animals which received the lethal dose of amethopterin (Group VIII) the mean 24-hour hepatic uptake was reduced from 19.9 ± 0.95% to a mean of 9.5 ± 1.41% by day 7 while the mean 24-hour renal uptake had increased from 8.0 ± 0.43% to 24.2 ± 4.03%. The 24-hour hepatic and renal uptake of this radioactive material was significantly different than in the controls ($p < 0.05$) on days 5, 6 and 7. Two animals from Group VIII died at the times indicated in the table so that the animals assayed at these periods are survivors.

Discussion. Interpretation of these data may be considered in the light of findings relating to an hepatotoxin, carbon tetrachloride. Experimentally-induced hepatic damage by this agent results in a release of vit. B_{12} from the liver with an initial rise in serum B_{12} and a slight increase in renal excretion (6). The decreasing storage and 24-hour uptakes of this radioactive vitamin by the livers of animals which received the lethal dose of amethopterin probably reflected progressive hepatic damage. The increased renal storage could be due to its preferential uptake of bound B_{12} released by the damaged liver or to its increased storage of this vitamin because of the liver's progressive inability to do so. The last alternative seems to be most likely since the 24-hour renal uptakes were also increased. The diminished binding capacity of a damaged liver may lead to utilization of the kidney as another important binding site for the vitamin(7). It would appear that the kidney, at least in some species, has a more fundamental role in vit. B_{12} metabolism than simply that of excretion.

Summary and conclusion. The effects of 2 doses of amethopterin on hepatic and renal storage and 24-hour uptake of $Co^{60}B_{12}$ were contrasted in the mouse. A nonlethal dose had little effect on these measurements of $Co^{60}B_{12}$ metabolism. Following administration of a lethal dose of amethopterin, a progressive decrease in storage and 24-hour uptake of $Co^{60}B_{12}$ by the liver occurred while concomitant increases were observed in the kidney. It is suggested that the lethal dose of amethopterin was hepatotoxic and decreased the ability of the liver to metabolize $Co^{60}B_{12}$. In such a situation the kidney may become a more important binding site for this vitamin in the mouse.

1. Sauberlich, H. E., *J. Nutrition,* 1959, v68, 141.
2. Ellis, L. N., Duncan, B. J., Snow, I. B., *ibid.,* 1959, v67, 185.
3. Banerjee, S., Dravid, A. R., *Ind. J. Med. Res.,* 1959, v47, 46.
4. Colsky, J. Greenspan, E. M., Warren, T. J., *A. M. A. Arch. Path.,* 1955, v54, 198.
5. Snedecor, G. W., *Statistical Methods,* State College Press, Ames, 1956.
6. Stein, O., Stein, Y., Aronovitch, J., Grossowicz, N., Rachmilewitz, M., *J. Lab. Clin. Med.,* 1959, v54, 545.
7. Heller, P., Henderson, W., Bowser, E., Levitsky, J. M., Zimmerman, H. J., *J. Lab. Clin. Med.,* 1960, v55, 29.

Received September 5, 1961. P.S.E.B.M., 1962, v109.

Tracer Studies of Vitamin C Utilization in Men: Metabolism of D-Glucuronolactone-6-C^{14}, D-Glucuronic-6-C^{14} Acid and L-Ascorbic-1-C^{14} Acid. (27324)

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Recent studies(1) indicated that D-glucuronolactone caused increased blood ascorbic acid levels as well as increased urinary excretion of ascorbic acid in men, whereas D-glucuronic acid did not do this. To check the possible conversion of D-glucuronolactone to L-ascorbic acid, it was decided to study the metabolism of the lactone in two ways. One was to give D-glucuronolactone-6-C^{14} orally and then isolate urinary ascorbic acid to determine if any of the labeled lactone had been converted to L-ascorbic acid. The other was

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first to label the total body ascorbic acid pool with L-ascorbic-1-C^{14} acid and then test with various loads of D-glucuronolactone to see if any changes would take place in the specific activity and rate of excretion of ascorbic acid. Further, an attempt was made to see if total body ascorbic acid and its rate of utilization were related to the fat-free body weight.

Experimental. The D-glucuronolactone-6-C^{14} and D-glucuronic-6-C^{14} acid were obtained from the National Bureau of Standards, Washington, D.C. The L-ascorbic-1-C^{14} acid was obtained from the California Corp. for Biochemical Research, Los Angeles. These compounds were checked for purity prior to use by melting point measurement and by paper chromatography. The activity of the above compounds was checked by radioassay. D-glucuronolactone-6-C^{14}, 4.11 μc/mg, or D-glucuronic-6-C^{14} acid, 3.72 μc/mg, were freshly dissolved in distilled water and swallowed immediately by healthy men in quantities of 20 μc of each appropriate compound for each man together with a cold carrier load of 2 g of D-glucuronolactone or D-glucuronic acid. For the men receiving L-ascorbic-1-C^{14} acid, each swallowed 20 μc of L-ascorbic-1-C^{14} acid, having a specific activity of 1.35 mc/mM. No cold carrier was given to these subjects. Following this, each man swallowed 2 g of freshly dissolved D-glucuronolactone on the 3rd or 9th day.

Total daily urine was collected and measured from all subjects; these samples were refrigerated and 2.0 ml of each were taken for counting.

Immediately after receiving the tracer quantity of D-glucuronolactone, D-glucuronic acid or L-ascorbic acid, the men were made to expire directly through a $CaCl_2$ drying train into a 5-liter Cary-Tolbert ionization chamber connected to a vibrating reed electrometer. The $C^{14}O_2$ activity, total CO_2, and the volume of flow were recorded automatically on a 6-channel recorder.

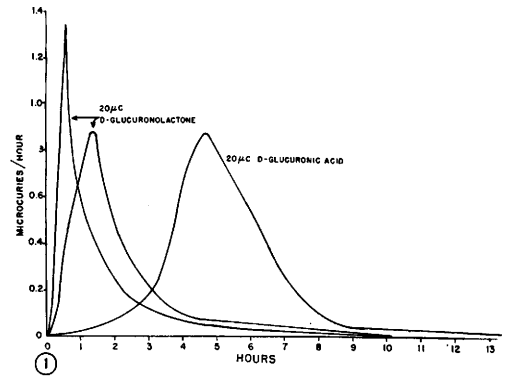
The total activity of each urine sample was determined by use of a liquid scintillator counter using P-dioxane-toluene. Oxalate in selected samples was isolated as calcium oxalate, recrystallized 4 times, and dissolved in 1 N hydrochloric acid for counting in the liquid scintillator. Quantitative determination of the total oxalate was done with the Archer method(2). Efficiencies for all liquid scintillator counting samples were determined individually by use of added standard C^{14} samples.

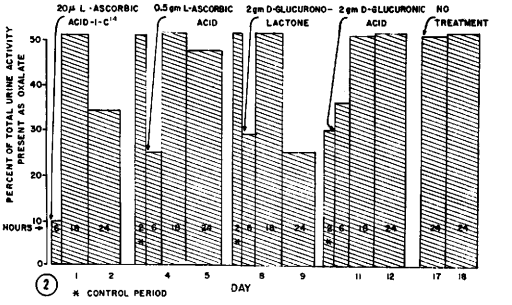
Urinary ascorbic acid levels were chemically determined by the Schaffert method(3). Urinary ascorbic acid was then isolated by the method described by Jackel *et al.*(4). After the dinitrophenylhydrazone (DNPH) derivatives were recrystallized, they were dissolved in P-dioxane and applied to weighed planchets and counted in a gas flow counter. All DNPH derivatives were recrystallized to constant activity which usually required 4 to 6 recrystallizations.

Total body tissue volume (V) was estimated in duplicate tests using a body volumeter based on displacement of water(5). From body weight (M) and V, fat (F) in kg was calculated according to an equation developed in this laboratory: $F = 4.834\ V - 4.366\ M$.

Results. Fig. 1 shows the rate of $C^{14}O_2$ expiration in 3 men, as measured with an ionization chamber electrometer immediately following the swallowing of 20 μc of D-glucuronolactone-6-C^{14} or D-glucuronic-6-C^{14} acid. The cumulative excretion of $C^{14}O_2$ after ingestion of D-glucuronolactone was 44% of the total carbon-14 dose in 2 experiments, whereas after ingestion of D-glucuronic acid it was 68%. The peak rate of excretion occurred within 25 minutes in the lactone experiments, whereas in the glucuronic acid experiments it did not occur until after the 4th hour following ingestion. No $C^{14}O_2$ activity could be detected in the expired air of the subjects receiving L-ascorbic-1-C^{14} acid even using a 15-liter ionization chamber for greater sensitivities. A fraction of 1% oxidation to CO_2 during the first 8 hours could have been easily detected by this technic.

The urinary ascorbic acid which was isolated as the DNPH derivative had no radioactivity in the labeled D-glucuronic acid experiments on 2 men. However, the DNPH derivatives isolated from the urinary ascorbic acid of the 3 men who had received D-glu-





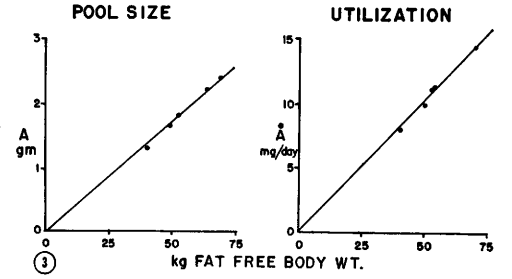


FIG. 1. Rate of $C^{14}O_2$ expiration following ingestion of 20 μc quantities of D-glucuronolactone-6-C^{14} and D-glucuronic-6-C^{14} acid.

FIG. 2. Per cent of total activity excreted by the kidneys as oxalate in Subject 1 on the days following ingestion of 20 μc of L-ascorbate-1-C^{14}. Hours of duration of each collection period are indicated in each block of figure. See text for indicated treatments.

FIG. 3. Total body ascorbic acid and its rate of utilization are directly related to fat-free body wt of healthy men.

curonolactone-6-C^{14}, the specific activities ranged from 2.1×10^{-3} to 3.6×10^{-3} μc/mg ascorbic acid. This, of course, was dependent upon the amount of ascorbic acid excreted daily, which ranged from 26.4 to 39.0 mg.

Further, an experiment was made with 20 μc of D-glucuronolactone-6-C^{14} added to freshly voided urine together with cold carrier D-glucuronolactone and L-ascorbic acid. From this mixture the ascorbic acid was isolated as the DNPH derivative, recrystallized at least 4 times, and counted to determine the degree of contamination, if any, by the C^{14} labeled lactone. Less than 0.04% of the initial 20 μc could be detected in the ascorbate derivative, indicating that in the experiments on ingestion of D-glucuronolactone-6-C^{14} that less than 10% of the ascorbate derivative activity could have been from contamination under the most unauspicious set of conditions.

Fig. 2 shows the percent of total activity excreted in the urine as oxalate from one of 2 men who had received 20 μc of L-ascorbic-1-C^{14} acid. This was done to see if the oxalate excretion arising from the C^{14} ascorbate was constant and, further, to study the effect of cold D-glucuronolactone, D-glucuronic acid, and L-ascorbic acid on oxalate and ascorbic acid excretion. The oxalate activity was, indeed, remarkably constant, remaining at a level of 50 to 52% of total activity excreted and changed only when the body ascorbate pool was overloaded or otherwise affected. Both subjects, who received cold D-glucuronolactone on the 3rd or 9th day following the L-ascorbic-1-C^{14} acid, excreted less total urinary oxalate activity whereas the urinary ascorbate total activity increased and was of a lowered specific activity. On the following day, the excretion of oxalate radioactivity returned to the 50% level. On the 2nd day following overloading with the cold lactone, the proportion of total radioactivity excreted in the form of oxalate decreased, for unknown reasons, and did not return to the 50% level until after a further 2 days. Also shown (Fig. 2) is a simliar effect exhibited by overloading with 0.5 g of cold L-ascorbate. Further, note that 2 g of cold D-glucuronic acid, if anything, caused increased oxalate excretion. The above effects, at the least, indicate a marked dilution of the body ascorbate pool presumably by ascorbate arising from glucuronolactone.

The half-life of the L-ascorbic acid was 16 days in the 3 subjects receiving labeled ascorbate. This was obtained by plotting the percent of the initial dose remaining in the body as a logarithmic function of time. An aver-

TABLE I. Body Ascorbic Acid in Healthy Men.

Subject	Day of collection*	(1) Spec. activ. of urinary ascorbic acid, μc/mg	(2) Activity remaining at end of collection, μc	(3) Total body ascorbic acid, mg	(4) Total body ascorbate / Body wt (mg/kg)	(5) Total body ascorbate / Fat-free body wt (mg/kg)
1	1	.0077	18.61	2416	29.4	34.0
2	3	.0079	12.95	1640	20.5	32.9
3	1	.0110	20.04	1823	29.7	34.4
				$\left(\frac{8800}{4}\right)$		
4†	1	.0021	18.50	2200	22.5	34.0
				$\left(\frac{5200}{4}\right)$		
5†	1	.0036	18.70	1300	27.6	31.8
				$\left(\frac{6600}{4}\right)$		
2†	1	.0028	18.00	1650	20.5	32.9

* All collections were for 24 hr except in Subject No. 3 which was for 8 hr.

† Assuming 25% conversion of D-glucuronolactone-6-C^{14} to body ascorbic acid.

Subjects 1, 3 and second test on Subject 2: L-ascorbic-1-C^{14} acid. Subjects 4, 5 and first test on Subject 2: D-glucuronolactone-6-C^{14}.

age biological half-life of 16 days for ascorbic acid has been reported to occur in 3 diseased patients(6).

Table I shows the body pool size of ascorbic acid in 5 men. To calculate this, the specific activity of urinary ascorbic acid (Column 1) was divided into the total activity remaining in the body at the end of the collection period (Column 2) to obtain the total body ascorbic acid (Column 3). In the last 3 tests the pool size was estimated by assuming that one-fourth of the C^{14}-labeled D-glucuronolactone supplied ascorbic acid which, in turn, was incorporated into the ascorbic acid pool. On Subject No. 2 the estimated pool size was 1.65 g compared with 1.64 g when this subject was measured using L-ascorbic-1-C^{14} acid, thus showing that, indeed, one-fourth of the lactone went to ascorbate.

From comparison of Columns 4 and 5 of Table I, it appears as though the fat-free body weight contains a uniform proportion of ascorbic acid in the quantity of 33.3 mg/kg. Further, the rate of utilization of ascorbic acid also is directly related to the fat-free body weight (Fig. 3). The utilization rate was obtained from 2 assumptions: (1) sufficient time had elapsed for the C^{14}-labeled ascorbic acid to become uniformly distributed in the body and (2) the proportion of excreted oxalate-C^{14} arising from labeled ascorbic acid was constant. Rate of utilization was obtained by dividing the specific activity of the ascorbic acid into total activity excreted as oxalate during the 24-hour collection period. For example, in Subject 2, 0.112 μc/day was present as oxalate and the specific activity of the ascorbate isolated that day was 0.0078 μc/mg ascorbic acid, giving a rate of 14.4 mg ascorbic acid utilized on that day. The same subject who, on the 3rd day had received 500 mg of cold ascorbic acid, continued to show an ascorbate utilization of only 15.0 mg/day.

Discussion. Studies of body composition and the use of C^{14} isotopes has here resulted in a method for stating the actual utilization of ascorbic acid by healthy men. After ingesting a single 20 μc quantity of D-glucuro-

nolactone-6-C^{14}, the urine of healthy men contains C^{14} activity which can be isolated and found in the highly purified phenylhydrazine derivative of ascorbic acid. Such activity is not found following the ingestion of D-glucuronic-6-C^{14} acid, indicating that the above lactone, but not its acid, can function as a source of ascorbic acid.

In subjects who ingest 20 μc of L-ascorbic-1-C^{14} acid, the daily urinary oxalate arising from metabolism of the labeled ascorbate is subsequently excreted as a constant proportion of total C^{14} activity remaining in the body. Thus, it can be inferred that the portion of the daily oxalate which arises from metabolism of ascorbate is formed and excreted at a constant rate. Note, since Crawhall et al.(7) showed that about 40% of the daily oxalate came from breakdown of glycine and here it is shown that another 50% comes from ascorbate, it is possible to speculate that nearly all of the urinary oxalate comes from the metabolism of glycine and ascorbate.

Ingestion of a single, comparatively large 0.5 g quantity of "cold" ascorbic acid or its precursors by a subject whose body ascorbic acid pool has been previously labeled, as described above, results in increased excretion of C^{14} ascorbate of lowered specific activity. These effects are transitory in that within 2 days total ascorbate excretion returns to previous levels and ascorbate specific activity is lower than it was prior to dilution of the body ascorbate pool.

Simultaneously, the total activity and the specific activity of the oxalate decrease, but the proportionality of total oxalate activity to specific activity of the ascorbate remains the same. From these effects, it can be inferred that the utilization breakdown of ascorbic acid in the body occurs at a constant rate irrespective of an increased rate of supply of ascorbate to the body.

Further, in 6 men of diverse body weight and degree of fatness, it was found that ascorbate utilization, as expressed in terms of C^{14} oxalate excretion, occurred at a rate of 0.207 mg per day per kilogram of fat-free body weight. Rarely, if ever, do adult males exceed 90 kg in lean body mass. Therefore, 18 mg per day intake would match the greatest quantity of ascorbate metabolized by the largest healthy man.

Summary. 1. Results of studies with healthy men revealed that close to one-fourth of D-glucuronolactone-6-C^{14} was converted to L-ascorbic acid whereas, on the other hand, no activity could be detected in the ascorbate derivative isolated from the urine of subjects receiving D-glucuronic-6-C^{14} acid. 2. One-half of the urinary oxalate arises from the breakdown of ascorbic acid and is excreted at a constant rate. 3. The size of the ascorbic acid pool and its rate of utilization were directly related to the size of the fat-free body weight.

1. Baker, E. M., Bierman, E. L., Plough, I. C., *Metabolism,* 1960, v9, 478.

2. Archer, H. E., Dormer, A. E., Scowen, E. F., Watts, R. W. E., *Clin. Sci.,* 1957, v16, 405.

3. Schaffert, R. R., Kingsley, G. R., *J. Biol. Chem.,* 1955, v212, 59.

4. Jackel, S. S., Mosbach, E. H., King, C. G., *Arch. Biochem. and Biophys.,* 1951, v31, 442.

5. Allen, T. H., Krzywicki, H. J., Worth, W. S., Nims, R. M., *U. S. Army Med. Res. Nutr. Lab. Rpt. No. 250,* 24 Sept. 1960.

6. Hellman, L., Burns, J. J., *J. Biol. Chem.,* 1958, v230, 923.

7. Crawhall, J. C., Mowbray, R. R., Scowen, E. F., Watts, R. W. E., *Lancet,* 1959, Nov. 14, 810.

Received January 8, 1962. P.S.E.B.M., 1962, v109.