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Flavin Requirement and Partial Separation of Enzymes Catalysing the Reduction of Folic Acid to Tetrahydrofolic Acid in Liver

By U. W. KENKARE AND BEATRIZ M. BRAGANCA

Enzyme Chemistry Department, Indian Cancer Research Centre, Bombay 12, India

(Received 26 February 1962)

The conversion of folic acid into tetrahydrofolic acid by liver enzymes is mediated by NADPH₂ (Futtermann, 1957). Many of the enzymes reoxidizing reduced nicotinamide dinucleotides are flavo-proteins (Mahler, 1956), and recent observations (Braganca & Kenkare, 1959) have indicated that flavins are also components of the system transforming folic acid into tetrahydrofolic acid in mammalian liver.

The question whether more than one enzyme protein participates in the reduction of folic acid to tetrahydrofolic acid is still controversial. Earlier observations (Futtermann, 1957; Osborn & Huenekens, 1958; Peters & Greenberg, 1958) suggested that the reduction of folic acid to tetrahydrofolic acid in chicken liver is mediated by two different enzymes catalysing the reduction of folic acid and dihydrofolic acid respectively [5,6,7,8-tetrahydrofolate-NAD(P) oxidoreductase, EC 1.5.1.3, and 7,8-dihydrofolate-NADP oxidoreductase, EC 1.5.1.4]. Recently the failure to separate the two component activities in chicken-liver preparations and the similarity in some of the properties of the systems reducing folic acid and dihydrofolic acid have led Zakrzewski & Nichol (1960) to claim that a single enzyme catalyses the reduction of both these substrates.

We now describe methods for the separation of the enzyme system converting folic acid into dihydrofolic acid from that which reduces dihydrofolic acid. Studies on the selective effects of flavins and flavin analogues also conform to the view that the two-stage conversion of folic acid into tetrahydrofolic acid requires two separate

enzymes. One of these steps may be mediated by a flavoprotein in which FMN or FAD can function as the flavin moiety.

MATERIALS AND METHODS

Isocitrate dehydrogenase from pig heart. The procedure of Ochoa (1955) was followed up to the end of the first stage of purification.

Acetone-dried powder of sheep liver. This was prepared according to the method of Kaplan & Lipmann (1948).

Assay of enzymes reducing folic acid and dihydrofolic acid

Diazotizable-amine method. This is described by Futtermann & Silverman (1957). It depends on the fact that solutions of tetrahydrofolic acid are unstable and quantitatively liberate *p*-aminobenzoylglutamic acid in the presence of air. This is estimated, as aromatic amine, by the method of Bratton & Marshall (1939).

Spectrophotometric method. NADP, sodium citrate, phosphate buffer, pH 5.5, and an extract containing isocitrate dehydrogenase were added to a 1 cm. cuvette in a Beckman spectrophotometer, model DU. The total volume was 2.7 ml. The reaction was allowed to proceed at 24° until about 70% of the NADP was reduced (ΔE_{340} approx. 0.26). At this stage 0.3 ml. of a mixture of CaCl₂ and ascorbic acid was added. The CaCl₂ trapped the excess of citrate and so prevented further reduction of NADP; ascorbate prevented the spontaneous decomposition of tetrahydro- and dihydro-folic acids (Futtermann & Silverman, 1957; Futtermann, 1957). During the following 5 min. E_{340} remained constant. The substrate (folic acid or dihydrofolic acid) was then added, when a rapid fall in E_{340} ensued. A blank cuvette was set up containing everything except NADP. The concentrations of the components in the reaction mixture are given in Table 1. Enzyme activities

were calculated from the final rapid decrease in E_{340} on the basis of an ϵ_{340} value of 6220 for NADPH₂ (Horecker & Kornberg, 1948).

Units of enzyme activity. The unit in the system reducing folic acid to tetrahydrofolic acid is defined as the amount of enzyme which can bring about the reduction of 1 μ mole of folic acid (estimated as *p*-aminobenzoylglutamic acid) in 1 hr. under the specified conditions. The unit for preparations with dihydrofolic acid as substrate as well as for measuring the conversion of folic acid into dihydrofolic acid is defined as the amount of enzyme which causes a rate of change in E_{340} of 0.100/15 min. Specific activity in either case is defined as the number of units/mg. of protein, the latter being estimated by the method of Kalekar (1947).

Partial purification of the enzyme system reducing folic acid to 5,6,7,8-tetrahydrofolic acid (fraction A)

Extraction with phosphate buffer, pH 5.5. The initial extract was prepared by stirring 40 g. of sheep-liver powder for 30 min. in 400 ml. of 0.15M-phosphate buffer, pH 5.5. The mixture was centrifuged 2 hr. later and the supernatant solution was dialysed for 20 hr. against 12 l. of water in two lots of 6 l. each.

Fractionation with ammonium sulphate. The dialysed extract (320 ml.) was treated with 112 g. of (NH₄)₂SO₄ to give 50% saturation, and the precipitate which had formed after 30 min. was removed by centrifuging. The supernatant fluid was treated with 56 g. of (NH₄)₂SO₄ to make it 75% saturated. The precipitate which formed after 30 min. was separated by centrifuging, dissolved in a minimum quantity of water and dialysed as above. The dialysed material (80 ml.) generally contained a small amount of inactive residue which was centrifuged off.

Removal of inert proteins with calcium phosphate gel. The active material was adjusted to pH 5.2 with 0.2M-acetic acid, and Ca₃(PO₄)₂ (900 mg. dry wt.) (Keilin & Hartree 1938) was added. The mixture was stirred for 5 min. and the gel was removed by centrifuging and discarded.

First fractionation with calcium phosphate gel. The supernatant solution was adjusted to pH 5.2 with 0.2M-acetic acid, treated with Ca₃(PO₄)₂ (1350 mg. dry wt.) for 5 min. and centrifuged. The active protein fraction was then eluted from the gel by stirring it with 10 ml. of 0.1M-phosphate buffer, pH 7.4, and centrifuging. This procedure was repeated thrice. The eluates were pooled and dialysed for 20 hr. against 6 l. of cold water (gel eluate no. 1).

Second fractionation with calcium phosphate gel. The dialysed eluate (50 ml.) was adjusted to pH 5.2 with 0.2M-acetic acid, Ca₃(PO₄)₂ (225 mg. dry wt.) was added and the mixture was centrifuged after 30 min. The adsorbed material was eluted from the gel with three portions (3 ml.) of 0.1M-phosphate buffer, pH 7.4. The eluates were pooled and dialysed for 20 hr. against 6 l. of cold water. This extract (fraction A) was brownish yellow in colour. It was stable for 1 month at -15°, but half the activity was lost after 1 week at 0°. Repeated freezing and thawing resulted in a progressive loss of activity.

Partial purification of the enzyme system reducing 7,8-dihydrofolic acid to 5,6,7,8-tetrahydrofolic acid (fraction B)

The phosphate buffer extract of sheep-liver powder (see the preparation of fraction A) was fractionated with

(NH₄)₂SO₄ and the material precipitating between 70 and 100% saturation was collected. This precipitate was dialysed and treated with Ca₃(PO₄)₂ by the same procedure as that described for the preparation of fraction A. The quantities of Ca₃(PO₄)₂ (mg. dry wt.) employed at different stages in the preparation were as follows: 112 mg. for the removal of inert protein, 560 mg. for the first fractionation and 112 mg. for the second fractionation. The dialysed product (fraction B) was colourless. It was stable for at least 2 months if stored at -15°. Repeated freezing and thawing or storage at 0° led to loss of activity in a few days.

Partial purification of the enzyme system reducing folic acid to 7,8-dihydrofolic acid (fraction C)

Extraction with phosphate buffer, pH 7.4. Sheep-liver powder (20 g.) was extracted with 200 ml. of 0.1M-phosphate buffer, pH 7.4. The mixture was centrifuged after 2 hr. and the supernatant fluid was dialysed for 20 hr. against 12 l. of cold water in two lots of 6 l. each.

Fractionation with ammonium sulphate. The fraction precipitating between 30 and 50% saturation with (NH₄)₂SO₄ was dissolved in water at 0° to give a volume of 25 ml.

Treatment with ammonium sulphate at pH 1. The solution was cooled in a bath to -5°, treated with 8.75 g. of (NH₄)₂SO₄ (50% saturation) and adjusted to pH 1 by the gradual addition of 2.5 ml. of cold 2N-HCl. The suspension was stirred for 10 min. and centrifuged. The precipitate was treated with sufficient 0.1M-phosphate buffer, pH 7.4, to bring the pH to 5.5. Insoluble material was removed by centrifuging and the supernatant fluid was dialysed for 20 hr. against 6 l. of cold water. The dialysed solution was stable for 3 weeks at -15°.

Chemicals

Dihydrofolic acid was prepared according to the method of Futterman (1957). Folic acid, NADP and FAD were products of Nutritional Biochemicals Corp., FMN was from L. Light and Co. Ltd., riboflavin from E. Merck, atebirin from Bayer and aminopterin from Lederle Laboratories.

RESULTS

Reduction of folic acid to 5,6,7,8-tetrahydrofolic acid

Table 1 shows the activity at different stages of purification of the enzyme system converting folic acid into tetrahydrofolic acid in sheep liver. The specific activity of the final fraction A, determined with either folic acid or dihydrofolic acid as the substrate, was 20 times that of the crude extract. The activity of the enzyme component reducing dihydrofolic acid paralleled that of the overall conversion of folic acid into tetrahydrofolic acid through most stages of purification. The final product was devoid of isocitrate-dehydrogenase activity which was considerable in the starting material. The optimum pH was 5.0-5.8 (Table 3).

The system converting folic acid into tetrahydrofolic acid was not inhibited by cyanide, azide, EDTA or *p*-chloromercuribenzoate, which suggests that neither metal ions nor thiol groups are essential for activity.

Table 1. Fractionation of sheep-liver extract leading to fractions A and B

At each step activities were measured in terms of (1) reduction of folic acid to 5,6,7,8-tetrahydrofolic acid and (2) reduction of 7,8-dihydrofolic acid to 5,6,7,8-tetrahydrofolic acid. Reaction (1) was followed by determination of diazotizable amine. The system consisted of 0.225 μ mole of folic acid, 125 μ moles of phosphate buffer, pH 5.5, 0.02 μ mole of NADP, 2 μ moles of sodium citrate, pig-heart isocitrate dehydrogenase (5 mg. of protein) and 1 ml. of enzyme extract. The total volume was 2 ml. Incubation was for 60 min. at 37°. Reaction (2) was studied by the spectrophotometric method. The system consisted of 0.1 μ mole of dihydrofolic acid, 15 μ moles of phosphate buffer, pH 5.5, 0.16 μ mole of NADP, 2 μ moles of sodium citrate, 125 μ moles of CaCl₂, 57 μ moles of ascorbic acid, pig-heart isocitrate dehydrogenase (1 mg. of protein) and 0.1 ml. of enzyme extract. The total volume was 3 ml. Incubation was for 15 min. at 24°.

Step	Vol. (ml.)	Protein (mg./ml.)	Substrate			Folic acid			Dihydrofolic acid			Sp. activity with folic acid	
			Total units	Yield (%)	Specific activity	Total units	Yield (%)	Specific activity	Total units	Yield (%)	Specific activity	Sp. activity with dihydrofolic acid	Sp. activity with dihydrofolic acid
(a) Phosphate (pH 5.5) extract	320	21.8	15 616	100.0	2.2	4800	100.0	0.7	4800	100.0	0.7	3.3	3.3
(b) 50-75% saturated (NH ₄) ₂ SO ₄ fraction	80	28.5	8816	56.0	3.9	1800	38.0	0.8	1800	38.0	0.8	4.9	4.9
(c) Gel eluate no. 1	50	5.7	3825	24.5	13.4	975	20.3	3.4	975	20.3	3.4	3.9	3.9
(d) Fraction A	15	2.0	1350	9.0	45.0	405	8.4	13.5	405	8.4	13.5	3.3	3.3
(a) Phosphate (pH 5.5) extract	320	22.0	16 544	100.0	2.4	4800	100.0	0.7	4800	100.0	0.7	3.5	3.5
(b) 70-100% saturated (NH ₄) ₂ SO ₄ fraction	112	10.0	2766	16.7	2.5	840	17.5	0.8	840	17.5	0.8	3.3	3.3
(c) Gel eluate no. 1	42	5.0	659	3.9	3.1	441	9.2	2.1	441	9.2	2.1	1.5	1.5
(d) Fraction B	25	1.2	95	0.6	3.2	262	5.5	8.8	262	5.5	8.8	0.4	0.4

Table 2. Fractionation of sheep-liver extract leading to fraction C

The reduction of folic acid was determined by both the spectrophotometric and diazotizable-amine methods. The reduction of dihydrofolic acid to tetrahydrofolic acid was determined by the spectrophotometric method. In each case the assay systems were as in Table 1.

	Vol. (ml.)	Protein (mg./ml.)	Substrate			Spectrophotometric method			Diazotizable-amine method			Dihydrofolic acid		
			Total units	Yield (%)	Specific activity	Total units	Yield (%)	Specific activity	Total units	Yield (%)	Specific activity	Total units	Yield (%)	Specific activity
(a) Phosphate (pH 7.4) extract	180	18	306	100.0	0.10	5256	100.0	1.6	2700	100.0	0.83	2700	100.0	0.83
(b) 30-50% saturated (NH ₄) ₂ SO ₄ fraction	25	22	105	34.3	0.19	562.5	10.6	1.0	225	8.3	0.41	225	8.3	0.41
(c) Fraction C	23	8	75.9	24.8	0.41	0.0	0.0	0.0	50.6	1.9	0.28	50.6	1.9	0.28
(d) Fraction C assayed in the presence of 66 μ M-FMN	—	—	103.5	33.8	0.56	0.0	0.0	0.0	48.3	1.7	0.26	48.3	1.7	0.26

Table 3. *Effect of pH on the activity of folate reductases*

Activity was determined by the spectrophotometric method (Table 1). The buffers employed in the cuvettes were as follows: pH 4, glycine-HCl buffer (15 μ moles); pH 4.5-5.2, acetate buffer (15 μ moles); pH 5.5-6.5, phosphate buffer (15 μ moles); pH 7.0-8.0, tris buffer (15 μ moles). The activity of fraction C was determined in the presence of 66 μ M-FMN.

Enzyme fraction	Total protein (μ g.)	Substrate	pH										
			4.0	4.5	5.0	5.2	5.5	5.8	6.0	6.5	7.0	7.4	8.0
			$10^3 \times \Delta E_{340}/15 \text{ min.}$										
A	200	Folic acid	31	48	60	62	64	59	53	22	13	—	—
B	120	Dihydrofolic acid	—	37	90	—	136	—	144	147	148	146	82
C	800	Folic acid	26	35	41	42	42	39	36	19	10	—	—

Reduction of 7,8-dihydrofolic acid to 5,6,7,8-tetrahydrofolic acid

During the preparation of fraction B there was a progressive increase of the activity with dihydrofolic acid as substrate relative to that with folic acid (Table 1). Thus with dihydrofolic acid fraction B was 13 times as active as the starting material whereas the activity with folic acid remained almost constant. The pH-activity curve for the reduction of dihydrofolic acid showed a broad optimum in the range pH 5.5-7.4 (Table 3).

Reduction of folic acid to 7,8-dihydrofolic acid

During these studies it became apparent that at low pH dihydrofolate reductase was considerably inactivated whereas re-oxidation of NADPH₂ in the presence of folic acid could still be detected. The greater stability of folate reductase in acid was utilized to separate this component from the one reducing dihydrofolic acid to tetrahydrofolic acid. Fraction C catalysed the oxidation of NADPH₂ in the presence of folic acid but *p*-aminobenzoyl-glutamic acid was not liberated (Table 2). Under our experimental conditions liberation of the aromatic amine is quantitatively related to the formation of tetrahydrofolic acid (Futterman & Silverman, 1957), whereas dihydrofolic acid is more stable and does not give rise to the amine in significant amounts. Thus by determining the activity in the presence of folic acid by the two procedures it was possible to infer that fraction C was enriched with respect to the enzyme system converting folic acid into dihydrofolic acid. During the course of fractionation, the specific activity measured in the presence of folic acid by the reoxidation of NADPH₂ increased fourfold whereas the liberation of diazotizable amine was reduced to nil in the final product. In the presence of dihydrofolic acid, however, the activity of fraction C was 60% lower than that of the initial phosphate extract. Fraction C could be activated by FMN. Apparently the flavin moiety was partially split during treatment with acidified ammonium sulphate, which is known to dissociate the flavin group from flavoproteins.

Table 4. *Liberation of p-aminobenzoylglutamic acid from folic acid in the presence of fractions B and C*

Assay was by the diazotizable-amine method (Table 1). Tests were carried out with 0.8 mg. of C, 0.8 mg. of B, or 0.4 mg. of B plus 0.4 mg. of C.

Enzyme fraction	FMN (μ mole)	<i>p</i> -Aminobenzoyl-glutamic acid formed (μ moles)
C	—	2.5
C	0.2	6.3
B	—	10.1
B	0.2	12.1
B+C	—	43.9
B+C	0.2	67.5

Fig. 1. Effect of aminopterin on folate-reductase activity measured by the spectrophotometric method. The assay system was as in Table 1. O, Fraction A (200 μ g. of protein) and folic acid (0.1 μ mole); ●, fraction B (120 μ g. of protein) and dihydrofolic acid (0.1 μ mole); ▼, fraction C (800 μ g. of protein) and folic acid (0.1 μ mole); ▽, fraction precipitated with 50-75% saturated (NH₄)₂SO₄ (2 mg. of protein) and folic acid (0.1 μ mole).

Table 4 demonstrates the effect of combining fraction B with fraction C on the liberation of aromatic amine. Though the two fractions were relatively inert individually they were able in combination to bring about a considerable conversion of folic acid into tetrahydrofolic acid.

Table 5. *Flavin requirement of folic acid-reducing systems*

Assays were carried out by the spectrophotometric method (Table 1) except that tris buffer, pH 7.4, was employed when dihydrofolic acid was the substrate. Dialysis was against water saturated with CO₂ (see text). Control values are those of untreated fractions in the absence of supplement.

Expt. no.	Supplement	Concn. of supplement (μM)	Folic acid A			Folic acid C			Dihydrofolic acid B		
			Activity		Activity expressed as % of control	Untreated fraction	Activity expressed as % of control	Untreated fraction	Activity		Activity expressed as % of control
			Untreated fraction	Dialysed fraction					Untreated fraction	Dialysed fraction	
1	None	—	0.45	0.30	66	0.35	100	1.74	1.37	79	79
	FMN	66	0.47	0.42	93	0.51	146	1.72	1.38	79	79
	FMN	33	0.45	0.39	87	0.46	131	—	1.38	79	79
	FMN	6.6	0.45	0.35	78	0.45	130	—	1.35	78	78
2	None	—	0.43	0.28	65	0.35	100	1.74	1.37	79	79
	FAD	66	0.43	0.43	100	0.48	137	1.77	1.40	80	80
	FAD	33	0.45	0.40	93	0.47	134	—	1.39	80	80
	FAD	6.6	0.44	0.37	86	0.42	120	—	—	—	—
3	None	—	0.45	—	100	0.46	100	1.89	—	100	100
	Atebrin	100	0.14	—	31	0.18	39	1.85	—	98	98
	Atebrin + FMN	100 } 66 }	0.42	—	93	0.40	87	—	—	—	—
	Atebrin + FAD	100 } 66 }	0.42	—	93	0.43	93	—	—	—	—
4	None	—	0.46	—	100	0.36	100	1.89	—	100	100
	Riboflavin	66	0.29	—	63	0.24	66	1.82	—	96	96
	Riboflavin + FMN	66 } 66 }	0.43	—	93	0.37	103	—	—	—	—

Inhibition by aminopterin of the enzymes converting folic acid into 5,6,7,8-tetrahydrofolic acid

Aminopterin is known to inhibit non-competitively the conversion of folic acid into tetrahydrofolic acid (Futtermann & Silverman, 1957) as well as the component reducing dihydrofolic acid to tetrahydrofolic acid (Osborn, Freeman & Huennekens, 1958). Fig. 1 demonstrates inhibitions by aminopterin of the component activities reducing folic acid and dihydrofolic acid. As little as $10^{-2}\mu\text{M}$ -aminopterin inactivated by more than 50% the overall conversion of folic acid into tetrahydrofolic acid by fraction A, as well as the reduction of dihydrofolic acid to tetrahydrofolic acid by fraction B. On the other hand, the reduction of folic acid to dihydrofolic acid by fraction C was inhibited only 8% by $10^{-2}\mu\text{M}$ -aminopterin. Since fraction C had a much lower specific activity than fraction B, it was necessary to eliminate the possibility of partial removal of aminopterin by non-specific adsorption on proteins. Experiments with crude liver extract containing 2 mg. of protein (curve D) showed that aminopterin caused the same order of inhibition on the overall system in the crude preparation as it did on fraction A, which contained only $200\mu\text{g}$. of protein. Thus it is not likely that the lower inactivation observed with fraction C arises from adsorption of aminopterin by the inert protein present.

Flavins as components of folic acid-reducing systems

Preliminary observations (Braganca & Kenkare, 1959) have indicated that folate reductases in some mammalian livers are flavoproteins in which FMN can function as the flavin moiety. Flavoproteins have been resolved into flavin and apoenzyme by treatment with acidified ammonium sulphate (Negelein & Brömel, 1939) or by dialysis against water saturated with carbon dioxide (Mueller & Miller, 1950). The first method irreversibly inactivated fractions A and B. However, fraction C, which was the product of such treatment, was partially resolved: i.e. an increase in activity of about 45% could be obtained on the addition of $66\mu\text{M}$ -FMN or -FAD. The second, milder procedure inactivated A by 34% and B by 20%. However, only in the former case could reactivation by FMN or FAD be obtained. Fractions A and C, but not B, were inhibited by atebirin or riboflavin, and these inhibitions could also be reversed by FMN or FAD. These effects are illustrated for fraction C in Table 5.

DISCUSSION

By fractionation of sheep-liver extracts we have obtained three types of preparation which catalyse

the reduction of folic acid: A, which reduces folic acid and dihydrofolic acid to tetrahydrofolic acid and shows comparable activities with both substrates; B, which actively reduces dihydrofolic acid to tetrahydrofolic acid but has less activity towards folic acid; C, which is more active in the reduction of folic acid than in the reduction of dihydrofolic acid. Combination of B and C gives a system with the same catalytic properties as A. From these results it may be concluded (i) that the reduction of folic acid to tetrahydrofolic acid in liver extracts occurs in two steps with the intermediate formation of dihydrofolic acid and (ii) that the two steps are catalysed by different enzymes or enzyme systems.

The pH optima of the three fractions were different although, as pointed out by Zakrzewski & Nichol (1960), these differences may be dependent not only on the properties of the enzymes involved but also on the state of ionization of the two substrates. As both folic acid and dihydrofolic acid were reduced at pH 5.5, this pH was chosen for all determinations of activity.

The antimetabolite aminopterin preferentially inactivates the component reducing dihydrofolic acid (fraction B); fraction C was considerably more resistant to inactivation. As reported by Zakrzewski & Nichol (1960), the degree of inhibition produced by aminopterin on the overall system was the same as that observed for the partial reaction: the reduction of dihydrofolic acid to tetrahydrofolic acid. These experiments suggest that the component which reduces dihydrofolic acid is the rate-limiting step in the overall transformation of folic acid into tetrahydrofolic acid.

The flavin nature of the component reducing folic acid to dihydrofolic acid is apparent from the observation that partially resolved preparations of this component could be activated by FMN and FAD. Braganca & Kenkare (1959) used pig heart as the source of FAD, but these preparations apparently contained impurities which inhibited the system; hence the conclusion was drawn that FAD was an inhibitor of the flavoprotein in which FMN functioned as the active moiety. The experiments described here were carried out with pure FAD and have consistently demonstrated that FAD is as effective as FMN in activating the reduction of folic acid to tetrahydrofolic acid as well as in reversing the inhibition produced by atebirin and riboflavin. The enzyme preparations employed in these studies were not sufficiently pure to allow a conclusion as to which of the nucleotides is in fact the prosthetic group. The apoenzyme prepared from the purified nitrate reductase of *Neurospora* is reported to be activated equally by both FMN and FAD (Nicholas & Nason, 1954). The interconversion of FAD and FMN, catalysed by enzymes

present in fraction C, is a possible explanation of the equal activation by both nucleotides. Atebrin and riboflavin inhibit many flavoprotein enzymes (Mahler, 1955). Thus the reversible inhibitions produced by these compounds provide supplementary evidence for the participation of a flavin in the biological reduction of folic acid. No indication could be obtained that fraction B was a flavin enzyme.

SUMMARY

1. A soluble enzyme system which reduces folic acid to tetrahydrofolic acid has been extracted from acetone-dried sheep liver. The component that reduces folic acid to dihydrofolic acid has been partially separated from that which reduces dihydrofolic acid to tetrahydrofolic acid.

2. The step that converts folic acid into dihydrofolic acid is mediated by a flavin enzyme in which either FMN or FAD can function as the active moiety.

3. Aminopterin preferentially inhibits the reduction of dihydrofolic acid to tetrahydrofolic acid.

4. It is concluded (a) that the conversion of folic acid into tetrahydrofolic acid is brought about by a two-step reduction, in which dihydrofolic acid is an intermediate, and (b) that each step is catalysed by a distinct enzyme.

The work was aided by a grant from the International Division, Lady Tata Memorial Trust. We are grateful to Lederle Laboratories Division for a gift of aminopterin.

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Transport and Phosphorylation of Sugars in Adipose Tissue

By A. HERNÁNDEZ AND A. SOLS

Department of Enzymology, Instituto Marañón, Centro de Investigaciones Biológicas, C.S.I.C., Madrid, Spain

(Received 17 July 1962)

A wide interest is now centred on white adipose tissue as a major target of insulin action. The primary effect of insulin appears to be a stimulation of the transformation of extracellular glucose into intracellular glucose 6-phosphate (Jeanrenaud & Renold, 1959), although evidence has also been reported pointing to some further effect on metabolic patterns (Leonards & Landau, 1960). Insulin also stimulates the utilization of mannose and fructose by the epididymal fat pad (Ball & Cooper, 1960; Wood, Leboeuf, Renold & Cahill, 1961). Attempts to ascertain whether a mechanism of

sugar transport occurs in adipose tissue have been hindered by the lack of definite information on the enzyme(s) involved in the phosphorylation of sugars in this tissue (Ball & Cooper, 1960; Wood *et al.* 1961).

The present paper reports the identification of a hexokinase in the adipose tissue of the rat and the study of its substrate specificity. The uptake of metabolizable hexoses by the intact tissue has a substrate specificity different from that of its hexokinase, in a way that indicates the occurrence of a stereospecific transport step before phos-