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THE FATE OF INTRAVENOUSLY INJECTED FOLATE IN RATS

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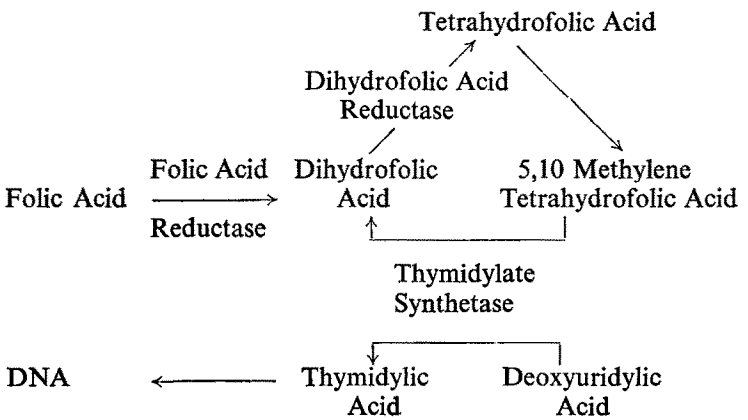
Abstract—The rates of removal of intravenously injected folate, dihydrofolate and tetrahydrofolate from blood serum of rats have been found to be rapid and similar. Over the wide concentration range examined it was found that the proportions of the injected doses of folate and reduced folate retained in the body varied only between 25 and 50 per cent, the remainder being excreted in the urine. 'Flushing' injections of folate or methotrexate were found to be equally effective in removing a small proportion of the retained folate. The suggestion is made that the removal of injected folate from the blood stream into the body is not associated with a specific uptake mechanism nor with folate-utilizing enzymes. It has been found that folate and dihydrofolate are capable of being bound onto serum, but that this binding can be reversed by electrophoresis in a density gradient column. The results could indicate that the removal of folate from the blood is by means of a non-specific binding onto cellular proteins similar to the observed binding to serum.

INTRODUCTION

DERIVATIVES of tetrahydrofolic acid act as carbon donating substances in enzymic carbon transfer reactions. One such carbon transfer, which uses 5–10 methylenetetrahydrofolate, is carried out by thymidylate synthetase[1] during the process of DNA synthesis. The formation of tetrahydrofolate would presumably depend on the activities of folate and dihydrofolate reductases. The reactions, according to present theories, are summarized in the following scheme.



The chemically induced rat breast tumours[2] transplanted into liver in this laboratory have been found to possess barely detectable levels of dihydrofolate reductase, in contrast to the liver tissue itself which is a rich source of the enzyme. The possibility therefore arises that DNA synthesis in these tumours might be dependent upon a supply of reduced folate from the liver. This reduced folate would presumably be transported via the bloodstream. It appeared from the literature that whilst the fate of intravenously injected folate had been examined in various animals,[3, 4] the fate of the reduced folate had received little or no attention. It was desirable therefore to examine and compare the fates of folate and reduced folates introduced into the bloodstream.

MATERIALS AND METHODS

Chemicals

Generally tritiated folic acid, specific activity 154 mc/mM was purchased from the Radiochemical Centre, Amersham, Bucks, England. Commercial folic acid was purified on a DEAE cellulose column essentially by the method of Johns *et al.*[3] and was used as the potassium salt. Methotrexate was a gift from the Lederle Laboratories Ltd., London, W.C.2.

Dihydrofolic acid (FH_2)* was prepared by the reduction of folic acid with dithionite in the presence of ascorbate.[5] The product was washed 4 times with 0·005 M HCl. Tritiated FH_2 was prepared in two ways: (a) By reduction of generally tritiated folic acid; and (b) by reduction of unlabelled folic acid with dithionite in the presence of tritiated water. The former procedure yielded tritiated FH_2 of similar specific activity to the original unreduced folate. The latter procedure in which unlabelled folate was reduced in tritiated water (10 mc/ml), yielded tritiated FH_2, presumably 7–8 tritiated, with a specific activity of 0·02 $\mu c/\mu M$ dihydrofolate. The reason for this low specific activity of the resultant FH_2 which has also been observed by other workers[6] has not been examined in the present study. Omission of the ascorbate from the reaction mixture did not affect the specific activity of the resultant FH_2 and it thus appeared that it did not contribute to the reduction of the folate. The positions of tritiation in the reduced forms of folate are inferred from the structures assigned to these compounds by Rabinowitz.[7] No work has been carried out in the present study to examine the position of tritiation. Recently the structures originally assigned to the products of reduction of folate by dithionite have been questioned,[8] and in view of this uncertainty no account has been taken in the present report of the possibility of tritium exchange.

Tetrahydrofolate (FH_4) was routinely prepared by the following method. Folate (20 mg) was reduced with dithionite as above, and the resultant FH_2 was precipitated and washed in 0·005 M HCl. The yield of FH_2 was 60–70 per cent. The FH_2 was suspended in 2 ml 0·05 M 2-mercaptoethanol and dissolved by addition of solid sodium bicarbonate. Sodium borohydride (10 mg) was added and the solution stirred in an ice bath for 10 min. Complete decolourisation of the solution could be obtained by this method in contrast to direct reduction of the folate with borohydride. The solution was added to the top of a DEAE cellulose column (5 × 1·25 cm) which had previously been washed with tris buffer, 0·005 M, pH 7·5 containing 0·05 M mercaptoethanol and a further 100 ml of the buffer was passed through the column. The FH_4

* FH_2—Dihydrofolic Acid, FH_4—Tetrahydrofolic Acid.

was then eluted with 10 ml of tris buffer (0·5 M, pH 7·5) containing 0·05 M mercaptoethanol. The product was freeze dried and stored under argon in a deep freeze. Yields of 40–50 per cent were obtained.

Tritiated FH_4 was prepared by the following methods:

(1) Generally tritiated folic acid was reduced to FH_2 using dithionite and then to FH_4 using non-labelled borohydride.

(2) 7–8 tritiated FH_4 was prepared from the 7–8 tritiated FH_2 by reduction with non-labelled borohydride.

(3) 5–6 tritiated FH_4 was prepared from unlabelled FH_2 using tritiated sodium borohydride.

(4) 5–6–7–8 tritiated FH_4 was prepared either by reducing 7–8 tritiated FH_2 with tritiated borohydride or by a direct reduction of folate by dithionite in tritiated water at 75° for 90 min.[8] The presence of ascorbate in the latter reaction mixture was found to reduce the retention of the FH_4 on the DEAE-cellulose and a larger column (30 × 1 cm) was used. The FH_4 was eluted by a gradient obtained by adding 0·8 M tris buffer to 1 l 0·005 M tris pH 7·8; all solutions contained 0·05 M 2-mercaptoethanol. 5-ml fractions were collected and the FH_4 was collected in fractions 54–60 The product was freeze dried and stored under argon in a deep freeze.

Assay of radioactivity

Radioactivity assays were carried out using a liquid scintillation counter (Isotope Developments Ltd., Reading, Berks, England, Type 6012). The efficiency for tritium was 19 per cent. Liquid scintillators were obtained from Nuclear Enterprises (G.B.) Ltd., Edinburgh, Scotland. Liquid scintillator designated NE 220 was used for water soluble samples and NE 213 for organic soluble samples after solution in dimethylformamide. Internal standards of tritiated folate were always used. NE 210 scintillator gel was also used for samples which precipitated in the other two scintillator liquids.

Uptake of radioactive folate from serum

0·5 ml of the folate solutions, in physiological saline, were injected into the jugular vein. Solutions of reduced forms of folate also contained 0·05 M ascorbate. To determine the removal of folate at various time intervals rats were killed, the heparinised blood was collected and the serum collected by centrifugation. The levels of serum radioactivity were then estimated directly and no attempt was made to isolate the folate from the serum. An average total blood serum value of 7·5 ml has been assumed in calculating the results.

Assay of excreted radioactivity

After intravenous injections of tritiated folic acid, rats were placed on peforated plates in large glass funnels. Urine samples were collected over certain periods, the urine made up to a standard volume, and radioactivity was assayed directly.

Animals

Male Sprague-Dawley rats weighing 280–300 gm were used throughout. Small differences in body weights were not taken into account when injections were made.

Assay of binding of folate or methotrexate on to serum

5 ml of serum plus aliquots of folate or methotrexate were placed in dialysis bags, and the bags placed on sintered glass discs supported on rubber rings approximately 1 in. above the bottom of 50 ml centrifuge tubes. The tubes were spun at 500 *g* and 4° until approximately 0·25 ml of solution had collected in the bottom of the tube (1–2 hr). The folate or methotrexate content of the dialysate was then determined by radiochemical or spectrophotometric means and compared with controls in which serum was replaced with 0·005 M tris buffer (pH 7·5) in the dialysis bags. In parallel experiments the solutions were filtered under pressure through membrane filters. ('Ultracella' type, Membranfiltergesellschaft). Similar experiments were carried out in which 2·5 ml aliquot of serum was buffered to pH = 8·6 with 2·5 ml 0·05 M barbitone buffer.

Zonal density gradient electrophoresis studies

These were carried out in an apparatus designed and constructed by Dr. D. M. Robertson of this Laboratory. A density gradient range of 0–50 per cent sucrose was used. The buffer used was 0·025 M barbitone (pH 8·6).

FH_2 reductase

This was prepared from normal rat liver homogenised in 0·05 M tris HCl (pH 7·8). The protein was fractionated with ammonium sulphate and the 40–80 per cent saturation fraction was used after dialysis against distilled water for 24 hr in the cold.

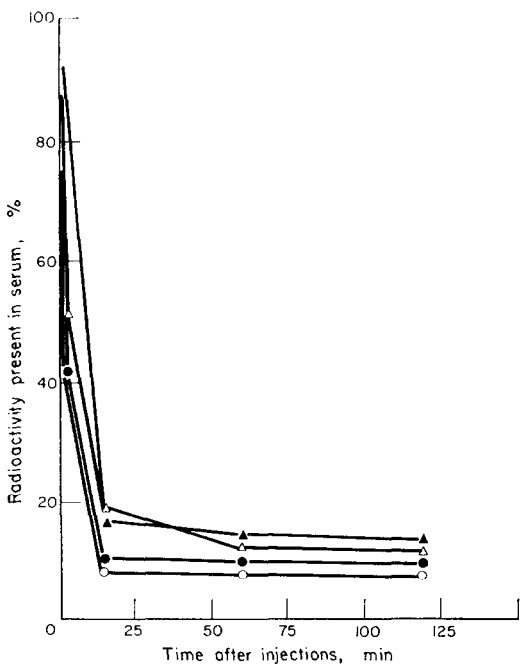


FIG. 1. The levels of radioactivity in the serum after intravenous injections of various amounts of tritiated folic acid. ●———● 0·42 μM folic acid injected, ○———○ 0·73 μM, ▲———▲ 1·1 μM, △———△ 1·8 μM.

RESULTS

Disappearance of injected folate from serum

When tritiated folate was injected intravenously into rats it was found that the radioactivity rapidly disappeared from the serum (Fig. 1). After 15 min more than 80 per cent of the injected dose had been removed. Similar results were obtained over a range of injected doses of 0·42–2·5 μM. Irrespective of the actual amounts of folate injected in this range, only 10–15 per cent of the injected doses remained in the serum after two hr and it was found that this percentage fell only slowly during the following 24 hr. The extreme rapidity of the disappearance is indicated in Fig. 1 in the case of the 0·42 and 1·8 μM doses, in which blood samples were removed 2 min after the injections were made. In this time 58 and 46 per cent respectively of the injected doses had been removed from the serum.

A comparison of the rates of disappearance of folate and reduced forms of folate from the serum was made (Fig. 2). The overall pattern of uptake of FH_2 andFH_4

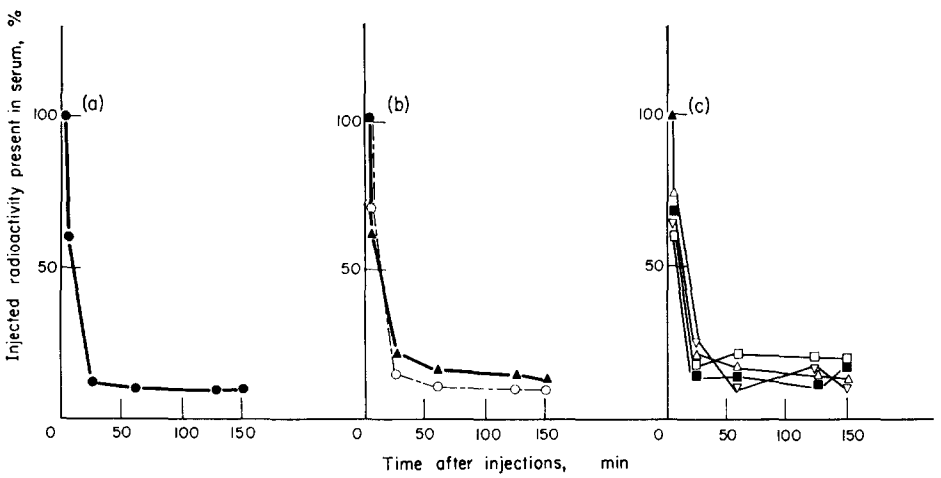


FIG. 2. The levels of radioactivity in the serum after intravenous injections of 1·2 μM amounts of various tritiated folates.

2 (a) ●——● tritiated folic acid

2 (b) ○——○ FH_2 prepared from tritiated folic acid. ▲——▲ 7–8 tritiated FH_2

2 (c) △——△ FH_4 prepared from tritiated folic acid. □——□ 7–8 tritiated FH_4 ■——■ 5–6 tritiated FH_4. △——△ 5–6–7–8- tritiated FH_4

from the serum appeared to be similar to that of folate. An initial very rapid uptake was followed by a prolonged period of gradual removal of the injected radioactivity from the serum.

Excretion of radioactivity following injection of tritiated folate

Rats were injected intravenously with various doses of tritiated folate and placed in the glass tanks for collection of urine. Urine samples collected after the 18 hr experimental period contained radioactivity only slightly above the background count. It appears from Table 1 that large percentages of the injected doses of radioactivity

are excreted even at the lowest dose level employed. FH_2 and FH_4 (obtained by reduction of generally tritiated folic acid) yielded similar results. The effect of 'flushing' doses of methotrexate or folate on the excretion of injected folate was examined and the results are given in Table 2.

TABLE 1. EXCRETION OF RADIOACTIVITY FOLLOWING INTRAVENOUS INJECTIONS OF TRITIATED FOLIC ACID*

Amount of folate injected (mμM)	Radioactivity injected (μC)	% of injected radioactivity excreted 0–5 hr after injections	% of injected radioactivity excreted 5–18 hr after injections	Amount of injected folate retained after 18 hr (mμM)
50	5	44	9	24
100	5	37	9	54
500	5	63	7	150
2,500	5	69	6	625

* (Mean of 4 experiments using 1 rat at each dose level. Results ± 15 per cent) Chromatography on DEAE-cellulose showed that > 80% of the radioactivity present in all samples of urine was in the form of folic acid.

TABLE 2. EFFECT OF 'FLUSHING' INJECTIONS OF FOLATE OR METHOTREXATE ON THE EXCRETION OF RADIOACTIVITY FOLLOWING INJECTION OF TRITIATED FOLIC ACID*

Amount of folate in initial injection (mμM)	% of injected radioactivity excreted in subsequent 5 hr	Nature of injection made 5 hr after folate injection	% of injected radioactivity excreted during period 0–13 hr after 2nd injections	Amount of radioactivity retained 18 hr after initial injection (mμM)
50	43	Saline	9	24
50	50	6,000 mμM folate	21	14
50	46	6,000 mμM methotrexate	20	17

* (Mean of 3 experiments. Results ± 10 per cent)

TABLE 3. RADIOACTIVE CONTENT OF VARIOUS BODY ORGANS 24 HOURS AFTER INTRAVENOUS INJECTION OF 0·1 μM TRITIATED FOLATE

	% of injected radioactivity
Liver	5
Kidney	6
Spleen	8
Testes	3
Duodenum	7
Erythrocytes	<1

Fate of absorbed folate in the body

0·1 μM of folate containing 5 μc was injected into a rat and the animal was killed 24 hr later. The radioactive content of various body organs was determined. The results are given in Table 3.

Effect of methotrexate and partial hepatectomy on the rate of removal of injected folate from the serum

Eight rats were partially hepatectomised, 8 animals acting as controls and 2 hr after the operation, 4 of the partial hepatectomised and 4 of the control animals received intravenous injections of 1 mg of methotrexate in a volume of 0·5 ml. The remaining animals received intravenous injections of 0·5 ml of physiological saline. 24 hr later 1·1 μM of tritiated folate was injected intravenously and the rate of disappearance of radioactivity from the serum determined (Fig. 3). It did not appear that

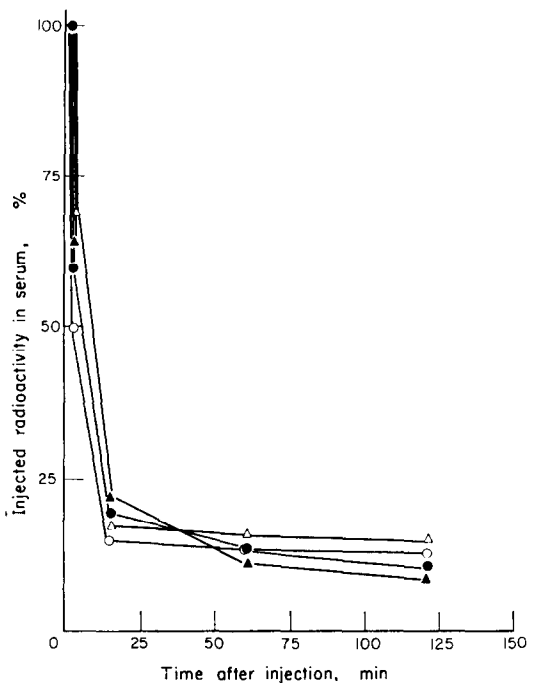


FIG. 3. The effect of partial-hepatectomy and methotrexate injection on the removal of injected folate from the serum. 1·1 μM tritiated folate injected into each rat. ●———● control animals. Non-hepatectomised. Saline injection. ○———○ Non-hepatectomised. 1 mg methotrexate injected. △———△ Partially-hepatectomised. Saline injection. ▲———▲ Partially-hepatectomised. 1 mg methotrexate injected.

either methotrexate or partial hepatectomy or both had any effect on the rapid rate of removal of folate from the serum. Similar results were obtained using FH_2 and FH_4.

Binding of folate onto serum proteins

Various amounts of tritiated folate were added in a volume of 0·5 ml to 5 ml serum. The serum was then centrifuged in a dialysis bag as described in the method section and the radioactivity in the dialysate determined. The percentage of the added folate which was non-dialysable was determined.

The ultra filtration method was also used to determine the percentage of folate which was non-dialysable (Table 4). Similar levels of binding were observed in the experiments in which the system was buffered to pH 8·6.

The effect of methotrexate on the binding of folate to serum was examined. Even at high levels of methotrexate there was a comparatively small effect on the level of binding (Table 5).

TABLE 4. PERCENTAGE OF ADDED FOLATE 'BOUND' TO SERUM*

Amount of folate mμM/2 ml serum	% of added folate found to be non-dialysable	
	Centrifugal method	Membrane filter method
1	46	—
25	54	49
100	50	—
375	40	46
1,650	56	51

* (Mean of 3 experiments. Results ± 12 per cent)

TABLE 5. EFFECT OF METHOTREXATE ON BINDING OF FOLATE TO SERUM*

Amount of folate (mμM) added to 2 ml serum	Amount of methotrexate (mμM) added after 10 min	% of added folate found to be bound by centrifugal method
40	0	53
40	2,300	50
20	0	57
20	2,300	54

* (Mean of 3 experiments. Results ± 15 per cent)

TABLE 6. EFFECT OF ORDER OF ADDITION OF METHOTREXATE AND FOLATE ON BINDING OF FOLATE TO SERUM*

Addition made to 10 ml serum	Addition made 10 min later	% of Folate found to be bound to serum by centrifugal method
230 mμM folate	—	56
230 mμM folate	2,300 mμM methotrexate	48
2,300 mμM methotrexate	230 mμM folate	50

* (Mean of 3 experiments. Results ± 10 per cent)

Experiments were carried out to determine if prior additions of methotrexate had any effect on the subsequent retention of folate by serum and vice versa (Table 6).

Experiments were designed to examine the retention of methotrexate by serum. The results indicated that over a wide range an approximately uniform percentage of the added methotrexate (54 per cent) was bound to serum in a non-diffusible form. This result was similar to that obtained using folate (Table 4).

The binding of folate to serum *in vivo* was also examined. The levels of bound folate present in serum following injection of 0·5 μM of folate were determined (Fig. 4).

It appeared that the percentage of folate which was bound remained constant during the process of disappearance of radioactivity from the serum.

The possible attachment of FH_2 to serum proteins was also examined. 0·05 μM of FH_2 (0·5 ml) were added to 5 ml serum and dialysed against 0·005 M tris buffer (pH 7·8) for 48 hr, in the cold. 5 ml of serum plus 0·5 ml of buffer were placed in

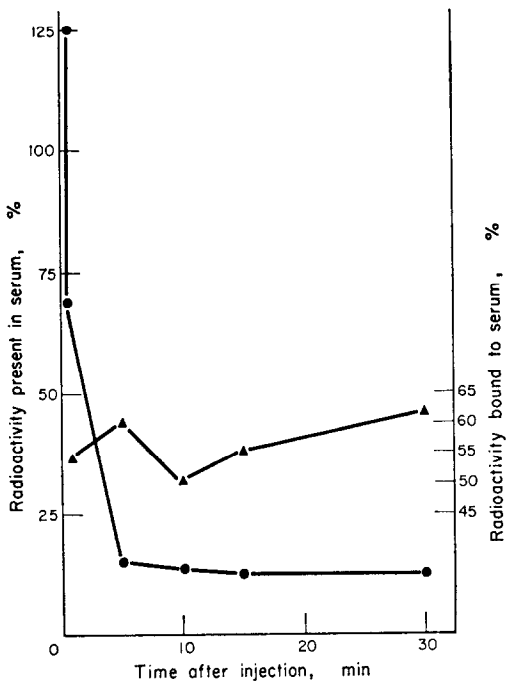


FIG. 4. The distribution of radioactivity in the serum between free and bound forms following intravenous injections of 1·1 μM tritiated folic acid. ●———● percentage of injected radioactivity present in the serum. ▲———▲ percentage of the radioactive content of the serum found to be non-dialysable.

another dialysis bag and 0·05 μM FH_2 (0·5 ml) plus 5 ml tris buffer in a third, and dialysed for a similar period against the tris buffer. 0·2 ml aliquots of the dialysed solutions were then assayed as substrates for FH_2 reductase (Fig. 5).

It has been reported that folate is found not to be bound to human serum when examined by paper electrophoresis.[10] In contrast to this a high level of binding has been reported as a result of ultra filtration studies.[3] In the present study experiments were carried out to examine the binding of folate to rat serum by means of zonal density gradient electrophoresis. 5 ml of serum were equilibrated with 0·05 μM tritiated folate. The serum was then subjected to electrophoresis (Fig. 6). Folic acid migrated faster towards the anode than the albumin fraction and only small amounts of radioactivity were found to be present in the protein fractions, clearly much less than would be expected in view of the results of the ultra-filtration studies referred to above. Prior dialysis of the serum-folate complex was found to yield a similar pattern of results on subsequent density gradient electrophoresis. The FH_2 serum complex was

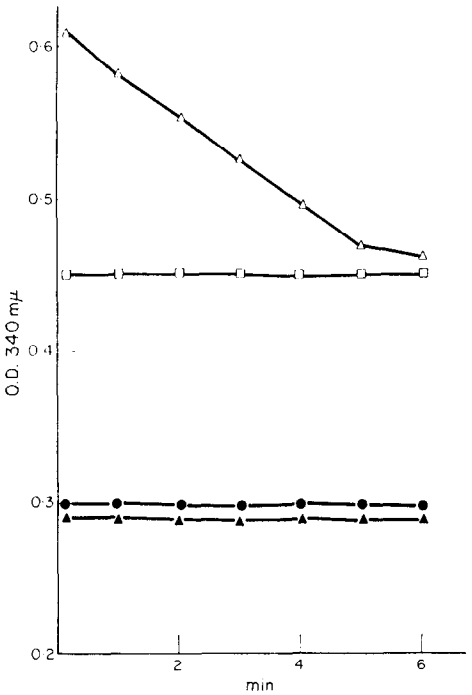


FIG. 5. An examination of the non-dialysable fraction of the FH_2-serum complex for the presence of FH_2-reductase. System for spectrophotometric assay at 340 mμ. 0·1 ml FH_2-reductase preparation, 0·1 μM NADPH, 0·2 ml. of dialysate listed below, 0·05 M tris pH 7·5 to a total of 2 ml. Dialysate obtained by dialysis of following solutions: ●———● tris buffer pH 7·5. ▲———▲ 0·05 μM FH_2. □———□ 5 ml serum. △———△ 5 ml serum + 0·05 μM. FH_2.

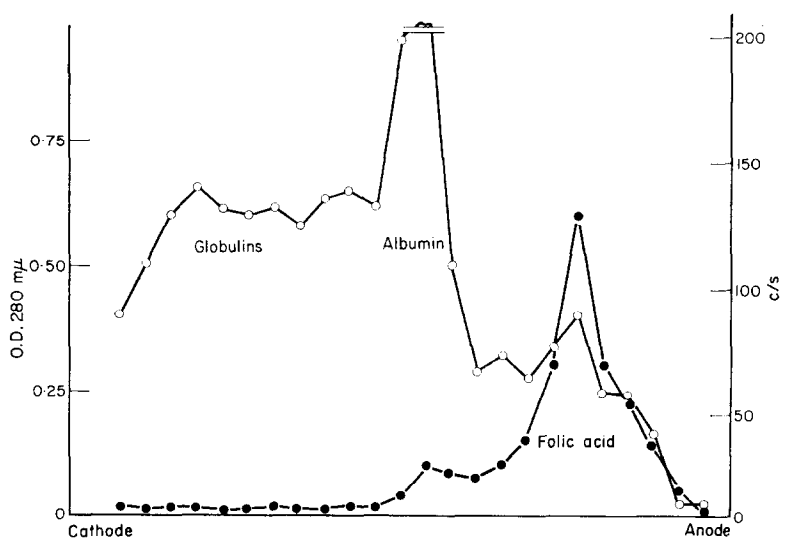


FIG. 6. The density gradient electrophoresis separation of the folate-serum complex obtained by adding 0·5 μM tritiated folic acid to 5 ml serum. Electrophoresis carried out for 48 hr at approximately 300 V and 25 m A. ○———○ 0.D at 280 mμ. ●———● c.p.s.

run on the density gradient electrophoresis apparatus and the fractions examined for the presence of FH_2 by means of FH_2-reductase. Unbound FH_2 was found to run in advance of the albumin, occupying a similar zone to that of folate. Traces of FH_2 were found to be present in the albumin fractions but not in the globulin fractions.

DISCUSSION

Intravenously injected folate disappears rapidly from the blood stream. The rates of disappearance of radioactivity from the serum appear to be independent of the state of reduction of the folate or the position of tritiation and indicate that a similar removal mechanism operates irrespective of the degree of reduction of the folate. A similar rapid removal of injected folate from the blood stream has been found to occur in humans[3] and dogs.[4]

Despite the high level of renal excretion of the injected folate it would appear that there is a large body-capacity available for the uptake of folate from the blood stream. The fact that folate which has been taken up from the blood stream can be 'flushed' out as unmetabolised folate[3] indicates that the uptake mechanism is not associated with the further metabolism of folate. The lack of effect of methotrexate on the uptake of folate from the blood stream found in the present study supports this idea. Johns *et al.*[3] have suggested that the uptake of folate is due to a membrane transport mechanism which is specific for an unchanged pteridine moiety, although later work in the same laboratory[11] appears to indicate a similar pattern of rapid uptake and retention of antifolic drugs having a modified pteridine structure.

The results of the present study appear to indicate that over a very wide range of concentrations the proportion of the injected folate absorbed into the body varies only between 25 and 50 per cent. It is possible that such a result could be obtained if the folate was taken up from the blood stream by a non-specific binding onto cellular proteins. This would be in accordance with the apparent non-metabolism of the absorbed folate and would account for the apparent large capacity of the body for injected folate. The ability of folate to combine with proteins is illustrated by the results of the experiments in which folate was added to serum, in which it was found that over a very wide concentration range, a constant proportion of folate became non-diffusible. Quantitatively similar results have been reported for human[3] and dog[4] serum. The weak nature of this binding was revealed when the serum-folate complex was subjected to density gradient electrophoresis, and suggests that the reason for the conflicting reports concerning the ability of folate to bind to serum proteins lies in the varying ability of the different methods used to split the serum-folate complex[3, 10]

The ability of folate and anti-folate drugs at high concentrations to inhibit enzymes which are unrelated to folate metabolism[12] might be associated with the non-specific binding of folate onto proteins.

It would appear, in view of the results of the present study, that if reduced forms of folate formed in the liver circulate to acceptor sites *via* the blood stream they must be protected against this non-specific cellular uptake. That at least one reduced form of folate injected into the blood stream can penetrate to sites where it can be metabolised, is indicated by the results of Mead *et al.*,[13] in which citrovorum factor is shown to be effective in overcoming the toxic effect of antifolate drugs in mice. These results have been repeated in this laboratory using rats.

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