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THE RENAL EXCRETION OF FOLIC ACID *

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The urinary excretion of tritiated folic acid after a single intravenous injection in man has been measured by Johns, Sperti, and Burgen (1). Plasma concentrations of the labeled compound fell sharply initially, and then more slowly. Urinary clearance in terms of the later, slowly declining plasma levels was shown, at plasma concentrations above 10 μg per L, to be independent of plasma level and to average 51 ml per minute. The finding of an upper fixed limit lower than the glomerular filtration rate (130 to 140 ml per minute) was correlated with binding of folic acid to plasma proteins. Ultrafiltration and equilibrium dialysis demonstrated a practically uniform binding over the concentration range of 5 to 3,000 μg per L, with an average of 64%. The renal excretion of folic acid at these high plasma levels could be accounted for simply by glomerular filtration and urinary excretion of unbound folic acid. At plasma levels lower than 10 μg per L, the urinary clearance appeared to decline, suggesting some renal tubular reabsorption of folic acid, but no threshold plasma concentration was defined, suggesting a relatively ineffective renal mechanism for the conservation of folic acid. At these lower plasma concentrations, the accuracy of the data was not great because of the low levels of radioactivity involved.

Tritium-labeled folic acid of much higher specific activity has now become available. This material has been used in the present study to investigate more completely the renal excretion of folic acid by the single renal intra-arterial injection technique (2).

METHODS

General experimental procedure. In each experiment a mixture of folic acid and inulin was placed in a single syringe and rapidly injected into the left renal artery of a dog anesthetized with pentobarbital. Twenty-five or more samples of urine were collected in the ensuing 10 minutes, and the recoveries of test substances in each sample were determined. Within each experiment, collection periods of constant duration were used; this duration varied from one experiment to another, however, depending on the rate of urine flow, and ranged from 20 to 38 seconds per sample.

Materials and injection solution. The following special materials were used: tritiated folic acid,[1] mixed with carrier folic acid for some experiments, purified before each experiment by chromatography on DEAE cellulose (1), the SA after purification ranging from 13 μc per mg to 637 μc per mg; carrier folic acid; [2] inulin, recrystallized from ethanol; [3] inulin — COOH C^{14}, 2.09 mc per g; [4] and methotrexate (4-amino-N^{10}-methylpteroylglutamic acid).[5] The materials to be injected were mixed together in solution, and a sample was removed for the preparation of standard solutions. The mixture was then diluted with saline or plasma to yield 0.5 to 1.0 ml injection solution. When tritium and C^{14} were both present, the carbon activity utilized was adjusted so that it was approximately 3 to 5% of the tritium activity.

Operative procedure and collection of samples. Mongrel dogs, anesthetized with pentobarbital, weighing 13 to 26 kg were experimental subjects. The renal artery was exposed through a left flank incision, and Teflon catheters were placed in the ureters either through a split bladder or in an extravesicular position. To facilitate collection and analysis of specimens, urine flow was increased by using a 15% urea infusion [6] to levels of from 0.5 to 9.8 ml per minute. The injection mixture was introduced into the left renal artery with a bent needle, and subsequent urine samples were collected in vials placed in a timed urine-collection rack.[7] The volume of each sample was determined by weighing. At the end of each experiment, the left kidney was excised and weighed.

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[2] Charles E. Frosst and Co., Montreal, Canada.

[3] U. S. Standard Products Co., Mount Prospect, Ill.

[4] New England Nuclear Corp., Boston, Mass.

[5] Lederle Laboratories, Pearl River, N. Y.

[6] Mannitol precipitated out in the scintillation mixture used for counting.

[7] J. Armant, Baltimore, Md.

Analysis of samples. Standard solutions were prepared from the injection mixture by addition, in serial dilution, of blank urine obtained just before the experiment. Inulin was determined chemically by the method of Higashi and Peters (3). Radioactivity was determined in a Packard Tri-Carb liquid scintillation counter at 5° C. In each experiment uniform portions of each sample and of the diluted standard solutions were added to 10 ml scintillator made up as follows: 77 ml dioxane, 23 ml ethanol, 13.3 g naphthalene, 25 mg 1,4-bis-2-(5-phenyloxazolyl)benzene (POPOP), and 1 g 2,5-diphenyloxazole (PPO). Where the samples contained both tritium and C^{14} activity, double-channel counting was utilized. Two calibration samples of blank urine, one containing only tritium and the other containing only C^{14} activity, were used to adjust the two channels so that less than 5% of the first channel tritium counts appeared in the second channel and the carbon counts appearing in the first channel did not greatly exceed those appearing in the second channel. With this setting of the channels, counts of the calibration samples led to a pair of simultaneous equations that could be solved for the tritium and C^{14} activity present in each sample counted. Addition of tritiated toluene of known activity demonstrated that the quenching properties for tritium activity of the uniform portions of urine were the same throughout a given experiment.

In an attempt to identify the nature of the radioactive compounds present in the urine after a displacing dose of unlabeled folic acid, a 5-ml sample of urine was chromatographed by the procedure previously described (1).

RESULTS

Urinary excretion after a single intra-arterial injection. The results for a given experiment were calculated as follows (2). The amount of material excreted in each sample was expressed as a fraction of the amount injected. The material excreted in a given sample by the control right kidney was assumed to result from the recirculation of injected material, and was subtracted from the corresponding experimental left sample to yield the net first passage urinary excretion from the experimental side. The net urinary excretion of inulin and of folic acid was then plotted as a function of time.

The inulin curve rose to a peak and then decayed to zero, whereas the folic acid curve rose to a peak and then died away to some continued very low level of urinary excretion of activity, most evident when high doses were used. This was regarded as a steady state leak of urinary radioactivity independent of the first passage phenomenon, and the first passage excretion was

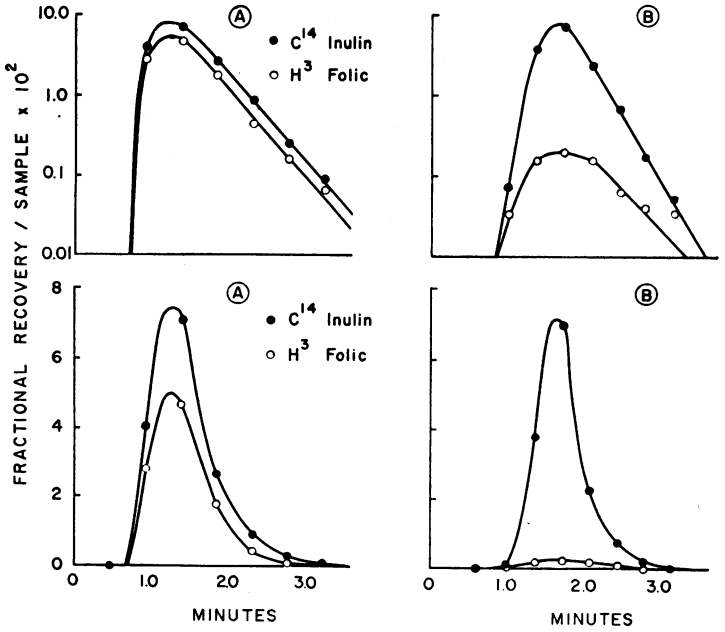

FIG. 1. SINGLE PASSAGE URINARY EXCRETION OF INULIN AND FOLIC ACID. A. High dose (experiment 8). B. Low dose (experiment 3). The upper panel illustrates the data on semilogarithmic plots, and the lower, on rectangular coordinates.

TABLE I

Transit times, folic acid dose, and recovery ratio

Exp't no.	Dose	Recovery ratio§	Mean transit time			Urine flow
			Inulin	Folic acid	Inulin / Folic acid	
	μg/10 g		*seconds*	*seconds*		*ml/min*
1	0.15	0.005	71.0	78.3	1.110	5.1
2	0.21	0.028	108.9	123.5	1.134	5.1
3	0.32	0.005	104.2	111.6	1.071	4.2
4*	0.38	0.11	121.0	131.2	1.084	3.8
5	0.59	0.62	139.8	143.6	1.027	4.6
6*	0.62	0.45	131.0	131.9	1.007	3.1
7	0.86	0.28	101.9	106.0	1.040	5.0
8	1.00	0.66	88.7	87.2	0.983	4.5
9*	1.04	0.51	101.3	103.6	1.023	3.2
10	2.16	0.61	90.8	89.9	0.990	5.9
11	2.60	0.75	122.5	124.5	1.016	2.5
12	4.73	0.70	111.0	107.9	0.972	9.8
13*	5.63	0.73	69.2	70.2	1.014	6.6
14†	470.00	0.71	189.9	196.5	1.035	0.5
15‡	0.10	0.81	217.7	219.5	1.008	1.5
16‡	0.12	0.73	111.9	113.0	1.010	2.3

* Folic acid was bound to plasma before injection.
† Nonradioactive inulin was used.
‡ The experiment was done 30 minutes after the administration of 30 mg of 4-amino-N^{10}-methylpteroylglutamic acid.
§ Ratio = single passage H^3 folic recovered in urine/single passage C^{14} inulin recovered in urine.

estimated as follows: the curve was plotted on semilogarithmic paper as a function of time, and the downslope of the folic acid curve was extended by linear extrapolation, generally beyond some value that was less than 10% of the peak value. The activity above the extrapolated line was excluded in calculating the recovery ratio.

At high doses of folic acid the semilogarithmic plots demonstrated symmetry in time of the folic acid and inulin curves (Figure 1A). At low-dose levels, where the urinary recovery was very small, the symmetry was less marked: the folic acid curve was flattened so that the peak was much lower, and the downstroke tended to approach the downstroke of the inulin curve (Figure 1B). At high-dose levels, where the inulin and folic acid excretion curves were symmetrical on a semilogarithmic plot, the mean transit times (cor-

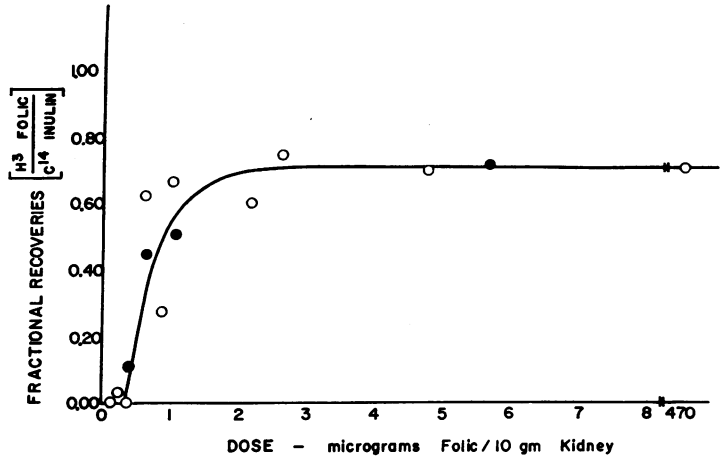

FIG. 2. RATIO OF THE CUMULATIVE FRACTIONAL RECOVERY OF FOLIC ACID TO INULIN AS A FUNCTION OF DOSE. The open circles represent experiments where the injected materials were suspended in saline; the filled circles, where they were suspended in plasma.

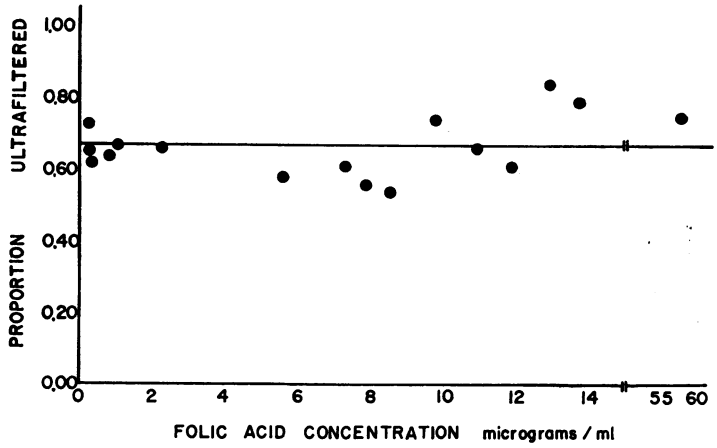

FIG. 3. PROPORTION OF FOLIC ACID ULTRAFILTERED AT VARIOUS CONCENTRATIONS OF TRITIATED FOLIC ACID.

rected for catheter lag) of inulin and folic acid were virtually identical (Table I). The curves of Figure 1, A and B, were plotted on rectangular coordinates in the lower panel of Figure 1. The time relationship of the curves is no longer evident from inspection.

To compare experiments in different animals, the dosage was expressed in terms of micrograms of folic acid injected per 10 g of experimental kidney. This will not allow for variations in blood flow or filtration fraction, but is a provisional way of obtaining a basis for comparison. Cumulative first passage urinary excretion of folic acid was calculated from the curves for each experiment and expressed as the ratio of the coincident urinary excretion of inulin. The data are illustrated in Figure 2, and it is evident that at small doses there was relatively complete reabsorption of the injected folic acid. As the dose was increased the proportion reabsorbed diminished, until at very large doses the amount appearing in the urine reached an asymptotic value of 71% of the cumulative inulin recovery.

The protein-binding of folic acid was examined by ultrafiltration of dog plasma containing folic acid at 37° C with CO_2 at a partial pressure of 40 mm Hg (Figure 3) by use of Toribara tubes (4). The proportion ultrafiltered was practically uniform over the concentration range explored and averaged 67 ± 8 SD%. The coincidence of this value with the asymptotic ratio of urinary fractional recoveries of folic acid to inulin indicated that at high values the folic acid appearing in the urine corresponded to that passing into the glomerular fluid. The line shown in Figure 2 was constructed on the premise that 71% of the folic acid is filtrable at the glomerulus, that all the folic acid filtered is reabsorbed up to a dose level of 0.32 μg per 10 g kidney, and that all the filtered folic acid above this dose level appears in the urine. It is evident that to a first approximation this fits the data.

The amount reabsorbed in each experiment may be calculated from the data. If the proportion ultrafiltered is assumed to be 0.71, then under these experimental conditions, the amount reabsorbed is essentially equivalent to the amount filtered up to a maximum of 0.04 μg per 10 g kidney, and thereafter the excess filtered is excreted in the urine.

Effect of methotrexate on the reabsorption of folic acid. Two experiments were performed 30 minutes after the administration of 30 mg of methotrexate to the anesthetized dogs. The dose levels used were 0.10 and 0.12 μg tritiated folic acid per 10 g kidney (no. 15 and 16, Table I). From the experiments described above, complete reabsorption of the folic acid would be expected at these dose levels (Figure 2). It was found, however, that the two urinary recovery ratios were 0.81 and 0.73, respectively, indicating complete inhibition of folic acid reabsorption. The first value is somewhat higher than the control asymptotic value and suggests that there may be some competition for plasma protein-binding sites at these very high levels of methotrexate. The

TABLE II

Displacement of tritiated folic acid

Exp't no.	H^3 folic acid dose	Recovery ratio*	Reabsorbed H^3 folic acid	Carrier folic acid	Interval	H^3 folic acid flushed	Peak lag
	μg/10 g		*μg/10 g*	*μg/10 g*	*minutes*	*μg/10 g*	*seconds*
17	0.35	0.066	0.11	78	20	0.0065†	140
18	0.47	0.13	0.06	45	24	0.0069	50
19	0.70	0.56	0.016	47	5	0.0040	60

* Ratio = single passage H^3 folic acid recovered, injection 1/single passage inulin recovered, injection 2.
† Collection of flushed activity was incomplete.

mean transit times of inulin and folic acid were found to be essentially identical (Table I).

Displacement of reabsorbed tritiated folic acid. The fate of the reabsorbed folic acid is not yet clear. If the tritiated folic acid reabsorbed by the tubule cells is not directly transported back into the blood stream but is stored within the cells, and if the stored folic acid is concentrated, a large dose of unlabeled folic acid might displace the activity from the cells (5). Experiments to test this hypothesis were carried out as follows. An initial dose of tritiated folic acid was injected into the left renal artery, and urine collection was begun and continued. Some time later a second dose containing inulin and a large amount of carrier folic acid was injected into the left renal artery. This simplified the counting procedure, since no C^{14} activity was present. The radioactivity excreted on the control side was subtracted from that on the experimental side as before. Activity displaced from organs other than the kidney and from the control kidney during recirculation of the unlabeled folic acid was subtracted as above from the experimental side. The results of a typical experiment (no. 18, Table II) are shown in Figure 4. After the injection of tritiated folic

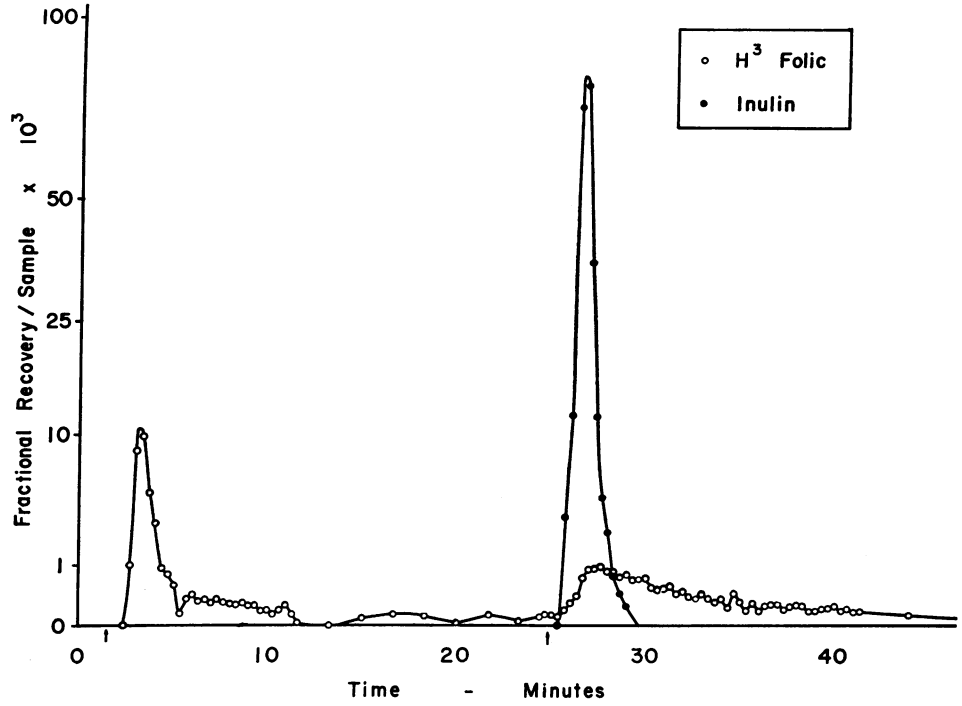

FIG. 4. DISPLACEMENT OF THE REABSORBED TRITIATED FOLIC ACID BY A LARGE DOSE OF NONRADIOACTIVE FOLIC ACID. The ordinate is a square root scale. This foreshortens the peaks and exaggerates values close to background.

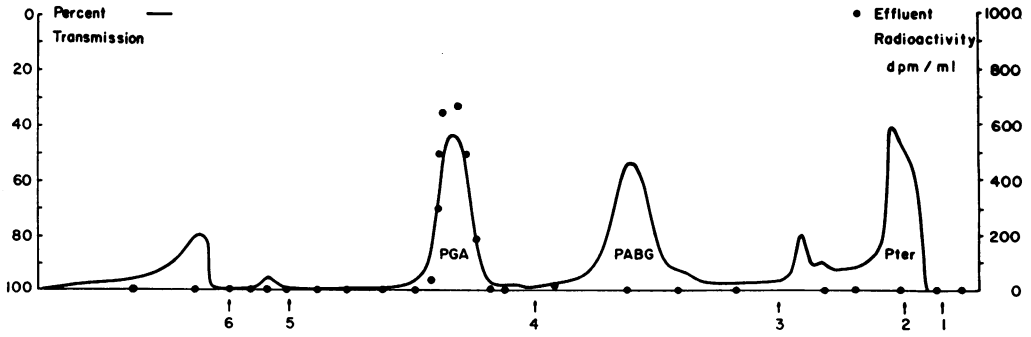

FIG. 5. ULTRAVIOLET ABSORPTIOMETER RECORD FROM COLUMN CHROMATOGRAPHY OF "FLUSH" URINE SAMPLES, WITH SUPERIMPOSED RADIOACTIVITY READINGS. At 1, sample was applied to DEAE-cellulose (5 ml urine with following marker compounds added: 1 mg 2-amino-4-hydroxy-6-methylpteridine (Pter), 0.5 mg *p*-aminobenzoylglutamate (PABG), and 1 mg pteroylglutamate (PGA)). At 2, application of the sample was complete, and 0.01 M sodium phosphate buffer, pH 6.9, was started. At 3, the strength of the buffer was increased (0.07 M), and again at 4 (0.2 M). At 5, ammonium hydroxide solution (1 M) was applied, and this was followed, at 6, by a solution of sodium hydroxide (0.5 M).

acid, the first passage excretion was followed by a steady state leak of small proportion. The inulin curve after the second injection represents the passage of material through the glomerular filter, and this is an index of the distribution of times taken by the unlabeled folic acid to appear in the urine. The concentration of tritiated folic acid that did appear rose more slowly than did the inulin concentration, and there was significant time lag between the inulin and the flushed tritiated folic acid peaks. There was prolonged flush of the tritiated folic acid from the tubular cells, in part owing to recirculation of the large dose of carrier folic acid. The essential data from three flush experiments are presented in Table II. The activity flushed, expressed in terms of the activity retained after the first passage, varied from 6 to 24%. Thus a considerable proportion of the reabsorbed folic acid was stored within the renal tubular cells for periods of up to 30 minutes, rather than transferred to the peritubular capillary blood.

The maneuver carried out above demonstrates the time lag and course for flush, but does not show conclusively that the excess radioactivity excreted from the left side originated from the material stored during the first passage. To show this, an initial dose of 1.18 μg labeled folic acid per 10 g kidney was injected into the left renal artery. The first passage excretion was 5.26% of the injected dose. A flushing dose of 0.79 mg unlabeled folic acid per kg body weight was administered 8 minutes later as a single rapid intravenous injection, and half-minute urine samples were collected for the next 30 minutes. During this time interval, the left kidney excreted 1.78% of the injected first dose, i.e., 0.02 μg per 10 g kidney, as excess radioactivity, while a total of 21.04% of the first dose was flushed from both kidneys and other body stores into the urine. The difference between the urinary excretion from the two kidneys was most evident during the first half of the collection. The excess radioactivity coming from the left side thus must have originated from material stored during the first passage.

A 15-minute collection of urine was pooled after a flushing dose in an experiment of the first type. Chromatography was carried out on this sample. Figure 5 illustrates that 97% of the effluent radioactivity was found in the folic acid peak.

DISCUSSION

Saturation of the reabsorptive mechanism. The results demonstrate that after a single intra-arterial dose, filtered tritiated folic acid is reabsorbed by the kidney. There is a maximum for initial reabsorption from tubular lumen to cell, and so this mechanism exhibits saturation. At high-dose levels the mean transit times of folic acid and inulin are virtually identical. This indicates that the folic acid which appears in the urine has taken a direct course along the tubular lumen with

the inulin and has spent no time within the cells, i.e., that the cells are relatively impermeable to folic acid. At low-dose levels, where the folic acid curve becomes relatively delayed on the semilogarithmic plot, the mean transit time of folic acid becomes longer than that of inulin (Table I). This delay is most evident at dose levels where almost all the folic acid is reabsorbed, the cellular luminal carrier sites being almost adequate to remove the folic acid completely. The difference in transit times could reflect some different kind of renal excretion of this delayed portion, but there is a more probable explanation. If the folic acid entering the tubule is visualized as a concentration wave one-half second long (the duration of injection), the first part of the concentration wave will encounter proportionately more available binding sites than will the last part and so much more of the last part of the wave may be expected to emerge because there are relatively more occupied sites. No delay of inulin will be apparent when a large proportion of the filtered folic acid passes down the tubule without reabsorption.

Inhibition of reabsorption. At the doses administered, methotrexate completely inhibits folic acid reabsorption. The virtual identity of the transit times for folic acid and inulin indicates that, under these circumstances, the filtered folic acid does not enter the renal tubular cell but passes directly down the tubular lumen. The methotrexate thus inhibits the binding of folic acid to what is presumably a membrane carrier-transport system situated at the luminal surface of the tubular cells, rather than to intracellular binding sites. The similarity in chemical structure of the methotrexate and folic acid molecules suggests that the inhibition may be due to competition at the binding sites. A similar phenomenon undoubtedly accounts for the higher cumulative urinary excretion of an intravenously injected dose of folic acid observed by Condit and Grob (6) after the administration of 4-aminopteroylglutamic acid and by Johns, Plenderleith, and Hutchison (7) after the administration of methotrexate.

Isotope countertransport (displacement phenomenon). Any tritiated folic acid flushed from the luminal surface of the tubular cells would presumably show a urinary excretion concentration time pattern similar to inulin. The demonstration of displacement of tritiated folic acid by carrier folic acid, delayed in time with respect to inulin excretion, implies that a part of the reabsorbed folic acid is stored in the tubular cells rather than rapidly transported to peritubular capillary blood. This behavior stands in marked contrast to the renal excretion of substances like glucose where there is rapid transport from the tubular lumen to peritubular capillary blood (2).

Tissue folic acid accumulation in the kidney has been examined in two ways. Grossowicz, Rachmilewitz, and Izak (8) administered 2,000 μg folic acid per kg body weight intravenously to rats and measured the folate activity in tissue using bacterial assay. With *Streptococcus fecalis* the control level measured in kidney was 1.4 μg per g. This rose to 5.1 μg per g at 1 hour, declined to 2.3 μg per g at 24 hours, and to 2.1 μg per g at 3 days. Similar results were obtained using *Lactobacillus casei.* Levels of folinic acid and related reduced compounds using *Pediococcus cerevisiae* showed little change. Watanabe (9) has examined the changes in tissue content of tritiated folic acid after a single intravenous dose of 29 μg per kg body weight. The kidney tissue counts at 5 minutes were equivalent to 441 mμg per g; at 1 hour, to 290 mμg per g; at 24 hours, to 90 mμg per g; at 3 days, to 70 mμg per g; and at 15 days, to 3 mμg per g. In this study the plasma tritiated folic acid activity at 5 minutes was 28.4 mμg per ml; at 1 hour, 13.8 mμg per ml; at 24 hours, 1.1 mμg per ml; and thereafter fell to amounts not easily detectable. Both these studies demonstrate the storage of folic acid in the kidney after a single intravenous injection, a smaller proportion of the higher dose being stored.

The present study demonstrates that there is a rather large initial maximal reabsorption rate (0.32 μg per 10 g kidney per single pass) after a single intra-arterial injection of tritiated folic acid. The experiments of Johns and associates, however, indicated a relatively low maximal tubular reabsorption rate (less than about 50 mμg per man per minute) after a single intravenous injection, when calculated at a time when plasma levels are no longer rapidly changing. Studies of the kinetics of sulfobromophthalein (BSP) transport may by analogy elucidate one way in which these observations may be reconciled with the present data. Combes, Wheeler, Childs, and Bradley (10) have shown that when BSP is in-

fused at such a rate that a constant plasma arterial level results, a steady state maximal rate of cellular uptake results, corresponding to transport into the bile. On the other hand, when a single injection of BSP is made into the portal vein, the initial maximal rate of uptake is many times greater than the steady state maximal biliary excretion level, implying storage (11). Wheeler, Meltzer, and Bradley (12) have shown that when the cells have been loaded, and after a period during which the transient response has died away, BSP transport in response to a steadily changing plasma concentration is the sum of the maximal transport rate into bile plus cellular storage that varies directly with the incremental change in plasma concentration. Thus during falling plasma concentrations, the maximal biliary excretion will be maintained until tissue stores are depleted, with the uptake from the blood diminished or even absent. The behavior of the renal tubular cells with respect to folic acid may be similar. At normal plasma levels, when tissue stores have not been expanded, and when the plasma levels are relatively stable, folic acid reabsorption from tubular fluid may be relatively complete, corresponding to some value below a steady state maximum for transport from tubular cells to blood. In response to a single intra-arterial injection, a maximal transport into the cells occurs, which is many times that of the maximal transport rate from tubular cells to blood, resulting in storage. In the same way, after a single intravenous dose, tissue stores are initially greatly expanded. Later, the plasma concentration falls to relatively low levels and then declines very slowly. At this time Johns and associates found that the calculated tubular reabsorption was low, a phenomenon analogous to the depression of BSP cellular uptake found at a time when plasma concentration is falling, but during which biliary transport is maintained at a maximum by tissue stores (12). The phenomenon of cellular storage thus complicates any attempt to estimate maximal transport from the tubular cell to the peritubular blood when some method is employed that entails transient changes in plasma concentration and expansion of tissue stores.

The renal tubular reabsorption of exogenously administered folic acid does not then resemble the kind of reabsorption usually inferred, i.e., a direct and rapid transport from tubular lumen to peritubular capillary blood, but rather resembles those processes in other tissues where a substance is rapidly taken up by the cells, stored and concentrated, metabolized slowly, and slowly transferred to the blood.

SUMMARY

The renal excretion of tritiated folic acid has been explored by using the single renal intra-arterial injection in the dog. Renal tubular reabsorption of folic acid was demonstrated. The initial reabsorptive mechanism from tubular lumen to cell exhibited saturation. It was inhibited by methotrexate. The reabsorbed folic acid was displaced into the urine from renal tubular cells by a large dose of unlabeled folic acid, demonstrating storage of folic acid in these cells under these experimental circumstances rather than rapid transport into the blood stream. The characteristics of this reabsorptive mechanism (saturation, inhibition, and countertransport) are compatible with a mobile, membrane, carrier-transport system for folic acid abutting upon the tubular lumen (5).

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