preparation of the enzyme

Active extracts of haemolysed cells were prepared as follows: fresh citrated (0.015 *M*) blood obtained from the Haffkine Institute blood bank was centrifuged to remove the plasma; it was then washed once with isotonic saline and the packed cells were haemolysed by addition of 5 volumes of water. In a typical experiment 26.5 ml of concentrated HCl were added dropwise to 110 ml of haemolysed cells under cold with continuous stirring. The final concentration was 2*N* with respect to HCl. The mixture was stirred for 5 minutes, then centrifuged for 10 minutes at 500 *g*. The precipitate containing the active material was washed twice with 55 ml of 2*N* HCl, and the suspension was centrifuged each time for ten minutes at 500 *g*. The washed precipitate was then suspended in 50 ml of water and brought to pH 5.5 by addition of approximately 52 ml of *N* NaOH. 26.5 ml of 0.5 *M* phosphate buffer of pH 7 were then added to the mixture with constant stirring. The total volume of fluid was made to 136.5 ml by addition of water. The final extract was at pH 7. The copious precipitate formed at this stage was removed by centrifugation for ten minutes at 500 *g*. The supernatant thus obtained was dialysed for 18 hours and formed the starting material for ammonium sulphate fractionation experiments.

Fractionation by precipitation with ammonium sulphate

The dialysed neutral extract obtained was fractionated by precipitation with ammonium sulphate at 0 °C. Solid ammonium sulphate was used for obtaining the percentage saturation required. The precipitates obtained at each stage were allowed to settle for 30 minutes, then centrifuged for 20 minutes at 11,000 *g*. These were then dissolved in distilled water and dialysed for 20 hours against 6 litres of cold distilled water with constant stirring. In a typical experiment precipitates obtained at each stage from 60 ml of the neutral extract were dissolved in 20 ml of water. After dialysis the volumes were usually made to 25 ml. The protein concentration of these extracts generally ranged from 1.5 to 3 mg per ml. The enzyme retained activity for at least 7 days if kept at 0 to 3° C. All centrifugations were carried out at 0° C in an International Refrigerated Centrifuge, PR-2.

Preparation of the supernatant (S) containing a cofactor for the enzyme

The supernatant obtained after removing the fraction precipitated by 70% $(NH_4)_2SO_4$ saturation was treated with a further quantity of $(NH_4)_2SO_4$ to make it 100% saturated. The precipitate was removed in the usual way and the supernatant dialysed in 3 litre portions of water (with one change) at 5°C for four hours. This material was found to contain the cofactor required by the fraction obtained by 55–70% $(NH_4)_2SO_4$ saturation. Longer dialysis led to loss of the active material.

Preparation of the heated extract from haemolysed cells

The heated extract was prepared from the same undialysed neutral supernatant which also formed the starting material for the experiments on fractionation with $(NH_4)_2SO_4$. This extract at pH 7 was heated for 30 minutes in boiling water, the coagulated proteins were then removed by centrifugation and the colourless solution thus obtained was used as the source of co-factors for the enzyme.

RESULTS

Degradation of folic acid by ammonium sulphate fractions prepared from blood extracts

The unfractionated, dialysed extracts, as well as the various fractions obtained by precipitations with $(NH_4)_2SO_4$, were consistently inactive, but the activity could be restored in the fractions containing the active protein by the addition of a heated extract and supernatant (S), prepared from haemolysed cells as described in the section on techniques. However, the unfractionated extract showed great variations in activity even when supplemented with the heated extract. In many of the preparations it was found to be inactive even if the incubation period was extended beyond 16 hours. Our experience indicates that if the temperature precautions were strictly adhered to, and the preparations were kept around 0°C throughout the experimental period, the unfractionated extracts were invariably inactive. This is evident from Table I.

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TABLE I

DEGRADATION OF FOLIC ACID BY AMMONIUM-SULPHATE FRACTIONS OF BLOOD CELL EXTRACTS

Components of the incubation mixture: Folic acid = 300 μg; 0.5 M PO_4^{---} buffer pH 7 = 0.1 ml; heated extract = 1.0 ml. Supplements: $(NH_4)_2SO_4$ fractions = 0.5 ml; Supernatant (S) = 1.0 ml. Total volume = 4 ml. Incubation time = 60 min. Temp. = 37° C. Non-enzymic degradation of folic acid = 10 μg.

Expt. No.	μg of folic acid degraded by 1.0 ml of unfractionated extract	F_1 0–55% Sat. with $(NH_4)_2SO_4$	F_2 55–70% Sat. with $(NH_4)_2SO_4$	F_3 70–100% Sat. with $(NH_4)_2SO_4$	S Supernatant from full saturation	$F_1 + S$	$F_2 + S$	$F_3 + S$	$F_1 + F_2$	$F_2 + F_3$	$F_1 + F_3$	$F_1 + F_2 + F_3 + S$
		μg of folic acid degraded by the above ammonium-sulphate fractions										
1	10.0 (18 hours)	9.7	10.0	10.7	9.3	10.3	36.0	10.0	10.7	10.3	11.0	—
2	10.0 (18 hours)	—	10.3	—	—	—	50.0	—	—	—	—	13.3
3	9.7 (1 hour)	—	—	—	—	—	58.0	—	—	—	—	38.5
4	11.5 (1 hour)	—	—	—	—	—	72.0	—	—	—	—	57.5

Figures in brackets indicate the time of incubation for the unfractionated extract.

Table I, experiment 1 shows the activity of various fractions when incubated with the heated extract and folic acid. It is apparent from these results that none of the individual fractions were able to degrade folic acid under the experimental conditions employed. Activity could be demonstrated only by combining the fraction F_2 precipitated by 55–70% sat. $(NH_4)_2SO_4$ with the supernatant (S). The presence of an active part in S suggests that this may have been split from the protein during the process of fractionation. Although the exact nature of S is not known at present, a study of some of its properties showed that it is lost if the dialysis was extended from 4 hours to 16 hours, whereas the control containing the material dialysed for 4 hours and kept under cold for the same period of time did not lose activity. The active material in S was stable when heated for thirty minutes at 100° C but lost activity on washing. These properties indicate that the substance is not a protein nor a metal ion. It may be noted that the activity of the different enzyme preparations varied considerably when the system was reconstituted with heated extract and S, possibly due to partial loss of the factor present in S during dialysis.

Observations in Table I, experiments 2, 3 and 4, show that the fraction F_1 precipitated by 0–55% saturated $(NH_2)_4SO_4$ inhibits the activity of F_2. Although the extent of this inhibitory action of F_1 on F_2 was not constant, it was consistently observed in all preparations. Preliminary experiments indicate that F_1 behaves like a protein in some of its properties. It is non-dialysable, can be precipitated by $(NH_4)_2SO_4$, it is unstable to heat and loses activity on keeping. The unstable nature of this inhibitor F_1, which separates from the active material during fractionation, may explain the occasional appearance of enzyme activity found in the unfractionated extracts when temperature precautions were not strictly observed.

Requirements and properties of the enzyme system

Since the fraction precipitated by 55–70% saturated $(NH_4)_2SO_4$ consistently showed activity when supplemented with the heated extract and supernatant S, the properties of the enzyme degrading folic acid were investigated using this fraction as the source of the enzyme. Table II shows the effects of the heated extract and S on the enzyme

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activity of F_2. The enzyme is completely inactivated in absence of S, whereas omission of the heated extract reduced the activity to approximately 60%.

TABLE II

EFFECT OF THE HEATED EXTRACT AND SUPERNATANT (S) ON THE ENZYMIC DEGRADATION OF FOLIC ACID BY FRACTION F_2.

Complete system consisted of 0.5 ml F_2; 1.0 ml heated extract; 1.0 ml (S); 0.5 M PO_4^{---}; Buffer pH 7.0 and 300 μg folic acid. Total volume of mixture = 4 ml. Incubation time = 1 hour. Temp. = 37°C. (Results corrected for non-enzymic blank)

Conditions	*µg folic acid degraded*
Complete system	48.0
Heated extract omitted	30.0
Supernatant (S) omitted	2.0
Heated extract and S omitted	Nil

In attempts to replace the cofactors supplied by the heated extract and S, it became apparent that maximal activity could be obtained in the presence of Mn^{++}, ATP and glutathione. Fig. 1 illustrates the effect of various concentrations of ATP, Mn^{++}, and glutathione on the activity of the enzyme. It is apparent that the optimum activity is obtained when $5 \cdot 10^{-4}$ M ATP, $1 \cdot 10^{-3}$ M Mn^{++} and $1 \cdot 10^{-3}$ M glutathione are present. Higher concentrations than these were found inhibitory to the system. The enzyme was completely inactive when Mn^{++} or glutathione was omitted, but 60% of the activity remained when ATP was left out. DPN has been found by SILVERMAN *et al.*[2] to aid the inactivation of folic acid by rat liver extracts. In our experiments with acetone-dried powders of rat liver it has been found that cleavage of folic acid at

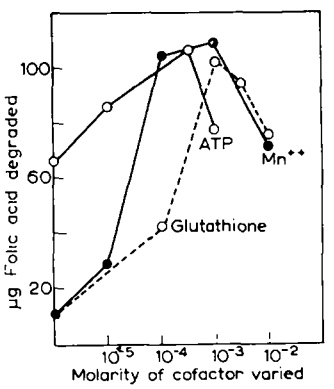

Fig. 1. Optimum requirements of ATP, Mn^{++} and glutathione for the enzymic degradation of folic acid by F_2. 0.3 ml of enzyme extract F_2, containing 30 μg protein were incubated with two of the cofactors in constant concentration and varying amounts of the third cofactor were added. Concentration of cofactors when not varied: ATP = $5 \cdot 10^{-4}$ M, Mn^{++} = $1 \cdot 10^{-4}$ M, glutathione = $1 \cdot 10^{-3}$ M. Other constituents: 0.5 M PO_4^{---} buffer pH 5.5 = 0.15 ml; folic acid = 300 μg. Total volume = 1.5 ml. Time of incubation = 1 h. Temp. 37°C.

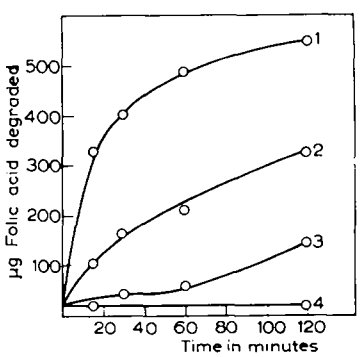

Fig. 2. Rate of enzymic cleavage of folic acid. Constituents of the incubation mixture. F_2 = 0.5 ml; 0.5 M PO_4^{---} buffer of pH 5.5 or 7.0 = 0.4 ml; Folic acid = 900 μg. Total volume = 4.0 ml. Temp. 37°C. Supplements: Curve 1: $5 \cdot 10^{-4}$ M ATP + $1 \cdot 10^{-3}$ M Mn^{++} + $1 \cdot 10^{-3}$ M glutathione + 0.5 M PO_4^{---} buffer, pH 5.5. Curve 2: $5 \cdot 10^{-4}$ M ATP + $1 \cdot 10^{-3}$ M Mn^{++} + $1 \cdot 10^{-3}$ M glutathione + 0.5 M PO_4^{---} buffer pH 7. Curve 3: Heated extract = 1.0 ml + S = 1.0 ml + buffer pH 7. Curve 4: Same constituents as curves 1 without addition of F_2.

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C_9–N_{10} linkage is reduced by 80% when DPN and nicotinamide are omitted[9]. In the enzyme system under discussion, however, DPN produced negligible effect.

The progress of folic acid degradation with time in presence of cofactors supplied by the heated extract and S, as well with known cofactors is illustrated in Fig. 2. It is evident from these results that the reconstituted system containing ATP, Mn^{++}, and glutathione is more active than the system in which the cofactors are supplied by heated extract and S. Furthermore, degradation of folic acid is much higher at pH 5.5 than at pH 7 as expected from the pH dependence curve. The initial rate of degradation was calculated to be approximately 0.029 μmoles of folic acid cleaved/minute/mg protein (1 mg of the F_2 protein having come from 0.6 ml of haemolysed cells).

Fig. 2 curve 4 represents the non-enzymic degradation of folic acid under the same experimental conditions. It is found to remain constant throughout the experimental period of two hours.

The effect of temperature on the activity of the enzyme system is given in Fig. 3. Under the experimental conditions employed the activity of the enzyme system was reduced by 50% at 35°C and 90% at 100°C. The relationship between pH and activity of the enzyme system is illustrated in Fig. 4. The curve shows a sharp pH maximum near pH 5 both with phosphate buffer as well as when tris(hydroxymethyl)aminomethane buffer was employed. At pH 7 the activity is only 15% of the activity at optimum pH. However, the experiments reported in Table I were carried out at pH 7 as the heated extract employed was buffered at this pH (see section on techniques). Heated extracts prepared in a similar manner but buffered at pH 5.5 or obtained directly by heating haemolysed cells have not been found effective.

In order to determine whether the activating role of glutathione on this enzyme system was specific, experiments were carried out in which glutathione was replaced by other compounds containing thiol groups. Results obtained as given in Table III show that cystein and thioglycollic acid in concentrations of 10^{-3} *M* produced only slight activation. CoA was found to be more effective but less efficient than glutathione In concentrations of 10^{-3} *M* it was about 75% as effective as glutathione.

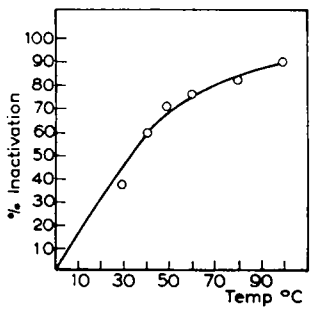

Fig. 3. Effect of temperature on folic acid degradation. 1 ml of enzyme solution (F_2) containing 100 μg protein was kept for 5 minutes at varying temperatures. An aliquot of 0.3 ml was then incubated with 300 μg of folic acid for 1 hour at 37°C. Constituents of the system: ATP $5 \cdot 10^{-4}$ *M*; Mn^{++} $1 \cdot 10^{-4}$ *M*; glutathione $1 \cdot 10^{-3}$ *M*; 0.5 *M* PO^{---} buffer pH 5.5 = 0.15 ml. Total volume 1.5 ml.

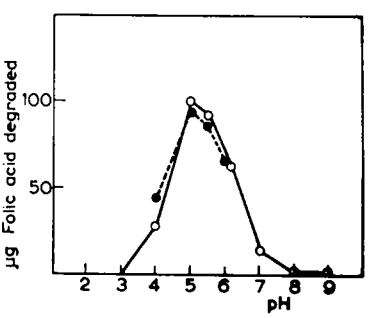

Fig. 4. Relationship between pH and enzymic cleavage of folic acid 0.3 ml. F_2 containing 26 μg protein was incubated in the usual system. (See Fig. 3) using buffers of varying pH at 37°C for 1 hour. Buffers employed: O—O pH 3–4: Sorensen's glycine/hydrochloric acid buffer (0.3 ml); pH 5–7: 0.5 *M* PO^{---} buffer (0.15 ml); pH 8: Sörensen's glycine/sodium hydroxide buffer (0.3 ml). ●—● 0.1 *M* tris(hydroxymethyl)-aminomethane.

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TABLE III

EFFECT OF SOME THIOL COMPOUNDS ON THE ENZYME ACTIVITY

The incubation mixture consisted of 0.3 ml F_2 (dil. 1:20) + $5 \cdot 10^{-4}$ *M* ATP + $1 \cdot 10^{-4}$ *M* Mn^{++} + 0.05 *M* PO buffer pH 5.5 + 300 μg folic acid. Time of incubation = 60 min. Temp. = 37° C. Thiol compounds were added in the concentrations specified. The results have been corrected for non-enzymic blank.

Expt. No.	*Thiol compound added*	*Concentration of thiol compounds*	*µg of folic acid degraded*
1	None		3
	Glutathione	$1 \cdot 10^{-4}$ *M*	56
	Glutathione	$1 \cdot 10^{-3}$ *M*	128
	Cysteine	$1 \cdot 10^{-4}$ *M*	6
	Cysteine	$1 \cdot 10^{-3}$ *M*	8
	Thioglycollic acid	$1 \cdot 10^{-4}$ *M*	5
	Thioglycollic acid	$1 \cdot 10^{-3}$ *M*	10
2	None	----	0
	Glutathione	$1 \cdot 10^{-4}$ *M*	52
	Coenzyme A	$1 \cdot 10^{-4}$ *M*	19
3	None		1
	Glutathione	$1 \cdot 10^{-3}$ *M*	111
	Coenzyme A	$1 \cdot 10^{-3}$ *M*	61

It is to be noted that in every case it was ascertained by control experiments that the particular thiol compound, in concentrations employed did not affect the BRATTON-MARSHALL test used for estimating the enzyme activity.

Inhibitory action of F_1 on the enzyme system containing known cofactors

In view of the inhibitory effect produced by the $(NH_4)_2SO_4$ fraction F_1 on fraction F_2 when heated extract and S were used as the source of cofactors (Table I), the role of F_1 on the enzyme system containing known cofactors was studied. The results as given in Table IV show that F_1 has no action on the system containing optimum amounts of ATP, Mn^{++} and glutathione (Table IV, column 2).

TABLE IV

EFFECT OF F_1 ON THE ENZYME SYSTEM CONTAINING KNOWN COFACTORS

The incubation mixture consisted of 0.5 ml F_2 + 300 μg folic acid + 0.05 *M* PO_4^{--} buffer pH 7 + the cofactors. Total volume = 4 ml. Time = 60 min. Temp. 37°C. The values have been corrected for non-enzymic blank.

Expt. No.	*Volume of F_1 added (ml)*	*Concentration of cofactors*					
		Heated extract = 1 ml S = 1 ml	*ATP = $5 \cdot 10^{-4}$ M, Mn^{++} = $1 \cdot 10^{-4}$ M, Glutathione = $1 \cdot 10^{-3}$ M*	*ATP = Nil, Mn^{++} = $1 \cdot 10^{-4}$ M, Glutathione = $1 \cdot 10^{-3}$ M*	*ATP = $5 \cdot 10^{-4}$ M, Mn^{++} = Nil, Glutathione = $1 \cdot 10^{-3}$ M*	*ATP = $5 \cdot 10^{-4}$ M, Mn^{++} = $1 \cdot 10^{-4}$ M, Glutathione = $1 \cdot 10^{-4}$ M*	*ATP = $5 \cdot 10^{-4}$ M, Mn^{++} = $1 \cdot 10^{-4}$ M, Coenzyme A = $1 \cdot 10^{-4}$ M*
		µg of folic acid degraded					
1	0	35	99		34	22	20
	1.0	9	96		36	19	22
2	0	79		77			
	1.0	11		74			

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As the activity of the system containing optimum amounts of known cofactors is much higher than the system containing heated extract and S, the possibility was considered that the lack of inhibition observed in the former system may be due to higher concentration of cofactors. Experiments in which the concentration of each individual cofactor was reduced below the optimum (Table IV, column 3–5), indicate that F_1 has no action on the enzymic activity in the system containing known cofactors even when these are present in concentrations below the optimum. It is therefore possible that the coenzyme complex present in blood may not be identical with the cofactors employed in the reconstituted system. In conformity with the observation that F_1 does not inhibit the enzymic activity in presence of ATP, Mn^{++} and glutathione, it is possible to demonstrate with the known mixture of cofactors, the activity of the enzyme in unfractionated extracts which are completely inactive when heated extract and S are employed as the source of cofactors.

Identification of the products of the enzymic degradation of folic acid

2-Amino-4-hydroxy-6-formyl-pteridine: the possibility that the pteridine derivative formed by the enzymic cleavage of folic acid may be 2-amino-4-hydroxy-6-formyl-pteridine was tested by treating the products of the incubation mixture with 2,4-dinitrophenylhydrazine[10]. It is evident from Fig. 6 that the degradation of folic acid by the usual enzyme system is accompanied by a rise in the liberation of a carbonyl derivative indicating that one of the products formed by cleavage of folic acid at C_9–N_{10} linkage may be 2-amino-4-hydroxy-6-formyl-pteridine. The identity of this derivative was confirmed by adsorption and selective elution from an ion exchange resin, and by paper chromatography.

Adsorption and elution from ion-exchange resin. Amberlite I.R.A. 410 (OH): Folic acid (1200 μg) was incubated with the enzyme extract F_2 containing 2.6 mg protein and the usual concentration of co-factors in a total volume of 6 ml for an hour at 37°C. The precipitate formed was removed by centrifugation and 5 ml of the supernatant were kept in contact with 1 g of the resin with constant stirring for about an hour. The contents were then centrifuged and the resin washed with 5 ml of water. The adsorbed

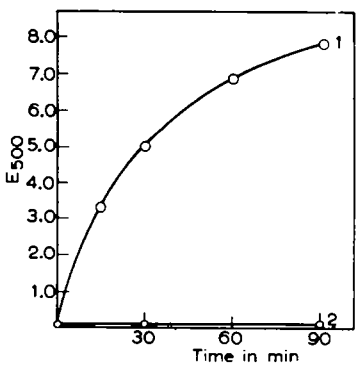

Fig. 5. Liberation of carbonyl derivative from folic acid. The incubation mixture consisted of $5 \cdot 10^{-4}$ *M* ATP + 10^{-4} *M* Mn^{++} + $1 \cdot 10^{-3}$ *M* glutathione + 0.05 *M* PO^{---} buffer pH 5.5 + 600 μg folic acid + F_2 containing 43 μg protein. Total volume = 1.5 ml. Temp. = 37°C.

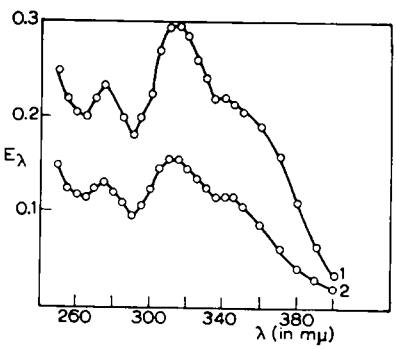

Fig. 6. Curve 1. Acetic acid eluate containing enzymic degradation products of folic acid (corrected for the blank). Curve 2. 2-Amino-4-hydroxy-6-formyl-pteridine in acetic acid.

Curve 1: Complete system. Curve 2: Enzyme omitted.

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pteridine derivative was then eluted from the washed resin with 5 ml of 1 *N* acetic acid. The absorption spectra of the eluate was determined in a Beckman Spectrophotometer, Model DU, with a silica cuvette of 1 cm light path. As it was found that acetic acid eluted materials from the resin which interfered with the spectrophotometric determinations of the experimental sample, a correction had to be applied for this extraneous absorption. The control cuvette contained materials which were treated in an identical way as the experimental sample, except that folic acid had been omitted during the incubation period. The absorption spectra of the pteridine derivative formed was then obtained by difference from that of the control.

In Fig. 6, curve 1, the absorption spectra are given of the acetic acid eluate, containing the enzymic degradation products of folic acid, after correcting for the absorption due to impurities from the resin. This is compared with the spectrum (Fig. 6, curve 2) obtained under similar conditions from a highly purified sample of 2-amino-4-hydroxy-6-formyl-pteridine, which was kindly sent to us as a gift by Dr. BROQUIST. It is apparent from these absorption curves, that both the spectra are similar and in agreement with that of 2-amino-4-hydroxy-6-formyl-pteridine as reported by LOWRY *et al.*[11].

Paper chromatography of the enzymic degradation products of folic acid

Paper chromatography of the enzymic degradation products of folic acid was carried out using the descending technique and 1 *N* ammonia as the solvent, followed by photography under ultraviolet light. From the results illustrated in Fig. 7 it is evident that when folic acid is incubated with the usual enzyme system, the chromatogram shows 3 spots having the R_F values corresponding to those of folic acid, 2-amino-4-hydroxy-6-formyl-pteridine, and *p*-aminobenzoic acid. In chromatograms developed in 1 *N* ammonia it was not possible to differentiate clearly between *p*-aminobenzoic acid and *p*-aminobenzoyl glutamic acid. It could be shown, however that the spot corresponding to *p*-aminobenzoic acid in Fig. 7 was diazotisable.

Identification of the diazotizable amine as p-*aminobenzoylglutamic acid*

The aromatic amine formed in the experimental mixtures was not extractable by ether from aqueous solutions at pH 3, while *p*-aminobenzoic acid was readily extracted as has been shown by STOKSTAD *et al.*[12].

The product of folic acid cleavage was identified as *p*-aminobenzoylglutamic acid by paper chromatography, using Whatman No. 4 with *n*-butanol (40 ml)-acetic acid (10 ml)–water (50 ml) mixture as the solvent. Th spots were developed by the procedure of EKMAN[13]. Results obtained are illustrated in Fig. 8 (Nos. 1–4). These show that no *p*-aminobenzoic acid was detectable among the degradation products of folic acid. The *p*-aminobenzoylglutamic acid present in the experimental mixture was isolated and hydrolysed according to a method described by WEBB[14]. Chromatograms of the hydrolysed and non-hydrolysed product as shown in Fig. 8 (Nos. 5–6) further confirm the presence of *p*-aminobenzoylglutamic acid which on hydrolysis liberated *p*-aminobenzoic acid. Glutamic acid could be identified in the mixture after hydrolysis.

From the experimental evidence given above it appears that the enzyme preparation from blood degrades folic acid only at C_9–N_{10} linkage, and there is no detectable cleavage of the *p*-aminobenzoylglutamic acid so produced.

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Fig. 7. Paper chromatogram of products formed on incubation of folic acid with F_2 in the usual system. 1. Enzyme system without folic acid. 2. Folic acid*. 4. 2-Amino-4-hydroxy-6-formyl-pteridine.* 5. *p*-Aminobenzoic acid.* 3. Folic acid incubated with enzyme system.

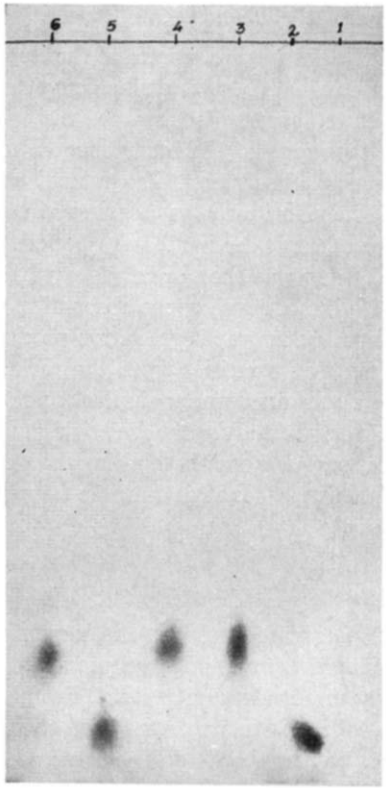

Fig. 8. Identification of the diazotizable amine as *p*-aminobenzoyl-glutamic acid. 1. Enzyme system without folic acid. 2. *p*-Aminobenzoic acid.** 3. *p*-Aminobenzoyl-glutamic acid.** 4. Folic acid incubated with the enzyme system. 5. Hydrolyzed.*** 6. Non-hydrolyzed.***

DISCUSSION

The experimental evidence presented in this communication demonstrates that extracts made from human blood cells can degrade folic acid at C_9–N_{10} linkage. This cleavage can account for the loss in growth promoting properties of the vitamin for *Streptococcus faecalis* organisms. From data available at present, it is not possible to determine the nature of the intermediate steps involved in this degradation of folic acid. Evidence from paper chromatography and the nature of the products identified, however, shows that the overall cleavage leads to the formation of *p*-aminobenzoyl-glutamic acid and 2-amino-4-hydroxy-6-formyl-pteridine. The necessity for several cofactors like ATP, Mn^{++}, and glutathione, indicates that the reaction mechanism is probably not a simple dehydrogenation followed by hydrolytic cleavage as has for example been suggested for the mechanism of amino acid oxidases[15]. It has not been

* Added to the enzyme mixture at the end of incubation period.
** Added to the enzyme system at the end of the incubation period.
*** *p*-Aminobenzoyl-glutamic acid isolated from the reaction mixture according to WEBB[14].

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possible to demonstrate cleavage of folic acid in haemolysed cells before acid treatment. This enzyme like many hydrolytic enzymes in blood, is apparently present in a masked form, and is liberated by treatment with acid, in which it is stable. Haemolysed cells also appear to contain other labile and proteinlike factors which depress enzymic cleavage of folic acid. These inhibit the activity of the enzyme when the cofactor requirements are supplied by extracts prepared from blood but not when ATP, Mn^{++}, and glutathione are employed. The inhibitors can be separated from the active protein by ammonium sulphate fractionation. The nature of these inhibitory substances is under investigation at present. Enzymes which are similar but may not be identical with the one in blood are widely distributed in animal tissues. Acetone dried powders of rat liver and sheep liver can actively degrade folic acid at C_9–N_{10} linkage. Although in these tissues as in blood, these enzyme systems are stable in cold HCl they are not present in an inactive form. Activity may be demonstrated in both homogenates and acetone dried powders of liver without pretreatment with HCl[9]. The existence in animal tissues of such active systems for the cleavage of folic acid, indicates this may be an important step in the pathway for the disposal of folic acid and also for the biosynthesis of some unconjugated pteridine derivatives known to occur in animal tissues. 2-Amino-4-hydroxy-6-formyl-pteridine which has been shown to be one of the products of enzymic degradation of folic acid has aroused biological interest in recent years as one of the most potent inhibitors of enzymes like xanthine oxidase[16,17].

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We wish to express our thanks to Dr. V. R. KHANOLKAR, the Director of the Institute for the keen interest shown in this work, to Dr. V. R. KULKARNI, Chief of the Haffkine Institute Blood Bank for the generous supply of blood and to Dr. A. P. BROQUIST of Lederle Laboratories Division for a generous gift of a highly purified sample of 2-amino-4-hydroxy-6-formyl-pteridine.

SUMMARY

1. Preparation is described of an enzyme system from human blood which can degrade folic acid to yield *p*-aminobenzoylglutamic acid and 2-amino-4-hydroxy-6-formyl-pteridine.

2. The enzyme system requires ATP, Mn^{++} and glutathione as cofactors and shows optimum activity at pH 5.

3. The enzyme is apparently present in blood in an inactive state. Activity is liberated on treatment with hydrochloric acid.

4. Evidence is given for the presence of heat-labile inhibitors which may be separated from the active fraction by $(NH_4)_2SO_4$ fractionation. These inhibitors depress the cleavage of folic acid when the cofactors requirements are supplied by extracts prepared from blood but not when ATP, Mn^{++} and glutathione are present.

5. Thiol compounds like cystein or thioglycollic acid cannot replace glutathione as a cofactor for the enzyme. Coenzyme A can partially serve this purpose.

6. The suggestion is made that these active mechanisms for cleavage of folio acid in animal tissues may be important for the metabolic disposal of folic acid, as also for the biosynthesis of biologically important unconjugated pteridines like 2-amino-4-hydroxy-6-formyl-pteridine which is a very potent inhibitor of xanthine oxidase.

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INFLUENCE DE L'INJECTION DE PYRIDOXINE SUR LA DÉCARBOXYLATION DE L'ACIDE GLUTAMIQUE ET DE L'ACIDE CYSTÉINESULFINIQUE PAR LE CERVEAU CHEZ LE RAT

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Il est maintenant bien établi que la décarboxylation enzymatique, chez les animaux supérieurs, de l'acide cystéinesulfinique[1,2,3], de l'acide cystéique[4] et de l'acide glutamique[5,6] implique la participation du phosphate de pyridoxal. Des travaux antérieurs ont montré que, chez le rat[2] et le lapin[7], l'activité décarboxylante de broyats de foie vis à vis de l'acide cystéinesulfinique et de l'acide cystéique n'est pas sensiblement accrue par addition, *in vitro*, de phosphate de pyridoxal, ce qui indique que, dans le foie, les systèmes enzymatiques responsables de ces décarboxylations sont pratiquement saturés en coenzyme. Ces travaux ont montré d'autre part que, chez les mêmes animaux[2,7], l'activité décarboxylante de broyats de cerveau vis à vis de l'acide cystéinesulfinique, de l'acide cystéique et de l'acide glutamique est au contraire fortement accrue par l'addition, *in vitro*, de phosphate de pyridoxal.

Il a paru utile de rechercher à quoi était due une telle différence entre les systèmes décarboxylants du cerveau et ceux du foie. *A priori*, on pouvait faire deux hypothèses:

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