ENZYMIC CLEAVAGE OF FOLIC ACID BY EXTRACTS FROM HUMAN BLOOD CELLS

II. OBSERVATIONS ON THE LIBERATION OF THE ACTIVE FORM OF THE ENZYME AND ITS SPECIFICITY

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A previous paper (I) reported the preparation and cofactor requirements of an enzyme system in extracts of normal blood cells which degraded folic acid at the C_9–N_{10} linkage with the liberation of 2-amino-4-hydroxy-6-formyl pteridine and *p*-aminobenzoyl-glutamic acid as the main products of reaction. As the untreated blood showed negligible activity, it was suggested that the enzyme system existed in blood in an inactive state. The observations reported herein show that this enzyme system may be activated by subjecting haemolysed cells to higher temperatures and aging. Studies relating to its specificity have demonstrated that the enzyme system in the blood can degrade aminopterin but does not attack A-methopterin or folinic acid. The latter two derivatives were found to inhibit the degradation of folic acid.

TECHNIQUES

Preparation of blood for studies on liberation of the active form of the enzyme

Fresh citrated blood (0.015 *M*) obtained from the Haffkine Institute blood bank was haemolysed as reported in Part I of this series. In order to remove the citrate, the haemolysed cells were dialysed with constant stirring in 3 l portions of distilled water (with two changes) for 24 hours at 0–2°C. It is important not to allow the temperature of the preparation to rise above 2°C throughout the experimental procedure. In experiments on heat treatment, 2 ml of the dialysed material was heated in a water bath at different temperatures. Except where otherwise specified, the uncentrifuged mixture containing the coagulated proteins was used as the source of enzyme.

Preparation of the active extract (F_2) employed in these studies has been described previously[1].

Assay methods. Routine determinations of enzyme activity were carried out by estimating the liberated aromatic amine by the BRATTON AND MARSHALL test[2] as described in[1]. Protein estimations were done by the spectrophotometric method[1].

Materials employed

Vitamin C, Purines, Pyrimidines, L-Proline, DL-Phenylalanine, DL-Tyrosine, DL-Methionine	Nutritional Biochemicals Corporation.
L-Glutamic acid, DL-Alanine	E. Merck Darmstadt
Benzylamine	Ward, Blenkinsop & Co., Ltd.
Folinic acid, Aminopterin, A-Methopterin	Lederle Laboratories Division, American Cynamid Company
Sulphanilamide	B.D.H.

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RESULTS

Studies on the liberation of the active form of the enzyme from haemolysed cells

In experiments designed to study the liberation of the active form of the enzyme, dialysed preparations of haemolysed cells were subjected to various procedures prior to incubation with folic acid. Technical details regarding the preparation of haemolyzed cells used in these studies are described in the section on techniques.

Effect of temperature and keeping on folic acid degradation by haemolysed cells

The effect of temperature on the activation of the enzyme system degrading folic acid was investigated by preheating the dialysed preparations of haemolysed cells at different temperatures for 10 minutes and 15 minutes respectively. Characteristic results obtained are given in Fig. 1a. It is seen from these that incubation for 10 minutes at 100°C resulted in highest activity. When the preincubation period was increased to 15 minutes, the enzyme apparently lost some activity as shown in Fig. 1a (II). It is also clear from these results that the activation is negligible up to temperatures of 50°C. Higher temperatures brought about a sharp increase in activity of the enzyme. Fig. 1b shows the slight increase in activity obtained on keeping the haemolysed cells at 0–2°C for several days. Maximum activation was obtained in eight days. At the end of two weeks the enzyme showed negligible activity under these conditions. In these experiments, controls were run along with the experimental tubes in which the preheated blood was incubated with all constituents of the mixture excepting folic acid. The latter was added to the system at the end of the incubation period. The results as given in Fig. 1a (III) show that about 26 μg of folic acid were degraded under these conditions.

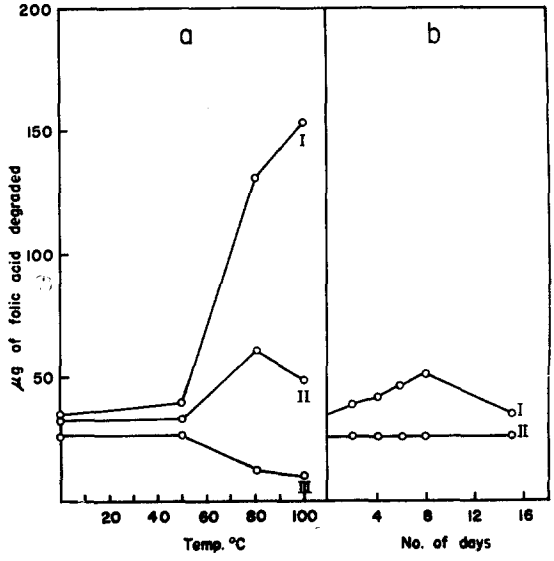

Fig. 1. Effect of temperature and keeping on the liberation of the active form of the enzyme from haemolysed cells. Constituents of the incubation mixture: $5 \cdot 10^{-4}$ *M* ATP + $1 \cdot 10^{-4}$ *M* Mn^{++} + $1 \cdot 10^{-3}$ *M* glutathione + 0.05 *M* $PO_4^{\equiv}$ buffer pH 5.5 + 300 μg folic acid + 0.5 ml haemolysed blood cells heated at various temperatures. Total volume = 1.5 ml. Temp. = 37°C. Time of incubation = 3 h. Fig. 1a. Curve (I) = Haemolysed blood heated for 10 min at various temperatures. Curve (II) = Haemolysed blood heated for 15 min at various temperatures. Curve (III) = Control (haemolysed blood heated for 10 min). Fig. 1b. Curve (I) = Experimental. Curve (II) = Control.

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This rather high blank could be due to the previously observed factor in blood[3] which degrades folic acid under acid conditions, and is heat-unstable. The cleavage probably takes place when the samples are acidified for protein precipitation in estimating the enzyme activity by the BRATTON AND MARSHALL test[2].

In the course of the above studies, it was observed that the extracts obtained by centrifugation of the haemolysed cells which had been subjected to heat treatment, were less active than the uncentrifuged mixtures containing the coagulated proteins. Experiments designed to study conditions for obtaining more of the active factor, in a soluble state, showed that greater amount of the active enzyme could be extracted from the precipitated proteins by further incubating the heat-treated mixture at 37°C. The scheme given in Fig. 2 illustrates the results obtained. In a typical experiment the supernatant obtained by centrifugation of the heat-treated material was only 33% as active as the uncentrifuged mixture. By further incubation of the whole mixture at 37°C for an hour, much more complete extraction of the active enzyme from the insoluble proteins could be effected. However, when the supernatant from the heated material was subjected to similar conditions, 35% of the original activity was lost. These results indicate that although the enzyme system in its inert state is stable to considerable heat treatment, the active and soluble form of the enzyme is much more susceptible to inactivation.

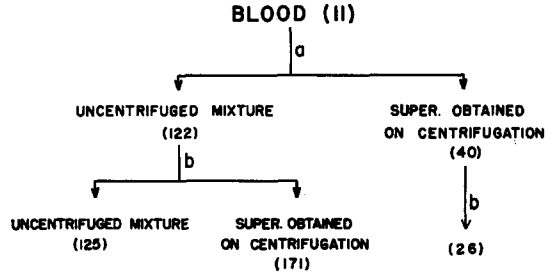

Fig. 2. Scheme illustrating conditions for extraction of the active enzyme from haemolysed cells. 0.5 ml extract incubated in the system given in Fig. 1. Temp. = 37°C. Time of incubation = 3 h. a = heat treatment for 10 min at 100°C. b = incubation for 60 min at 37°C. Figures in brackets show μg of folic acid degraded. (Values have been corrected for the degradation in control tubes.)

Effect of organic solvents and detergents on folic acid degradation by haemolysed cells

Experiments in which haemolysed cells were shaken with organic solvents, such as chloroform, ether, toluene, and olive oil, showed no increase in activity above that produced by haemolysed cells. Detergents such as: Cetavalon, Duponol, Tween 80, Shivapon TH/conc., Ultravon WX, Triton K-60, when present in concentrations of 0.5% were also ineffective in producing greater degradation of folic acid than found with haemolysed cells.

Co-factor requirements of the enzyme system liberated from haemolysed cells by heat treatment

In view of the evidence obtained mainly from investigations on blood and liver[1,4], which suggests that biological systems degrading folic acid, though widely distributed may not all be identical, the cofactor requirements of the enzyme obtained by heat treatment of haemolysed cells were studied. Results given in Table I show that this preparation is activated by the same cofactors as those required by extracts prepared from blood by acid treatment and ammonium sulphate fractionation (F_2). ATP was

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TABLE I

COFACTOR REQUIREMENTS OF THE EXTRACT OBTAINED FROM HAEMOLYSED BLOOD CELLS BY HEAT TREATMENT

0.5 ml extract incubated in the system given in Fig. 1. Temp. = 37°C. Time of incubation = 3 h. Results have been corrected for the non-enzymic blank.

Conditions	*μg of folic acid degraded*
Complete system	23
ATP omitted	28
Mn^{++} omitted	Nil
Glutathione omitted	Nil
ATP, Mn^{++}, glutathione omitted	Nil

found to decrease the activity of the system to a small extent, but a similar effect of ATP has also been observed on some preparations of F_2. As has been demonstrated previously[1] the cofactors when present in concentrations above the optimum were inhibitory to the system. It is possible therefore, that the variations in the effect of ATP can be due to its inadequate removal by dialysis from some of the enzyme preparations.

Effect of various ions on folic acid degradation by ammonium sulphate fraction F_2

Experiments on the effect of various ions, on folic acid degradation by F_2 have demonstrated that many of these substances are able to influence the activity of the enzyme in catalytic amounts. Higher concentrations were always found to be inhibitory.

The results listed in Table II show that Co^{++} in concentrations of $10^{-6}M$ activated the system by 20%, whereas concentrations of $10^{-3}M$ inhibited the activity by 50%. Fe^{++} and Cu^{++} ($10^{-5}M$) were potent inhibitors possibly by inactivation of reduced glutathione. Mg^{++} and Ca^{++} had no effect on the activity of the system. None of the metal ions tested could replace Mn^{++} as an essential cofactor for this enzyme system CN^- and azide produced strong inhibitory effects. $10^{-5}M$ CN^- inhibited the system by 80%, possibly by removal of Mn^{++} from the field of activity.

TABLE II

EFFECT OF VARIOUS IONS ON FOLIC ACID DEGRADATION BY AMMONIUM SULPHATE FRACTION (F_2)

0.3 ml F_2 containing 30–45 μg protein incubated in the system given in Fig. 1. Temp. = 37°C. Time of incubation = 60 min. Results have been corrected for the non-enzymic blank.

Expt. no.	*Ion added to the test system*	*Concentration of the ion added*							
		0	$1\cdot10^{-10}M$	$1\cdot10^{-9}M$	$1\cdot10^{-8}M$	$1\cdot10^{-6}M$	$1\cdot10^{-5}M$	$1\cdot10^{-4}M$	$1\cdot10^{-3}M$
		μg folic acid degraded (expressed in % of the control in absence of added ion)							
1	Mg^{++}	100	—	—	100	103	—	102	—
2	Ca^{++}	100	101	—	103	105	101	104	—
3	Co^{++}	100	—	—	113	122	114	106	55
4	Fe^{++}	100	—	—	102	105	72	3	—
5	Cu^{++}	100	—	—	—	46	10	0	—
6	Citrate	100	104	110	116	123	86	69	—
7	Azide	100	106	—	102	104	103	32	0
8	CN^-	100	—	—	—	77	19	3	0

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Effect of miscellaneous substances on folic acid degradation by F_2

In Table III are listed the various amino acids, purines, pyrimidines and amines tested for their effect on folic acid degradation by F_2. Among these only adrenaline was found to inhibit the system. The least concentration of adrenaline employed was half the molarity of folic acid used.

TABLE III

EFFECTS OF MISCELLANEOUS COMPOUNDS ON FOLIC ACID DEGRADATION BY F_2

0.3 ml F_2 containing 30–45 μg protein incubated in the system given in Fig. 1. Concentration of the compound added was $1 \cdot 10^{-3} M$ and $1 \cdot 10^{-4} M$ except where otherwise specified. Temp. = 37°C. Time of incubation = 60 min. Results have been corrected for the non-enzymic blank.

Substance added	*% inhibition*
Amino acids	
L-Glutamic acid	Nil
DL-Aspartic acid	Nil
L-Proline	Nil
DL-Alanine	Nil
DL-Phenylalanine	Nil
DL-Tyrosine	Nil
DL-Methionine	Nil
Purines	
Adenine	Nil
Guanine	Nil
Xanthine	Nil
Hypoxanthine	Nil
Pyrimidines	
Thymine	Nil
Uracil	Nil
Amines	
Benzylamine	Nil
Histamine (0.34 μmoles/tube)	Nil
Histamine (0.68 μmoles/tube)	Nil
Adrenaline (0.34 μmoles/tube)	93
Adrenaline (0.68 μmoles/tube)	97

Substrate specificity

The specificity of the enzyme system cleaving folic acid was studied by determining the degradation of some closely related compounds such as: aminopterin, folinic acid and A-methopterin.

Results given in Table V show that 37% of aminopterin was degraded in comparison with 43% of folic acid cleaved under similar conditions. It is to be noted that the non-enzymic blank for aminopterin was considerably higher than the corresponding value for folic acid, but the results reported in Table V have been corrected for the blank. Although folic acid is commonly found as an impurity in most commercial preparations of aminopterin[5], the relatively high degradation obtained with the latter substrate suggests that it is not likely to be due to the folic acid present as a contaminant.

A-methopterin was not cleaved by the enzyme system in blood. Since in A-methopterin, there is no free hydrogen attached to the aromatic amino nitrogen, the activity of the enzyme system in presence of this substrate could not be followed by the

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BRATTON AND MARSHALL test. It was therefore, estimated by the formation of 2,4-dinitrophenylhydrazone, as described in[1]. The values given in Table V are expressed as the Klett readings and showed no increase above the reagent blank, even after an incubation period of 16 hours. Folinic acid, which is one of the biologically active derivatives of folic acid, was not degraded by the enzyme system under study.

Inhibition of folic acid degradation by vitamin C and its reversal by glutathione

It was particularly interesting to study the action of vitamin C on the enzyme system degrading folic acid, in view of the activating action of this vitamin on biological systems converting folic to folinic acid[6]. In Table IV are given the results obtained.

TABLE IV

INHIBITION OF FOLIC ACID DEGRADATION BY VITAMIN C AND ITS REVERSAL BY GLUTATHIONE

0.3 ml F_2 containing 33 μg protein incubated in the system given in Fig. 1. Temp. = 37°C. Time of incubation = 60 min. Results have been corrected for the non-enzymic blank.

Concentration of Vitamin C	*$1 \cdot 10^{-3}M$ glutathione*		*$5 \cdot 10^{-3}M$ glutathione*	
	μg of folic acid degraded	*% inhibition*	*μg of folic acid degraded*	*% inhibition*
0	143	—	131	—
$1 \cdot 10^{-5}M$	142	0	134	0
$2.5 \cdot 10^{-5}M$	123	14	127	3
$5 \cdot 10^{-5}M$	30	76	126	4
$1 \cdot 10^{-4}M$	3	98	111	15
$5 \cdot 10^{-4}M$	3	98	2	98

TABLE V

SUBSTRATE SPECIFICITY

0.3 ml F_2 containing 30–45 μg protein incubated in the system given in Fig. 1 using 150 μg substrate. Temp. = 37°C. Results for folic acid and aminopterin have been corrected for the nonenzymic blank.

Time	*μg degraded Klett readings (2,4-dinitrophenyl-hydrazone)*			
	Folic acid	*Aminopterin*	*Folinic acid*	*A-methopterin*
0	0	0	3.2	30
15 m	50	17	—	—
30 m	61	41	—	—
60 m	65	53	5.2	34
16 h	—	—	4.5	36

Experiments in which varying concentrations of vitamin C were incubated with folic acid and the usual components of the system, showed that $10^{-4}M$ vitamin C completely inhibited the degradation of folic acid by F_2. The possibility that vitamin C may be producing this effect by inactivation of reduced glutathione[7,8] was tested by a study of the reversal of vitamin C inhibition, in systems containing higher concentrations of glutathione. Table IV shows that $10^{-4}M$ vitamin C produced 98% inactivation in the usual system, whereas in systems in which $5.10^{-3}M$ glutathione was added, the inhibition produced by similar concentrations of vitamin C was only 15%. As previously observed, higher concentrations of glutathione than $10^{-3}M$ inhibited the enzyme, and absolute values obtained with systems containing $5.10^{-3}M$

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glutathione are therefore on the whole lower. These findings lend support to the view that vitamin C inhibits the present enzyme system by interference with glutathione.

Effect of some structurally related compounds on folic acid degradation

In Table VI are listed the results of an investigation on the action of several structurally related compounds, on folic acid degradation by fraction F_2.

TABLE VI

EFFECT OF SOME STRUCTURALLY RELATED COMPOUNDS ON FOLIC ACID DEGRADATION

0.3 ml F_2 containing 30–45 μg protein incubated in the system given in Fig. 1. Temp. = 37°C. Time of incubation = 60 min. Results have been corrected for the non-enzymic blank.

Expt. no.	*Supplement added*	*μg of supplement added*	*μg of folic acid degraded*	*% inhibition*
1	A-methopterin	0	101	—
	A-methopterin	50	73	28
	A-methopterin	100	36	64
2	Folinic acid	0	119	—
	Folinic acid	75	65	45
	Folinic acid	150	33	72
3	Xanthopterin	0	127	—
	Xanthopterin	50	70	45
	Xanthopterin	100	25	80
4	*p*-Aminobenzoic acid	0	100	—
	p-Aminobenzoic acid	50	50	50
	p-Aminobenzoic acid	100	7	93
5	Sulphanilamide	0	100	—
	Sulphanilamide	50	69	31
	Sulphanilamide	100	47	53
6	PABGA*	0	105	—
	PABGA*	50	103	Nil
	PABGA*	100	104	Nil
7	6 FP**	0	108	—
	6 FP**	50	107	Nil
	6 FP**	100	111	Nil
	Mixtures			
8	6 FP + PABGA	50 + 0	94	—
	6 FP + PABGA	50 + 50	72	23
	6 FP + PABGA	50 + 100	42	56
	6 FP + PABGA	100 + 50	59	37
9	6 FP + L-glutamic acid	50 + 0	95	—
	6 FP + L-glutamic acid	50 + 25	83	13
	6 FP + L-glutamic acid	50 + 50	76	20
10	Products of reaction (expressed in terms of μg of folic acid degraded)	0	102	—
		147	54	47

* PABGA = *p*-Aminobenzoylglutamic acid.
** 6 FP = 2-Amino-4-hydroxy-6-formylpteridine.

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The three pteridine derivatives: A-methopterin, folinic acid and xanthopterin produced more than 50% inhibition of folic acid degradation, when 100 μg of the derivative was added. Results with *p*-aminobenzoglutamic acid, sulphanilamide and *p*-aminobenzoic acid indicate that the substitution in the carboxyl group of the aromatic amine reduces the inhibitory effect. It is to be noted that since the aromatic amines reacted with the BRATTON AND MARSHALL reagents, it was necessary to include controls containing all reactants except the enzyme, in experiments in which these derivatives were employed. The enzymic degradation was then found by difference.

It is also evident from these results that *p*-aminobenzoylglutamic acid and 2-amino-4-hydroxy-6-formyl pteridine — major products of the enzymic degradation of folic acid by F_2[1]—produced no effect on the system when added individually, but mixtures of the two components in varying proportions were found to be inhibitory. In accordance with these observations, the products of the enzymic reaction mixture also inhibited folic acid cleavage by the system under study.

Table VII illustrates the results of experiments in which varying concentrations of folic acid were incubated overnight with the enzyme system. Even though the incubation period was prolonged, the degradation of folic acid was found to be incomplete and the percentage degraded decreased with increasing concentrations of the substrate. A possible explanation for these findings may be that the products of enzyme reaction which inhibit the system compete with the substrate for the active centres of the enzyme.

TABLE VII

PERCENTAGE OF THE DEGRADATION OF FOLIC ACID IN RELATION TO SUBSTRATE CONCENTRATION

0.3 ml F_2 containing 30 μg protein incubated in the system given in Fig. 1 with various amounts of folic acid. Temp. = 37°C. Time of incubation = 17 h. Results have been corrected for the non-enzymic blank.

μg folic acid added	*μg folic degraded*	*% degradation*
100	65	65
600	350	58
900	364	40

DISCUSSION

Experimental results presented in this communication provide evidence showing that like many of the proteases, the biological system degrading folic acid in blood, exists in an inert state. The enzyme activity could be detected only after the proenzyme had been activated by heat treatment or by acid extraction. Extracts obtained by heat or acid treatment required the same cofactors and showed similar instability towards heat. 80% of the activity was lost on heating aliquots of either type of extract for 5 min at 70°C.

The ratio of folic acid degradation produced by the acid-treated and heat-treated enzyme extracts was 48.0, on the basis of enzyme activity obtained from 1 ml of blood. It appears from these findings that in the course of the activation produced by heating, considerable portion of the active form of the enzyme is lost presumably by simultaneous heat inactivation. This explanation is in agreement with the observations

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given in Fig. 1a (II) which show that most of the activity is lost by increasing the heating time to 15 min.

The presence of the inert form of the enzyme appears to be particular to the system in blood. Biological systems responsible for similar degradation of folic acid in liver and kidney[9,4] apparently exist in the free state, and their activity in these tissues can be demonstrated in homogenates prepared in aqueous solutions.

Observations reported in [1] have shown that F_2 obtained by precipitation with 55–70% ammonium sulphate was active when supplemented with a heated extract and another factor S, which separated in the supernatant obtained by 100% saturation with ammonium sulphate. The latter factor lost activity on prolonged dialysis, it was stable to 100°C for 30 min but was inactivated on ashing, indicating that it was not a metal ion or a protein. Blood cells were also shown to contain protein-like inhibitors which separated from the active material in the fraction precipitated by 0–55% ammonium sulphate (F_1). It was observed that F_1 depressed the activity of F_2 only when the cofactors were supplied by the heated extract and S, but not when ATP, Mn^{++} and glutathione were employed as cofactors. These findings suggested that the coenzyme complex present in blood may not be identical with the cofactors employed in the reconstituted system. The properties of the enzyme system degrading folic acid reported in the present communication have been investigated using F_2 supplemented with ATP, Mn^{++} and glutathione, since the latter system could efficiently replace the cofactors supplied by the heated extract and S.

Investigations relating to specificity indicate that the enzymes degrading folic acid in different tissues may not be identical. Aminopterin was cleaved by the system in blood, although not so actively as folic acid, whereas experiments with homogenates[9] and extracts from acetone-dried powders[4] have shown that rat liver enzymes cannot attack aminopterin. The latter derivative was a strong inhibitor of folic acid degradation by the liver enzymes. Folinic acid, which is one of the biologically active derivatives of folic acid, was not reacted upon by the system investigated. It is therefore improbable that the degradation of folic acid in blood proceeds through the formation of folinic acid. Such a mechanism has been suggested by DINNING and associates[10] to be operating in liver slices. These results, in addition to the earlier demonstration of an enzyme in horse liver[11] which specifically attacks folinic acid but has no effect on folic acid, rather indicate, that cleavage of folic and folinic acids in animal tissues is mediated by independent enzyme systems.

Among the various metal ions tested, only Co^{++} was able to activate the system when present in catalytic amounts and in addition to Mn^{++}. It could not, however, replace the latter as an essential cofactor.

The inhibitory effect of vitamin C on this enzyme system was particularly interesting, as on the basis of these results it is possible that vitamin C aids the biological conversion of folic to folinic acid[6] by protecting the substrate from destruction by tissue enzymes.

It was noteworthy that neither 2-amino-4-hydroxy-6-formyl pteridine nor *p*-amino-benzoylglutamic acid had any action on folic acid degradation, but combinations of these two major products of reaction[1] were definitely inhibitory. Although the evidence available is inadequate to explain this phenomenon, one may recall in this connection, the observations by ELION *et al.*[12] according to which many of the hydroxypteridines become strong inhibitors of the growth of *Lactobacillus casei* in the presence of

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p-aminobenzoylglutamic acid, even though they were inactive in its absence. The inactivation produced by combinations of the two compounds in the present system is, however, in accordance with the finding that addition of the products of enzymic reaction mixture inhibited the forward reaction.

ACKNOWLEDGEMENTS

We thank Dr. V. R. KHANOLKAR, the Director of the Institute for the facilities made available to us during the course of this work, and the Lederle Laboratories Division, for generous gifts of several folic acid derivatives.

SUMMARY

1. Haemolysed cells which can bring about only slight degradation of folic acid, can be activated by heat treatment for ten minutes at 100°C. The active form of the enzyme liberated by heat treatment has the same cofactor requirements as the ammonium sulphate fraction F_2.

2. Among the various ions tested for their effect on F_2, Co^{++} activated the system. Cyanide and azide, were strong inhibitors. Mg^{++} and Ca^{++} were without effect. None of the metal ions tested could replace Mn^{++} as an essential cofactor.

3. Vitamin C was found to be a potent inhibitor of the system and its effect could be partially reversed by increasing the concentration of glutathione. It is therefore suggested that vitamin C exerts its inhibitory effect by inactivating reduced glutathione.

4. Experiments relating to substrate specificity have shown that aminopterin can be degraded by the enzyme system in blood. A-methopterin and folinic acid are not attacked.

5. Among the various closely related derivatives tested for their effect on folic acid degradation: A-methopterin, folinic acid, xanthopterin, *p*-aminobenzoic acid, and sulphanilamide inhibited the system, *p*-aminobenzoylglutamic acid and 2-amino-4-hydroxy-6-formyl-pteridine when added individually were ineffective, but mixtures of the two derivatives were inhibitory. In conformity with this fact, the products of enzymic reaction also inhibited the folic acid degradation in the present system.

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Received July 11th, 1957