R. W. Hanson during the preparation of this manuscript are gratefully acknowledged.

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Received September 9, 1965. P.S.E.B.M., 1965, v120.

Uptake of Reduced Folic Acid by Chicken Liver Enzyme. (30675)

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The information whether folic acid in serum occurs free or in a combination with proteins is both meager and controversial. Condit and Grob(1) by using various methods (*e.g.*, electrophoresis and bioautography, paper chromatography, equilibrium dialysis), concluded that folic acid in plasma is not bound to protein, while Johns *et al*(2) using H^3PGA (H^3-pteroylglutamate) found that as much as 64% of PGA in plasma is protein-bound. A highly dissociable protein-bound PGA conjugate was found to occur in yeast(3).

In our laboratory, while investigating the binding by serum of vit. B_{12}(4), which is functionally related to folic acid, a liver extract preparation was found to restore the binding by serum which was inactivated by boiling(5). Experiments were thus designed to investigate whether a similar mechanism operates in relation to PGA. As an outcome of these studies we have found that liver extract, and not serum, is active in binding H^3PGA. This finding led us to investigate further the nature of the uptake. Some of our results are presented here.

Materials and methods. Tritiated pteroylglutamic acid (H^3PGA, specific activity 1 μg/0.263 μC, purchased from the Radiochemical Centre, Amersham, England) was used. Reduced nicotinamide adenine dinucleotide phosphate (NADPH) and its oxidized form (NADP), adenosine triphosphate (ATP) were obtained from the Nutritional Biochemical Corp., Cleveland, Aminopterin from Mann Research Laboratories, New York, Norit A from Fisher Scientific Co., and 2-mercapto-ethanol was obtained from Matheson, Coleman and Bell Co.

The chicken liver cell-free extract was prepared according to Chanarin and Bennett(6). The uptake of H^3PGA was determined in the Packard Tricarb Liquid Scintillation Spectrometer model 314E. A mixture of 2 scintillation solutions was used: a) a dioxane scintillation solution and b) a toluene scintillation solution. Their composition was as follows: a) 2,5 diphenyl-oxazole—7 g, naphthalene—50 g and 1-4-di-[2-(5-phenyloxa-

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zole)]-benzene—50 mg (purchased from Packard Instrument Co. Inc.), dissolved in 1000 ml of p-dioxane; b) 2,5-diphenyloxazole —3 g, 1-4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene—100 mg, and made up to 1000 ml with toluene. For radioactivity measurements the counting vials were filled with 9 ml of (a), 1.5 ml of (b) and 0.5 ml of the samples to be tested. All samples were stored at 8°C for 24 hours before counting.

Reaction mixture. Final volume—0.5 ml; the complete system contained liver extract (about 1.75 mg protein)—0.1 ml, H^3PGA—0.5 mμmole, NADPH—14 mμmoles, 2-mercaptoethanol—40 μmoles, $MnSO_4$—0.4 μmole, ATP—0.5 μmole and citrate-phosphate buffer —50 μmoles.

The reaction mixture was pipetted into small (16 × 50 mm) screw-capped test tubes and incubated in a water bath for 30 to 90 minutes at a temperature which will be specified later. After incubation, 1 ml of 0.5% suspension (in water) of activated charcoal (Norit A) was added and shaken vigorously for 15 minutes. One-half ml of the charcoal supernatant was added to the 10.5 ml of the scintillation mixture. Over 95% of the radioactivity was adsorbed to the charcoal in the controls (no enzyme, or complete system at 0°C) under comparable conditions. Uptake of H^3PGA was defined as the amount of radioactivity in the supernatant after charcoal treatment and centrifugation.

Results. An appreciable uptake of H^3PGA by the chicken liver preparation was found. The uptake was dependent on the presence of NADPH; without NADPH no incorporation of H^3PGA took place. With the enzyme preparation used, omission of ATP, Mn^{++} or 2-mercapto-ethanol reduced the uptake to some extent (Table I). Under the conditions described the uptake was about 40% of the radioactivity introduced. At 0°C the uptake was negligible in comparison to that obtained at the optimal temperature (Fig. 3, see below).

pH optimum. By using citrate-phosphate buffer the optimal pH for uptake was found to be between 4.8 and 5.6 (Fig. 1). In this experiment, controls for each pH value were taken by adding H^3PGA under ice and adsorbing immediately on charcoal and determining the radioactivity in the supernatant. The purpose of these controls was to find out whether different pH values and ionic concentrations have any effect on the adsorption capacity of the charcoal. No difference was found under the experimental conditions employed.

TABLE I. Effect of Omissions of Components on Uptake of H^3PGA by Chicken Liver Extract.

Complete system contained: chicken liver extract 1.75 mg protein; H^3PGA—0.5 mμmole (approx. 12,000 cpm), NADPH—14 mμmoles; 2-mercaptoethanol — 40 μmoles; $MnSO_4 \cdot 7H_2O$ — 0.4 μmole; ATP—0.5 μmole; citrate-phosphate buffer, pH 5.2—50 μmoles. Total volume—0.5 ml. The reaction mixture was incubated at 45°C for 30 min. Thereafter, 1 ml of a 0.5% charcoal (Norit A) was added, shaken vigorously for 15 min, the contents centrifuged for 15 min at 3000 rpm and 0.5 ml aliquot of the supernatant taken for radioactivity counts.

Omissions	Radioactivity (cpm)
None; complete system	4750
NADPH	950
NADPH; added NADP (14 mμmoles)	700
ATP	3890
Mn^{++}	4150
2-mercapto-ethanol	4500
NADPH, Mn^{++}, ATP and mercapto-ethanol	540
Liver extract	540

Effect of time and temperature. In Fig. 2 the effect of time on the uptake of H^3PGA is shown; the reaction reached completion between 30 and 45 minutes; no further increase was found on incubating the reaction mixture for up to 90 minutes.

The optimal temperature was found to be

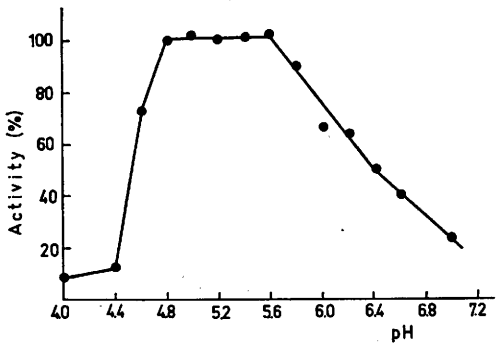
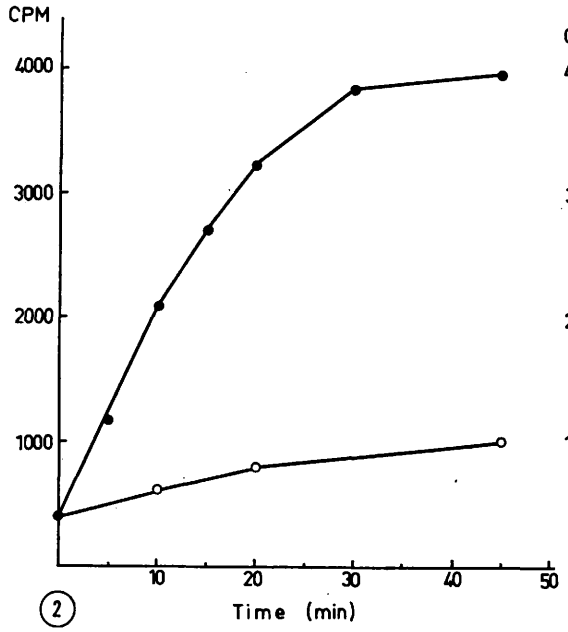
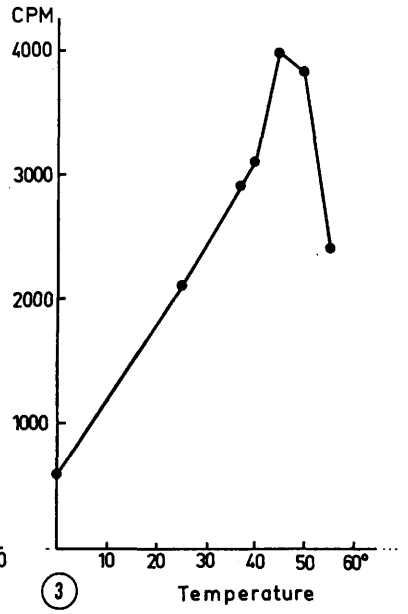


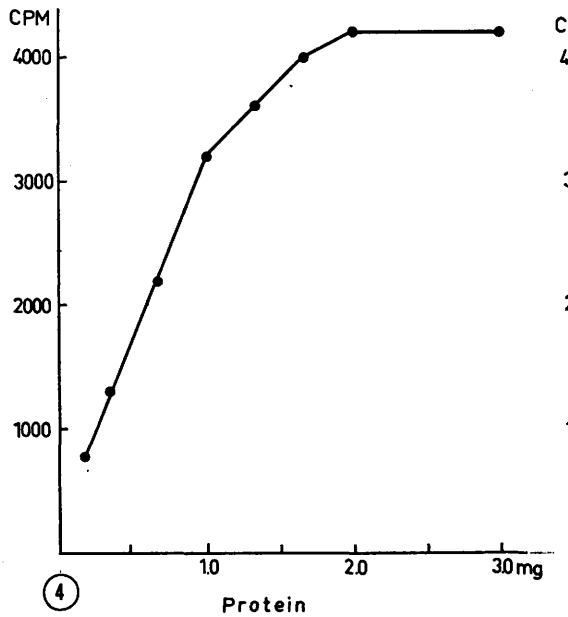
FIG. 1. Uptake of H^3PGA at different pH values; complete system was incubated at 45° for 30 min. Citrate-phosphate buffer, adjusted to the different pH values, was used.

at about 45°C; at 25°C and at 55°C, respectively, the uptake was about 50% of that obtained at 45-50°C (Fig. 3); almost no uptake occurred at 0°C (see also Fig. 2).

Effect of enzyme concentration. The uptake of H^3PGA by different amounts of liver extract is shown in Fig. 4. Under the experimental conditions used (concentration of H^3-







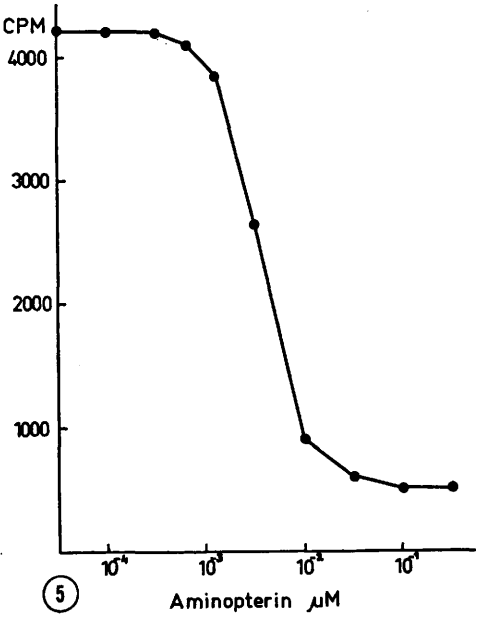


FIG. 2. Uptake of H^3PGA as a function of time. Complete system; pH of reaction mixture—5.2. Open circles—0°C, closed circles—45°C.

FIG. 3. Effect of temperature on H^3PGA uptake. Complete system (pH 5.2) was incubated for 30 min at the temperatures indicated.

FIG. 4. Effect of enzyme concentration on uptake of H^3PGA.

FIG. 5. Inhibition of H^3PGA uptake by increasing concentrations of aminopterin.

PGA) the uptake increased with increasing amounts of protein up to 1 mg and levelled off completely above 2 mg.

Inhibition by aminopterin. The dependence on NADPH suggested that the incorporation of folate involves an enzymatic reduction to the tetrahydrofolate level. To explore this possibility, the effect of aminopterin on the reaction was tested. Aminopterin is known (7-10) to prevent the formation of tetrahydrofolate by inhibiting the enzyme dihydrofolate-reductase. The results are shown in Fig. 5. A very marked inhibition was obtained at very low concentrations of aminopterin; 50% inhibition was obtained by addition of 2×10^{-3} μM and complete inhibition by 2×10^{-2} μM. These results suggest therefore very strongly that PGA is reduced to tetrahydrofolate or to a tetrahydrofolate derivative prior to, or during, the uptake.

Comment. The liver enzyme involved in the uptake of H^3PGA shows a remarkable resemblance to the folate-reductase. This is evident from the cofactors required, pH optimum and the high sensitivity towards inhibition by aminopterin. It remains to be established whether the reduced folate is bound directly to the dihydrofolate-reductase, to a related enzyme, or to a carrier protein that accompanies the enzymic preparation. Further purification of the chicken liver enzyme preparation might help to elucidate this problem. However, it is known that folate-reductase binds aminopterin and other structural analogues of folic acid(7-9). It seems therefore very plausible that tetrahydrofolate or a derivative thereof forms with the enzyme a kind of a complex which is resistant to adsorption by charcoal under the experimental conditions used. Very recently Rothenberg (11,12) has been able to separate quantitatively, by paper chromatography, tetrahydrofolate from PGA.

The main folate derivatives present in animal and human tissues and in blood are reduced forms of folic acid(13-17). If the various reduced folates bind equally well to the chicken liver preparation, as the reduced folate derivative described here, a convenient radio-assay for determination of folates in blood and tissues by isotope dilution could be developed(4). This possibility is being now explored.

Summary. An appreciable uptake of H^3-pteroylglutamate (H^3PGA) by chicken liver cell-free extract was found. The uptake was dependent on NADPH and was slightly stimulated by ATP, manganese ions and mercaptoethanol. Aminopterin at low concentrations (2×10^{-3} μM) inhibited the uptake of H^3PGA. The latter finding indicates the formation of a reduced folate derivative at the tetrahydrofolate level as a prerequisite for uptake. The temperature and pH optima of the reaction were studied. The possibility of utilizing the radio-assay for determination of folates in blood and tissues, by isotopic dilution, is discussed.

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Received September 9, 1965. P.S.E.B.M., 1965, 120.