DEPARTMENT OF THE ARMY
OFFICE OF THE SURGEON GENERAL
WASHINGTON, D.C. 20315

IN REPLY REFER TO:
MEDPS-PE

15 September 1966

Isotopes Branch
Division of Materials Licensing
U. S. Atomic Energy Commission
Washington, D. C. 20545

Gentlemen:

Attached are reports of nutrition and metabolism studies authorized by the Fitzsimons General Hospital License and requested by your letter of 4 August 1966.

Sincerely,

for HERSCHEL E. GRIFFIN
Colonel, MC
Chief, Preventive Medicine Division

1 Incl
as

2278

THIAMINE STUDIES

Attached is an abstract that was recently presented at the Federation of American Societies for Experimental Biology which summarizes the studies that have been carried out to date.

Current work is now in progress to attempt to identify the 27 labeled metabolites found in the urine. Future studies will include at least one more human study using the pyrimidine-^{14}C labeled thiamine to determine if the metabolic pattern is similar to that seen in the thiazole-labeled thiamine studies in man.

~~Enc 1 #3~~

ABSTRACT OF PAPER FOR PRESENTATION AT AMERICAN INSTITUTE OF NUTRITION, FEDERATION OF AMERICAN SOCIETIES FOR EXPERIMENTAL BIOLOGY, 50TH ANNUAL MEETING, 11-16 APRIL 1966, ATLANTIC CITY, NEW JERSEY

METABOLISM OF 2-^{14}C-THIAZOLE LABELED THIAMINE IN MAN

E. M. Baker, M. Balaghi, R. S. Pardini and H. E. Sauberlich
U. S. Army Medical Research and Nutrition Laboratory, Denver, Colorado

In order to study the metabolism of 2-^{14}C-thiazole labeled thiamine in man, a young male subject was fed a controlled diet providing about 1.4 mg thiamine per day. After three days on this diet, he received an oral dose of 0.592 mg 2-^{14}C-thiazole labeled thiamine (46.9 μc). The subject consumed the same diet for seventeen days after the administration of radioactive thiamine, and the excretion of the radioactivity in urine, feces and respiratory air was studied. The daily urinary excretion of radioactivity for the ten days following the dose was: 9.7, 6.7, 2.9, 2.5, 2.2, 2.0, 1.4, 1.5, 1.3 and 1.6% of the dose, respectively. The total radioactivity excreted in feces during the three days after the ingestion of the ^{14}C-thiamine, measured by combustion method, accounted for 3.8% of the dose. No measurable amount of radioactive CO_2 could be detected in respiratory air. The biological half-life of the vitamin, as calculated from the rate of excretion, was estimated to be about eighteen days. Fractionation of the desalted urine on an Amberlite CG-50 column gave a pattern similar to that obtained by Balaghi and Pearson for similarly labeled thiamine in the rat.

PYRIDOXINE STUDIES

An article which was published in the Journal of Clinical Nutrition in April 1966 summarizes the results of the pyridoxine studies to that time.

Since then, the methodology has been worked out for the isolation and characterization of the metabolites excreted in the urine from the ^{14}C-labeled pyridoxine. Future studies will be required to determine whether the pyridoxic acid is the chief metabolite and what changes in specific activity would be seen in subjects who were labeled with ^{14}C-labeled pyridoxine in terms of dietary and stress situations.

Incl #4

USE OF CARBON-14 LABELED VITAMINS IN HUMAN NUTRITION STUDIES: PYRIDOXINE AND L-ASCORBIC ACID

E. M. Baker, H. E. Sauberlich, W. H. Amos and J. A. Tillotson

U. S. Army Medical Research and Nutrition Laboratory
Fitzsimons General Hospital
Denver, Colorado 80240

Young, normal adult male human subjects received orally 30 μc of 2-^{14}C labeled pyridoxine. One subject received a constant intake of vitamin B_6 in the diet (1.5 mg/day) throughout the study; the other received an ad lib diet. Over 97% of the orally ingested labeled pyridoxine was absorbed, with no conversion to carbon dioxide, as noted by monitoring the radioactivity of the expired air. The results of these experiments indicated that the half-life of the ^{14}C-pyridoxine was 15 days in the subject who received the ad lib diet, and 20 days in the subject who received the controlled 1.5 mg of vitamin B_6 per day. The urinary metabolites of the ingested pyridoxine were separated by column and thin-layer chromatography and specific activities determined. Metabolites isolated and containing radioactivity included pyridoxal, pyridoxine, pyridoxamine and pyridoxic acid. Other radioactive compounds were noted but, thus far, have not been identified. Changes were noted with experimental time in the specific activity of the various metabolites, indicating the existence of several pools of pyridoxine. The best estimate of the total body pool of vitamin B_6 (expressed as pyridoxine) in the young adult is approximately 16-25 mg.

In a related study, the influence of a 30-day period of high pyridoxine hydrochloride supplementation (300 mg/day) on ascorbic acid pool size and utilization was investigated in two human subjects. L-ascorbic-1-^{14}C acid was employed to label the total body ascorbic acid pool. The results of this study indicated that although there was no change in the body ascorbic acid pool size, the daily utilization was markedly increased as a result of the high vitamin B_6 supplementation.

ASCORBIC ACID STUDIES

The current status of the ascorbic acid studies is summarized in the attached two articles. The first article concerning studies with ^{14}C-C_1 labeled ascorbic acid has been accepted for future publication by the Journal of Clinical Nutrition. The second article discusses the metabolism of L-ascorbic-4-^{3}H acid and will soon be published in the Biochemical and Biophysical Research Communications.

It is obvious from the results obtained from the ^{14}C-C_1 and the ascorbate-4-^{3}H that future experiments must be performed using the ^{14}C-C_6 labeled L-ascorbic acid. It would be anticipated that one would see the same results as were seen from the tritium-labeled experiments. The synthesis of the ^{14}C-C_6 L-ascorbic acid is almost completed and, upon purification and characterization, this material will be used for future animal and human studies.

Incl #5

ASCORBIC ACID METABOLISM IN MAN

E. M. Baker, J. C. Scarl and B. M. Tolbert

U. S. Army Medical Research and Nutrition Laboratory
Fitzsimons General Hospital
Denver, Colorado

and

University of Colorado
Boulder, Colorado

Short Title: Ascorbic Acid Metabolism

Send Proofs to: Lt. Col. E. M. Baker
Assistant Chief, Chemistry Division
U.S. Army Medical Research
and Nutrition Laboratory
Fitzsimons General Hospital
Denver, Colorado 80240

The catabolic fate of L-ascorbic acid in man has been studied using L-ascorbic acid labeled with either carbon-14 or carbon-13 in the number 1 position (1-3). On ingestion, ascorbic acid enters the body pool of ascorbic acid and is excreted in the form of urinary ascorbic acid (AsH_2), dehydroascorbic acid (dAs) and urinary oxalate. The oxalic acid contains the number 1 carbon of ascorbic acid and is therefore presumed formed by C_2-C_3 carbon cleavage. Whether glyoxalic acid is an intermediate in this process is unknown, and the site, function, mechanism and immediate precursor of this catabolic process are unknown. The comparative excretion of ascorbic and dehydroascorbic acid in the urine has been studied (4), but the origin of dAs is uncertain, since it could arise by chemical oxidation of AsH_2 in the urine.

A current question in the catabolism of ascorbic acid, which we believe to have an important bearing in future work on the role of ascorbic acid in biological systems, is whether $^{14}CO_2$ is a metabolic product of its degradation. Hellman and Burns' (1) study in 1958 showed that less than 5% of the number 1 carbon was converted to CO_2. Baker et al. (2), in a study in 1962, showed a conversion of a maximum of about 1%. Abt and co-workers (5), in 1963, showed a conversion of some 53 to 66% of the number 1 carbon to CO_2. In a study of the kinetics of ascorbate catabolism and turnover by Atkins (3) in 1964, the conclusion was made in the analysis of the data that 44 to 57% of the ascorbate was 'catabolized by pathways compatible with the production of CO_2 from the number 1 carbon.' An explanation for the discrepancy between the work of Baker or Hellman and Burns on one hand, and of Abt on the other, was proposed by Baker (6) in a paper published in 1963. In this study, it was shown that ascorbic acid rapidly decomposes in aqueous solution, and that the large conversion of ascorbic acid to CO_2 observed by some workers was probably due to impurities in the material used. In fact, the published radioautogram of the material used by Abt (5) showed an appreciable contamination of the

reduced ascorbate by an oxidized product, probably dAs. It is the purpose of this paper to present quantitative results on the catabolism of carefully analyzed labeled ascorbic acid in man, to show how the results are affected by oxidation impurities in the ascorbic acid and to calculate turnover times and pool sizes from the experimental data.

MATERIALS AND METHODS

Experimental

1. Ascorbic-1-^{14}C acid, purchased either from the California Biochemical Corporation or New England Nuclear Corporation, was used. When this material was used as fresh solutions, the sample was opened, dissolved in water and consumed by the subject within 5 minutes. Assays were also initiated at the same time.

2. Chromatographic analysis of labeled ascorbate samples was made by paper chromatography (6) or by thin-film chromatography (7) on silica gel plates using an acetonitrile-butyronitrile solvent system. In some cases, radioautographs were prepared and analyzed by an Anitrol densitomer. In others, the chromatogram was analyzed by a strip chart counter.

3. Continuous assay of the respired $^{14}CO_2$ was made by vibrating reed electrometer and ionization chamber, using a Cary Respiration Pattern Analyzer. The $^{14}CO_2$ analysis by this instrument was cross-calibrated against the liquid scintillation counting procedure used in the other assays, with the following results: 10-minute sample of subject RSH, by ionization chamber 0.0109 μc, by scintillation counting 0.0105 μc; 10-minute sample of subject TIN, by ionization chamber 0.0078 μc, by scintillation counting 0.0075 μc. $^{14}CO_2$ samples were absorbed in ethanolamine-methyl cellosolve solution (1:2).

4. The original ascorbate sample and the whole urine samples were counted in the liquid scintillation counter. For these assays, 0.2 ml raw urine was dissolved in 15 ml of dioxane-naphthalene-PPO-dimethyl-POPOP scintillation solutions, counted, spiked with toluene-^{14}C and recounted.

Calcium oxalate samples were dissolved in dilute hydrochloric acid and scintillation counted, spiked and recounted. The 2,4-dinitrophenylhydrazine derivative of ascorbic acid (DNPH-As) was counted as solid samples in a low background Nuclear-Chicago planchet counter.

5. Urinary oxalate was determined and isolated as calcium oxalate by the method of Archer et al. (8). Urinary ascorbate was analyzed by iodometric titration after isolation on a Dowex column, and was followed by preparation of a DNPH-derivative by the method of Searl et al. (9). This derivative was recrystallized to constant specific activity. No less than three recrystallizations from acetonitrile were needed.

6. Ingested ascorbic acid was calculated from dietary analysis using standard food table and, in some cases, was supplemented with 100 mg/day of crystalline vitamin C.

RESULTS

Figure 1 shows results on the analysis of labeled ascorbic acid used in the three subjects reported in detail in this paper. The first study, with subject RSH-2, used fresh ascorbic acid labeled with carbon-14 which had been stored in a sealed ampule. The results of the analysis were quite good; the sample was 95% ascorbic acid. The second study is reported on subject TIN-2; this study was also done with a sample of freshly-prepared ascorbic acid. However, this material was from a different supplier and had not been stored under vacuum. It contained an appreciable amount of dehydroascorbic acid, as well as unknown material which stayed at the origin. The third study to be reported in detail, subject CR-2, used a sample of ascorbic acid which was aged for 24 hours in a 0.05% solution. As seen in Figure 1, only 15% of the ascorbic acid remained after aging, and most of the material had oxidized to dehydroascorbic acid and its hydrolysis product, diketogulonic acid (DKG).

Subject RSH-2 ingested 43 μc of the ascorbic acid, whose analysis is shown in the top chromatogram of Figure 1. Following that, his total urinary-^{14}C, urinary ascorbate-^{14}C, urinary oxalate-^{14}C and breath $^{14}CO_2$ were monitored. Results are given in Figure 2. The urinary ascorbate-^{14}C includes both ascorbic acid and dehydroascorbic acid, which are analyzed simultaneously by the dinitrophenylhydrazine procedure. The initial breath excretion of carbon-14 was equal to or less than 0.16 μc and rapidly decreased to background. After the first 6 hours, no carbon-14 could be detected in the breath. The radioactivity in the urine oxalate and ascorbate decreased with a half-time of approximately 12 days. An excellent material balance was obtained, and the summation of the urine oxalate and the urine ascorbate equaled the total amount of radioactivity found in the urine, both in cumulative total and for each individual day. A material balance for the excretion of carbon-14 in subject RSH-2 is presented in Table I. The 24-hour excretion showed 2.6 μc of carbon-14 in the urine, of which 2.04 μc was ascorbic acid and 0.5 μc was urinary oxalate.

The accountability of the urinary-^{14}C as oxalate or ascorbate held not only for the first 24-hour excretion, but also for all other days analyzed through half-life. Total urinary-^{14}C, urinary oxalate-^{14}C and ascorbate-^{14}C values can be calculated by summing up the daily excretion through the half-life and multiplying this value by two. These results were analyzed for material balance. Total urinary-^{14}C accounted for 97% of the activity in the ingested ascorbic acid. This excellent material balance in the cumulative excretion, taken together with the very low breath $^{14}CO_2$, indicates that for this relatively pure sample of ascorbic acid, there is essentially no catabolism of the labeled ascorbic acid to CO_2, nor is there any need to infer such processes, as was done by Atkins (3). The small amount of $^{14}CO_2$ that was observed can be ascribed to the dehydroascorbate present in the ingested sample, as will be shown later in this paper. In Table I, cumulative urinary ascorbate excretion

is obtained by difference of total urinary-^{14}C and oxalate. However, as is seen in Figure 2, similar balance was obtained by direct assay of the DNPH derivative of the urinary ascorbate.

Subject RSH-2 received 300 mg/day of pyridoxine HCl for a period of 30 days immediately prior to this study. The high vitamin B_6 caused a higher oxalate excretion than normal, reflecting an increased utilization of ascorbic acid (10).

Table II presents the half-life and pool size analysis of the ascorbic acid study on subject RSH. From the specific activity of the ascorbic acid, RSH was shown to have a pool size of 2.3 g. The half-life, based on decrease in the specific activity of urine components as shown in Figure 2, gave a value of 12 days. On the basis of the pool size and the ingestion data for the ascorbic acid, which was 211 mg/day, a theoretical value for the turnover time would have been 8 days. A similar low result was obtained, making a calculation based on the pool size and the ascorbic acid (206 mg/day) that was excreted or catabolyzed to oxalate. It would thus appear that at this very high ingestion rate of ascorbic acid (200 mg/day), not all of the dietary ascorbic acid equilibrates with the vitamin C pool.

Table III presents the metabolic data on subject TIN-2. TIN-2 ingested 48.4 μc of labeled material, which contained 76% ascorbic acid, some 18% dehydroascorbic acid, relatively small amounts of diketogulonate and an unknown material remaining at the origin. Calculated cumulative excretion data showed a total of 29.7 μc of carbon-14 in the urine. In the breath, 2.76 μc of $^{14}CO_2$ were exhaled, all of which was excreted in the first day. This gives a total accountability in the subject of 67% of the material ingested. The fate of the remaining material is unknown. It could have been incorporated into body components with relatively long turnover times, such as carbohydrates, fats or proteins, and it may have been excreted at levels below the threshold of detectability in the breath and the feces. The total radioactivity ingested

as ascorbate itself is calculated to be 37 μc plus 10% of the dehydroascorbate, which is presumed reduced to ascorbate on the basis of data to be presented later for subject CR. The urinary carbon-14 of 29.7 μc corresponds to 79% of the ingested reduced ascorbate and ascorbate equivalent. We have no satisfactory explanation for this low recovery, except to say that when working in impure materials, it has not been possible to obtain good material balances. The half-life of the ascorbate pool, calculated from the rate of decrease of urinary and oxalate carbon-14, was found to be 22 days, and this checked quite well with the half-life calculated from the assay of the ascorbic acid in the urine and the pool size, which gave a value of 23 days. The pool size of ascorbic acid in subject TIN-2 was calculated as 2.80 g, using the known specific activity of the ascorbic acid and the amount of ascorbic acid that we could account for in the material ingested.

A study was made on subject CR, using labeled ascorbic acid which had been aged for 24 hours. Analysis of the ingested material showed that it contained mostly dehydroascorbic acid and DKG and only 16% ascorbic acid. Cumulative excretion of breath $^{14}CO_2$ from this material was 25.8%, consistent with the high dehydroascorbate and diketogulonate component (see Table IV). Analysis of the ascorbic acid excreted in the urine showed a specific activity that was higher than could be accounted for by the amount of ascorbic acid present in the ingested material. It was, therefore, not possible to calculate an ascorbic acid pool size. On the other hand, it is possible to make an estimation of how much dehydroascorbate was reduced back to ascorbic acid in the ingested material. If we assume that the ascorbic acid pool of CR was 2.7 g, which is consistent with the ascorbic acid pools observed for a number of other subjects of comparable size and lean body mass (2), we find that the specific activity data on the labeled ascorbic acid in the urine correspond to an ascorbic acid pool content of 9.2 μc. This means, then, that some 3.4 μc

of the dehydroascorbic acid must have been reduced back to ascorbic acid and entered the ascorbic acid pool. Thus, some 5 to 10% of the labeled dehydroascorbic acid entered the ascorbic acid pool when the material was administered orally. This is the origin of the dehydroascorbate contribution to the AsH_2 pool assumed in Table III. In the 24-hour excretion, the total urinary-^{14}C is much larger than the sum of the urine oxalate and the urine ascorbate excretion. Furthermore, the urine oxalate excretion is much larger than the ascorbic acid excretion. Normally, the labeled urinary oxalate excretion is approximately one-fourth of the urinary ascorbate excretion. These data indicate that an appreciable amount of the oxidized forms of ascorbic acid are converted to oxalic acid and other labeled urinary products in the first day. These data also indicate that most of the oxidized forms of ascorbic acid enter metabolic pathways other than that characteristic of AsH_2 itself, probably entering the pentose shunt through the formation of xylitol, an intermediate in the C-6 oxidative pathway of glucose (11).

DISCUSSION

Table V presents a summary of the ascorbic acid pool sizes and the turnover times for two studies each on subjects RSH and TIN. In all cases, the pool size was found to be between 2.3 and 2.8 g, calculated by dividing the amount of ascorbate activity ingested by the specific activity of the excreted ascorbate. The ascorbate pool remained essentially constant during the experimental period. This figure corresponds to 29 mg/Kg, a value quite consistent with our earlier results and those of Hellman and Burns (1). The higher and lower results of Atkins (3) and Abt (5) are probably due to impurities in the ascorbic acid, as is indicated by their implied or observed catabolism of ascorbate to CO_2.

Our data confirm that the pool size is limited by factors other than ascorbic acid intake. Our earlier results related the pool size to fat-free lean body mass. The variability in pool size of subject TIN is probably not significant and may arise from problems introduced by the impure nature of the labeled ascorbate.

In subject RSH, on a high ascorbate intake, the turnover of the labeled ascorbate pool is appreciably longer than would be expected by mathematical analysis of the pool size and ascorbate ingestion data. This indicates that part of the ingested ascorbate may not equilibrate with the labeled ascorbate pool because of rapid excretion. This problem did not occur during the labeling procedure because all subjects were labeled by ingesting high specific activity ascorbate (30 μc/mg) on a day when ascorbate was withheld from their diet. No cold carrier was given with the labeled material. Under these conditions, essentially no spillage of labeled ascorbate occurred, as confirmed by the first day's analysis and material balance.

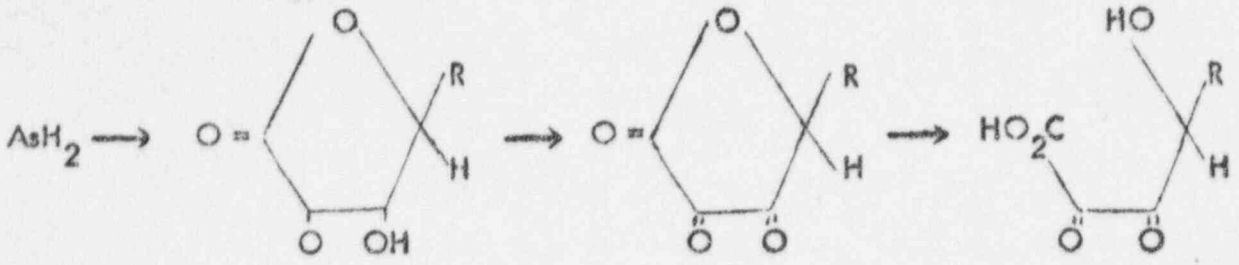
Turnover time. It is possible to calculate a half-time for turnover of the ascorbate pool by five methods. Three are by direct graphical analysis of the excretion data, either the total urinary-^{14}C, the ascorbate-^{14}C, or the oxalate-^{14}C. The two are based on mathematical analysis, using the pool size data and either the ascorbate ingestion or excretion, assuming a first order excretion process as shown in Figure 1, and as previously demonstrated by other studies (3). The equation used is as follows:

$$\frac{d(c)}{dt} = \frac{(0.693)(c)}{T_{1/2}} = \text{daily excretion or ingestion}$$

$$T_{1/2} = \frac{(0.693)(c)}{\frac{d(c)}{dt}}$$

where c = ascorbate pool in grams; t = time; $T_{1/2}$ = half-time for turnover. The half-times for turnover are comparable, whether calculated by direct analysis or by ingestion and excretion data.

Oxidative pathway. The presently accepted pathway for the oxidative decomposition of ascorbic acid in aqueous solution is as follows:



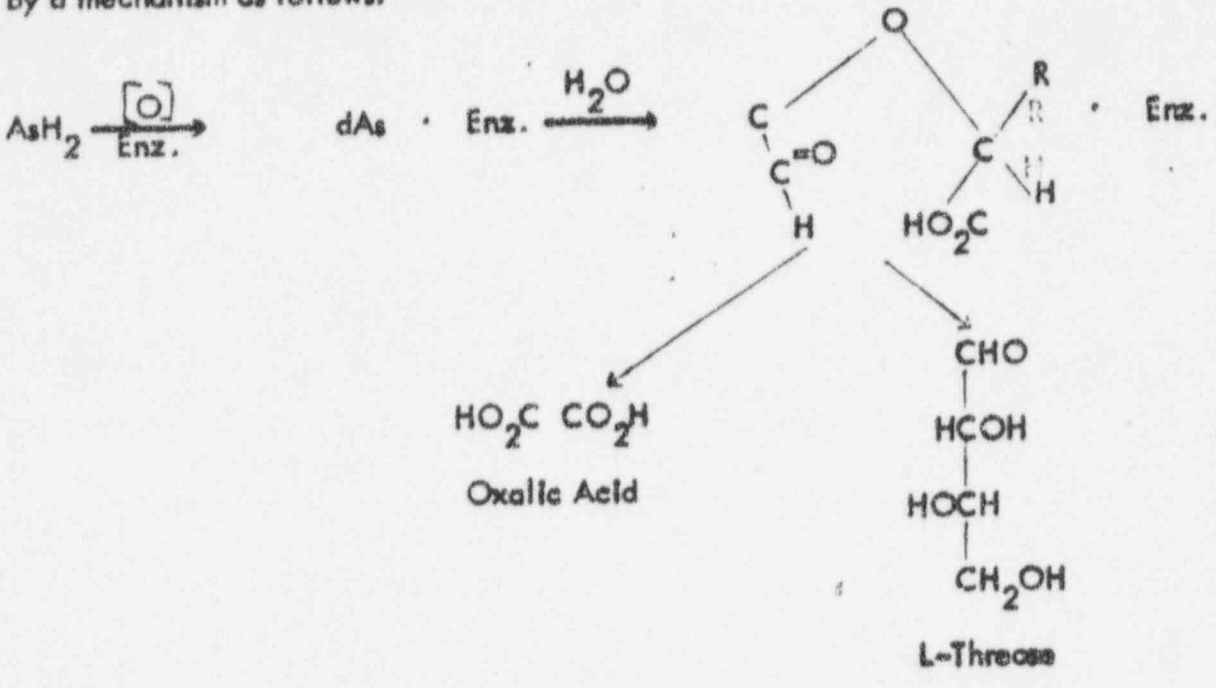
AsH
Monodehydro-
ascorbic Acid

dAs
Dehydro-
ascorbic Acid

DKG
2,3-diketo-
gulonic Acid

The ascorbic acid is oxidized to a monodehydroascorbic acid, a free radical intermediate which exists in very small concentrations but has been detected by electron spin resonance (12, 13). This substance is then oxidized to dehydro-ascorbic acid, a stable lactone, which is hydrolyzed to 2,3-diketogulonic acid. This moderately unstable diketo acid decomposes to a variety of compounds, as yet unknown. This reaction proceeds autocatalitically in dilute aqueous solution, and in experiments that we have made, an 85% loss of ascorbic acid was observed in 48 hours. The reaction requires oxygen and proceeds at a very slow or negligible rate in a nitrogen swept sample (14). It is very likely that the reaction involves intermediate peroxides, as has been discussed and suggested by Mason (15). This decomposition sequence can be studied using thin-film chromatography with acetonitrile-butyronitrile developing solvent on silica gel plates since this solvent system easily separates the ascorbic acid from the dehydroascorbic acid and the 2,3-diketogulonic acid (9). It is possible, by this process, to separate and quantitate the amount of each one of these materials in a given sample. The use of an analytical method such as this to check the purity of any compounds used in the study of ascorbic acid metabolism is absolutely essential to obtain reliable results.

One question yet remains. Are dAs and DKG intermediates in ascorbate metabolism? It is doubtful that DKG is an ascorbate metabolite, for then we would expect to find $^{14}CO_2$ in the breath and/or an incomplete material balance after ingesting labeled ascorbic acid. By a similar argument, it can be suggested the dAs in a "free" form is also not a normal metabolite, although it may well be formed as a bound molecule. Since DKG is not a likely metabolite, we then suggest that the cleavage of the C-2 to C-3 bond may occur enzymatically while the ascorbate lactone ring is still intact, perhaps by a mechanism as follows:



The L-threose would be an abnormal metabolite and could be directly excreted, excreted as a conjugate, or else converted to a normal metabolite and enter normal metabolic pathways. Further indications that this cleavage is enzymically controlled is furnished by recent work of Baker and Canham on high vitamin B_6 studies in man (10).

It is unlikely that free glyoxalate is a catabolic product of ascorbate metabolism in view of the lack of $^{14}CO_2$ excretion observed in this study. Major pathways in glyoxate metabolism (16) include this process, which is catalyzed by a B_1-NAD enzyme system.

In conclusion, then, we show that the only known metabolic products of vitamin C in man are oxalate, AsH_2 and dAs. In young, healthy male volunteers studied, the AsH_2 pool size is 2-3 grams and the turnover half-time is about 20 days on AsH_2 intakes of about 100 mg/day.

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TABLE I

Material Balance for Excretion of Carbon-14 in Subject RSH-2* Following Ingestion of Ascorbic-1-^{14}C Acid

Ingested: 42.7 μc		
AsH_2	40.8 μc	95.3%
dAs	1.8 μc	4.3%
DKG	0.04 μc	0.1%
24-hour Excretion:†		
Total Urine-^{14}C	2.59 μc	
Urinary AsH_2-^{14}C	2.04 μc	
Urinary Oxalate-^{14}C	0.54 μc	
Breath $^{14}CO_2$	≤ 0.16 μc	
Cumulative Excretion (based on half-life):		
Total Urinary-^{14}C	39.5 μc	97.0%
Urinary Oxalate-^{14}C	9.2 μc	22.6%
Urinary AsH_2-^{14}C ‡	30.3 μc	74.4%
Breath $^{14}CO_2$	≤ 0.16 μc	≤ 0.4%

*Subject received 300 mg/day pyridoxine HCl for a period of 30 days immediately prior to this study in conjunction with a separate study.

†Based on ingested AsH_2-1-^{14}C.

‡By difference, confirmed by direct assay.

TABLE II

Half-life and Pool Size Analysis of Ascorbic-1-^{14}C Study

Subject RSH-2*

AsH_2 Pool Size by Specific Activity of AsH_2-^{14}C	2.29 g
Half-life of AsH_2-^{14}C by Urinary AsH_2-^{14}C, Urinary Oxalate-^{14}C, or Total Urinary-^{14}C Data †	12 d
Half-life by Pool Size and Ingestion Data	
AsH_2 Ingestion = 211 mg/day	8 d
Pool Size = 2.29 g	
Half-life by Pool Size and Excretion Data	
AsH_2 Excretion and Catabolism = 206 mg/day	8 d
Pool Size = 2.29 g	

*Subject received 300 mg/day pyridoxine HCl for a period of 30 days immediately prior to this study.

† Data calculated assuming a first order process.

TABLE III

Ascorbic-1-^{14}C Study with Subject TIN-2

Ingested: 48.4 μc		
AsH_2	76.5%	37.0 μc
dAs	18.5%	9.0 μc
DKG	1.5%	0.7 μc
Unknown	2.9%	1.4 μc
Cumulative Excretion:		
Total Urinary-^{14}C	61.4%	29.7 μc
Urinary Oxalate-^{14}C	23.1%	11.2 μc
Urinary AsH_2-^{14}C*	38.3%	18.5 μc
Breath $^{14}CO_2$	5.7%	2.76 μc
Accountability:		
Of Total Ingested	$\frac{2.76 + 29.7}{48.4}$ = 67%	
Of AsH_2-^{14}C Ingested	29.7/(37 + 10% of 9.0) = 79%	
Kinetic Analysis:		
Pool Size	2.80 g†	
Half-life by ^{14}C Excretion	22 d	
Half-life by Excretion Assay‡	23 d	

* By difference.

† Based on accountability.

‡ Total excretion was 45 mg AsH_2 + 40 mg oxalate (AsH_2 equivalent) = 85 mg/day.

TABLE IV

24-Hour Aged Ascorbic-1-^{14}C Study on Subject CR

Ingested: 37.3 μc		
AsH_2	16.9%	5.8 μc
dAs	53.2%	20.0 μc
DKG	28.7%	10.7 μc
Unknown	2.1%	0.8 μc
Cumulative Excretion:		
Breath $^{14}CO_2$	25.8%	9.61 μc

dAs Labelling of AsH_2 Pool:

Assume AsH_2 pool = 2.7 ± 0.3 g. From specific activity data, this corresponds to 9.2 μc into AsH_2 pool. 9.2 - 5.8 = 3.4 μc from dAs. Thus, 5 to 10% of dAs-^{14}C must have entered the AsH_2 pool when administered orally.

24-Hour Excretion:

Total Urinary-^{14}C	2.90 μc
Urinary Oxalate-^{14}C	0.87 μc
Urinary AsH_2-^{14}C	0.14 μc

TABLE V

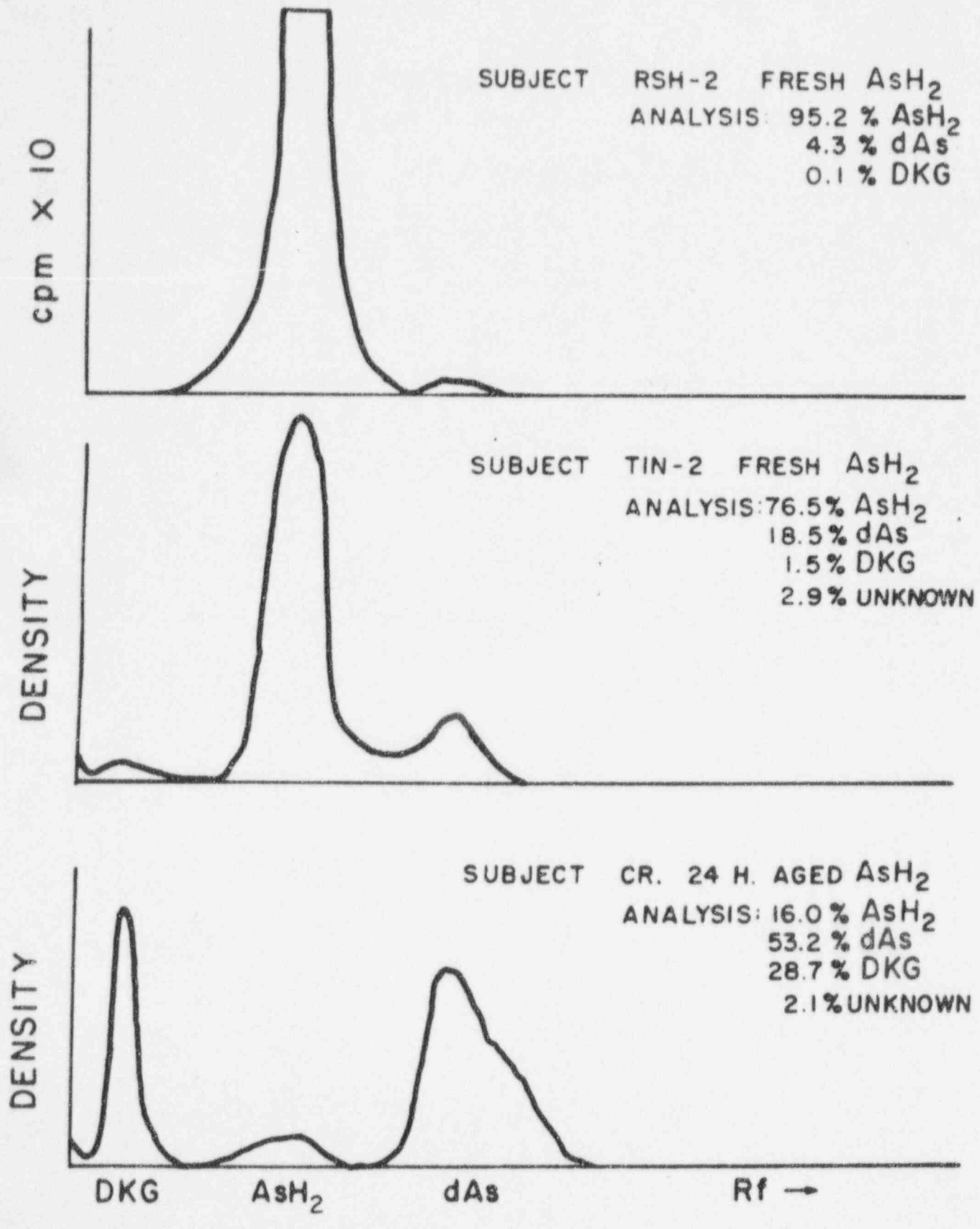
Summary of AsH_2 Pool Sizes and Turnover Times in Male Volunteers

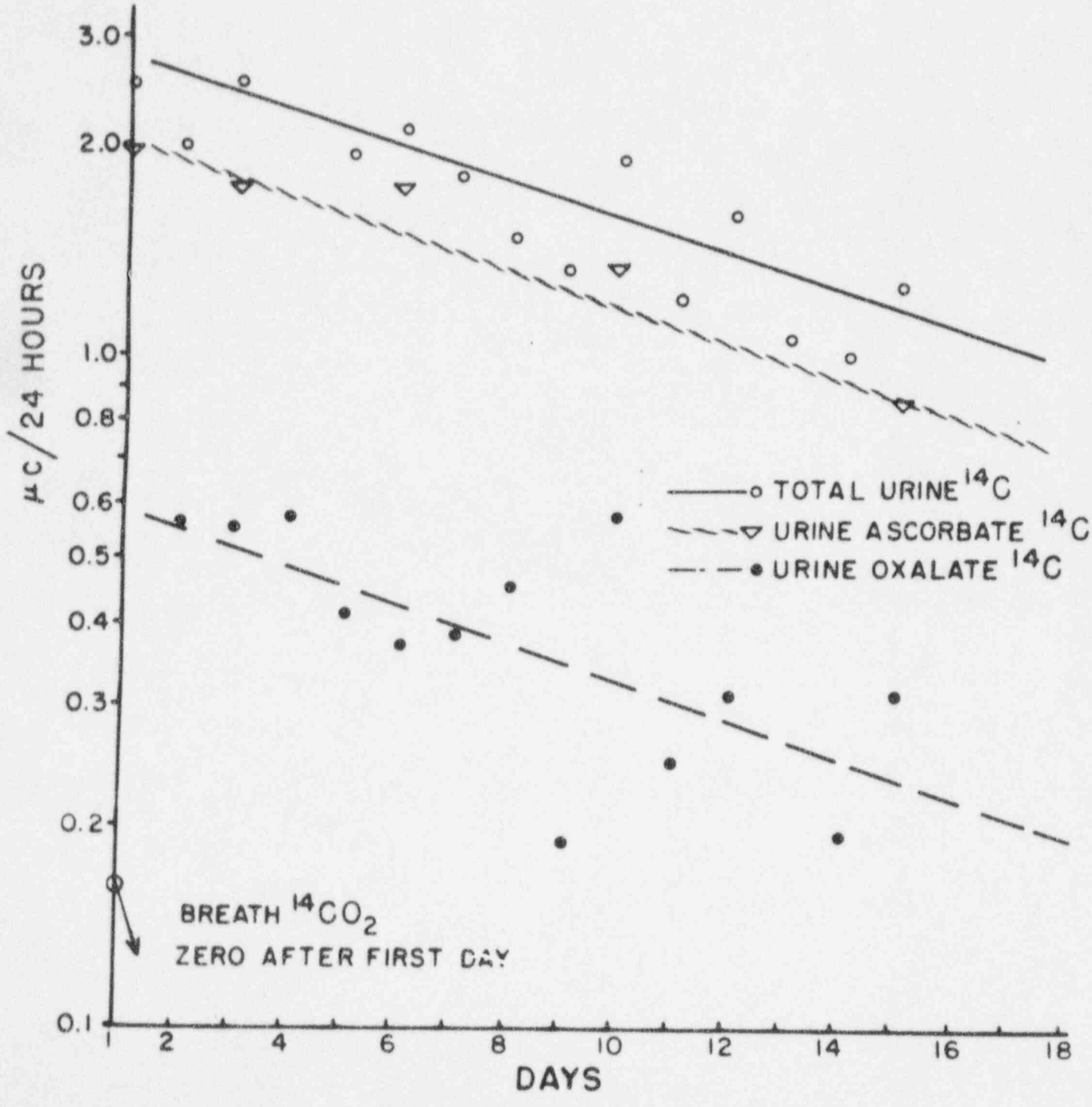
Subject	Pool Size by Sp. Act., g	$T_{1/2}$ by ^{14}C Sp. Act. by Decrease in Log Conc.	"Ascorbate" Plus Equivalent Ascorbate Excretion, mg/Day	$T_{1/2}$ by Excretion	$T_{1/2}$ by Ingestion
RSH-1	2.42	20 d	110	14.4 d	15.3 d
RSH-2	2.29	12 d	206	8 d	8 d
TIN-1	2.28	24 d	66	24 d	
TIN-2	2.80	22 d	85	23 d	

LEGENDS FOR FIGURES

Fig. 1: Analysis of ascorbic acid solutions that were ingested by subjects in this study. Radioactivity was analyzed either by a strip counter or by Anytrol densitometer readings of a radioautograph.

Fig. 2: Excretion of carbon-14 following oral ingestion of ascorbic-1-^{14}C acid in subject RSH-2. Half-time for urine radioactivity decrease is 12 days.


cpm X 10
SUBJECT RSH-2 FRESH AsH2
ANALYSIS 95.2 % AsH2
4.3 % dAs
0.1 % DKG
DENSITY
SUBJECT TIN-2 FRESH AsH2
ANALYSIS: 76.5% AsH2
18.5% dAs
1.5% DKG
2.9% UNKNOWN
DENSITY
SUBJECT CR. 24 H. AGED AsH2
ANALYSIS: 16.0 % AsH2
53.2 % dAs
28.7 % DKG
2.1 % UNKNOWN
DKG
AsH2
dAs
Rf →


3.0
2.0
1.0
0.8
0.6
0.5
0.4
0.3
0.2
0.1
μc/24 HOURS
TOTAL URINE ^{14}C
URINE ASCORBATE ^{14}C
URINE OXALATE ^{14}C
BREATH $^{14}CO_2$
ZERO AFTER FIRST DAY
1
2
4
6
8
10
12
14
16
18
DAYS

METABOLISM OF L-ASCORBIC-4-^{3}H ACID IN MAN(*)

Bert M. Tolbert, Agnes W. Chen, Ellen M. Bell and Eugene M. Baker

Department of Chemistry, University of Colorado, Boulder, and
U.S. Army Medical Research and Nutrition Laboratory, Denver, Colorado

The kinetic and metabolic fate of ascorbic-4-^{3}H acid has been studied in a human subject. The radioactive label does not enter the body water pool, but instead is excreted as organic-bound tritium. The excretion products were found to be ascorbic acid and its immediate oxidation products, and unknown organic compound(s). Kinetic analysis of the data shows half times of 2 days and 46 days for turnover of the labeled ascorbic acid and the unknown compound respectively. These results, combined with previous ascorbate-1-^{14}C studies indicate that the unknown metabolite(s) are probably derivative(s) of L-threose or L-threonic acid.

The metabolic fate of ascorbic acid (AsH_2) in man has been extensively investigated using ascorbic-1-^{14}C (Hellman and Burns, 1958; Baker et al., 1962). The carbon-14 studies show that the label is either excreted in AsH_2 or one of its oxidation forms -- dehydroascorbic acid (dAs) or diketogulonic acid (DKG) -- or as free oxalate. No other catabolic forms are observed. The fate of the 4-carbon piece formed by the presumed cleavage of the C-2,3 bond remained unknown.

The metabolic fate of orally ingested ascorbic-4-^{3}H acid has now been studied in a human subject. The subject received 105 μC of material con-

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*Supported in part by grant DA-49-193-MD-2611 from the Surgeon General's Office of the U.S. Army.

taining 10 mg ascorbic acid. His daily ascorbate intake was about 250 mg/day except on ingestion day, when he received no supplementary AsH_2 except that contained in the labeled material. The urinary excretion of the following items were followed: tritium in urine water; total urine tritium; urine AsH_2, dAs and DKG; tritium in urine AsH_2, dAs and DKG, and in undetermined organic compound(s).

Materials and Methods

AsH_2-4-^{3}H was prepared by exchange labeling of dipotassium ascorbate (Bell et al., 1966). It was chromatographically pure (Saari et al., 1966) except for traces of DKG, dAs and L-araboascorbic-4-^{3}H acid. Tritium assays were made in a dioxane-naphthalene-PPO-dimethyl POPOP solvent system or in a solubilized toluene-PPO-dimetnyl POPOP system by liquid scintillation counting.

Organic components of the urine were separated by thin layer chromatography (TLC) on silica gel plates. Areas on the plates corresponding to the labeled organic products were scraped off and eluted with water. Aliquots were counted for tritium and analyzed for ascorbate by a modification of the Shaffert-Kingsley method (Shaffert and Kingsley, 1955). Urine water was obtained by low temperature distillation of urine treated first with charcoal and then with dinitrophenylhydrazine to prevent decomposition of any labeled ascorbate.

Results and Discussion

Figure 1 shows the specific activity of tritium in urine water vs. time. The data follow a first order excretion process corresponding to a half-life of 13.7 days and extrapolate to an initial specific activity of

18.5×10^{-5} μC/ml. This value gives 8.2 μC as the amount of tritium initially incorporated into the body water, based on a subject weight of 88.6 Kg and an estimated body water of 50%.

The 13.7 day half-life is consistent with other studies on the half-life of body water. This result indicates that essentially none of the ascorbate label enters the body water pool after the first day. The 7.8 percent that entered the water pool initially is also consistent with the expected labeled impurities in the labeled ascorbate, which included a small amount of exchangeable hydrogen on the OH groups of the AsH_2 itself.

Figure 2 presents a log plot of the organic-bound tritium remaining in the subject plotted vs. time in days. These organic-bound tritium values were obtained by taking the tritium ascorbate ingested (105 - 8.2 μC) and subtracting the summation of organic tritium found in the daily urine. The data may be resolved into two first order processes -- one of 2.0 days and one of 46 days. The 46 day component is an unknown organic compound(s) that did not migrate in TLC system employed in the analysis. Some 68% of the ascorbate tritium entered this pool. Very high urine excretions of organic-bound tritium two months after ingestion day confirm that this highly labeled and long-lived pool cannot be an artifact of the analytical procedures.

The 2 day component is probably labeled ascorbate and its oxidation products. Analysis of direct assay data of the specific activity of ascorbate and its oxidation products gives a half-life of 3-4 days. The discrepancy between 2 days and 3-4 days is within analytical error. The initial specific activity of the urine AsH_2 gives a pool size in this sub-

ject of 2.84 g. This value agrees well with pool sizes found in previous studies with ascorbate-1-^{14}C in man (Baker et al., 1966; Hellman and Burns, 1958). An ascorbate intake of 250 mg/day into a 2.84 g pool gives a calculated half-life of 8 days. This calculated half-life agrees well with half-lives determined using one-labeled ascorbate (Baker et al., 1966). The discrepancy between 2-3 days and 8 or more days is quite interesting and appears outside of experimental error. It could arise if the one-labeled ascorbate studies measure turnover of the total ascorbate pool and our 4-labeled study measures a mobile or readily exchangeable pool. In any case, the data indicate the presence of more than one kinetically distinguishable pool in the metabolism of ascorbic acid.

Studies with ascorbate-1-^{14}C do not show any long half-life excretion of the C-1 label. We must therefore assume that the 46 day component represents the excretion of a metabolite which has lost the C-1 carbon and also, by implication from the oxalate data, the C-2 carbon (Baker et al., 1962). Cleavage of the C-2,3 bond of ascorbate could give L-threitol, L-threose or L-threonic acid. Oxidation of either the C-1 or C-4 carbon of these 4-carbon compounds should lead to further degradation of this entity and release of the organic-bound tritium into the body water pool. We therefore postulate that this long-lived component represents a 4-carbon metabolite of ascorbic acid held to some essential structure of the body by a covalent bond. It may represent a yet unknown and unsuspected indirect function of vitamin C. Studies of this long-lived metabolite are underway and may lead to interesting new aspects of the nutritional and physiological role of vitamin C.

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Saari, J. C., Baker, E. M., and Sauberlich, H. E., Anal. Biochem., in press, (1966).

Shaffert, R. R. and Kingsley, G. R., J. Biol. Chem., 212, 59 (1955).

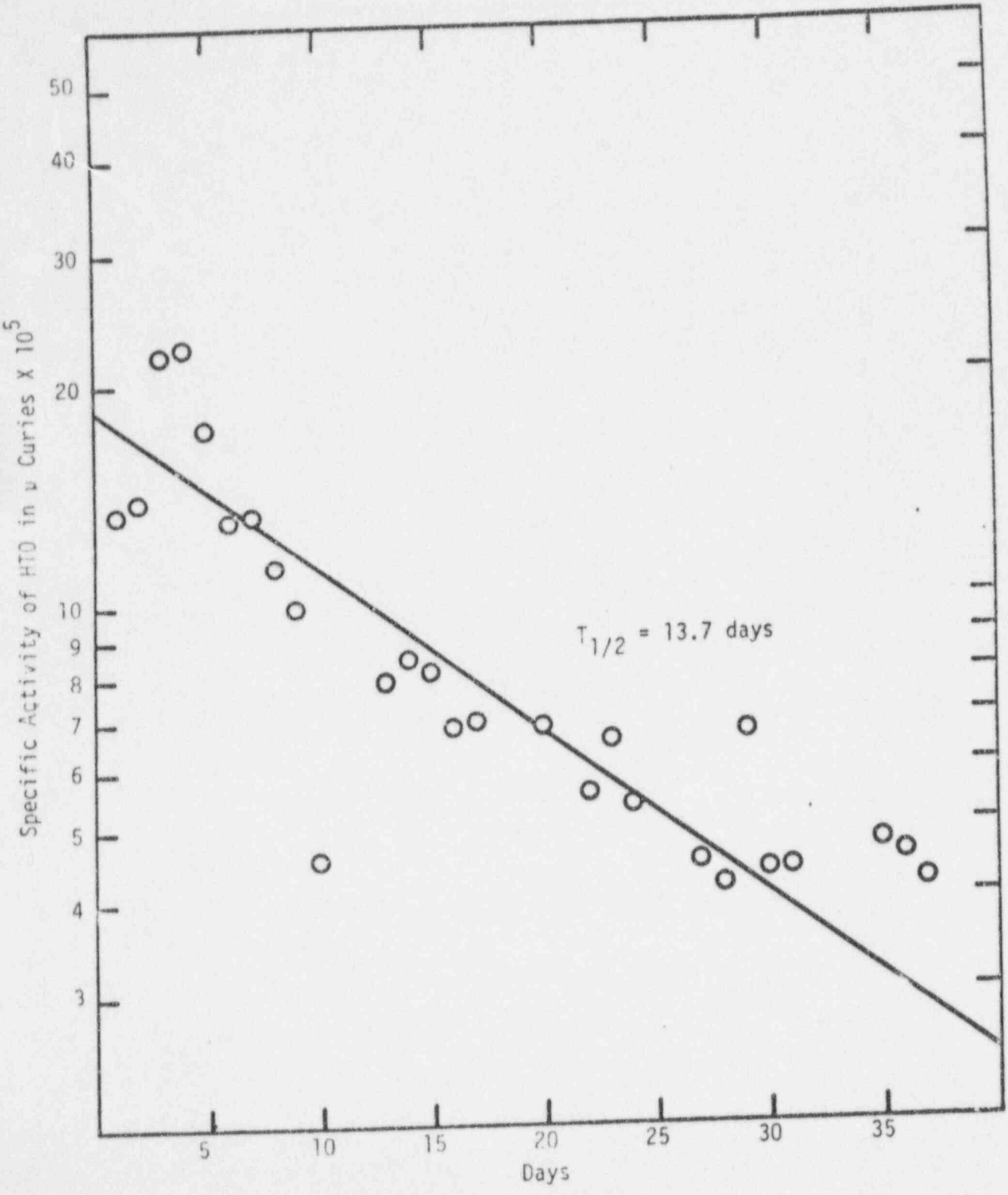

Fig. 1. Specific activity of HTO distilled from urine plotted against time in days after ingestion of ascorbate-4-^{3}H.

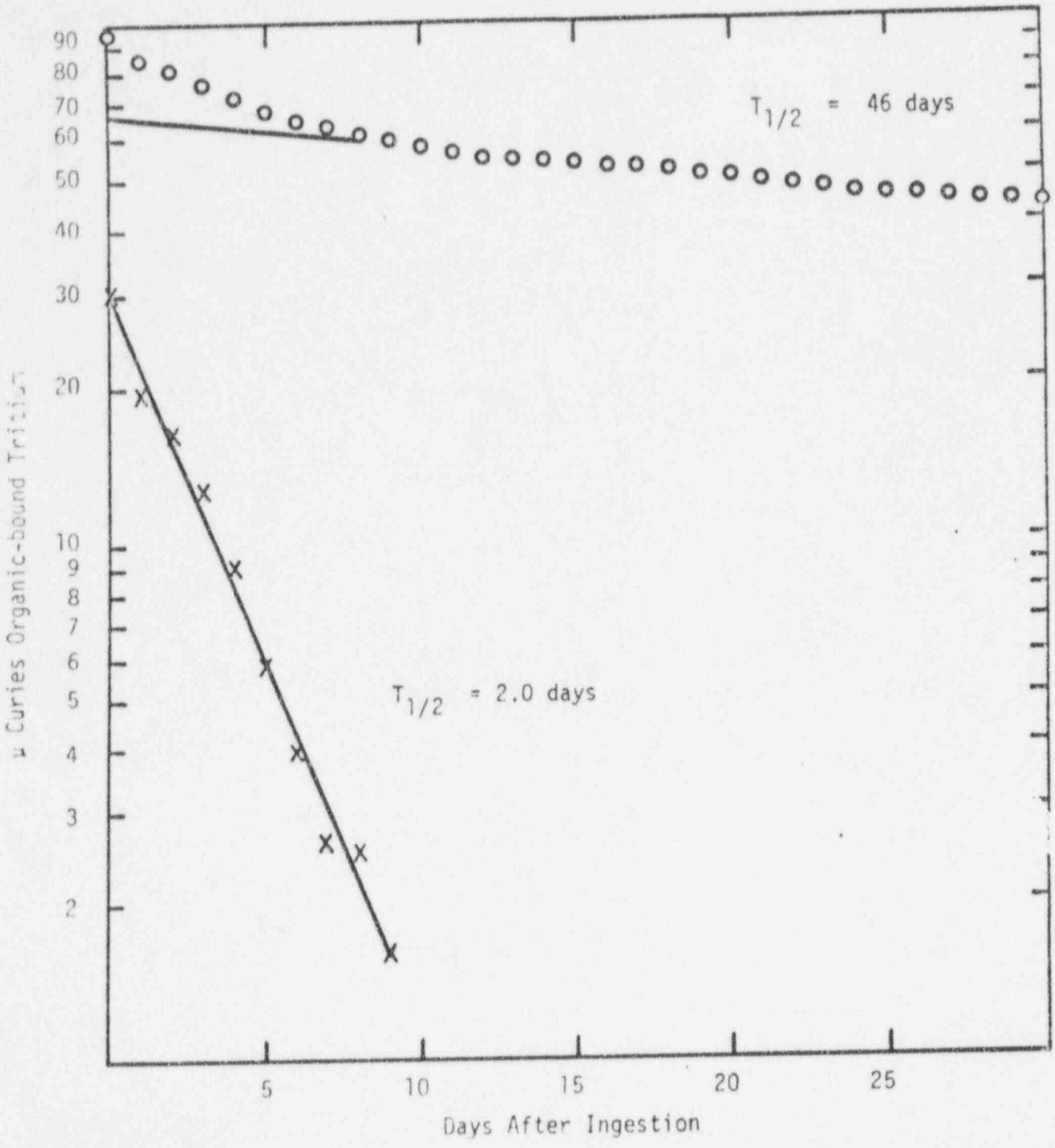

Fig. 2. Radioactivity excretion data vs. time in days. The human subject ingested 96.8 μC L-ascorbic-4-^{3}H acid. The data, circles, for the organic-bound tritium remaining in the subject are analyzed as two first order processes. The X values are differences between μC remaining organic-bound tritium and the extrapolated long lived component.

CARBOHYDRATE (CELLULOSE) STUDIES

Preliminary studies with ^{14}C-labeled cellulose have been carried out in the rat, but no human studies have been done to date.

^{14}C-labeled Avicel-R was prepared from ^{14}C-labeled cotton which was grown at this Laboratory. The preparation was made by American Viscose and returned to us. Thus far, the animal (rat) studies have been completed, showing that there is absolutely no degradation or digestion of the ^{14}C-labeled cellulose. It is anticipated that one human subject will receive the ^{14}C-labeled Avicel in the near future. One would expect similar results in man as was seen in the rat.

Incl #6

RIBOFLAVIN STUDIES

^{14}C-labeled riboflavin studies in man are planned in the future, contingent upon completion of isolation techniques and characterization of the urinary metabolites. The rat studies are now in progress and, as yet, are not completed.

Incl #7

REQUEST FOR APPROVAL FOR HUMAN USE OF RADIOISOTOPES IN TRACER AMOUNTS IN VOLUNTEER EXPERIMENTAL RESEARCH SUBJECTS

Submitted by:

U. S. Army Medical Research and Nutrition Laboratory
Denver, Colorado

M. E. McDowell, M.D., Lt. Col., MC, Commanding Officer and Director
J. E. Canham, M.D., Lt. Col., MC; Chief, Metabolic Division
J. E. Hansen, M.D., Lt. Col., MC; Chief, Physiology Division
E. M. Baker, Ph.D., Maj., MSC; Assistant Chief, Chemistry Division and Chief, Carbohydrate Branch
K. E. Kinnamon, DVM, Capt., VC; Chief, Radioisotope Branch, Physiology Division
J. R. Handy, M.D., Capt., MC; Chief, Cellular Physiology Section, Physiology Division
H. E. Sauberlich, Ph.D., (PL-313), Chief, Chemistry Division
G. A. Leveille, Ph.D., (GS-13), Chief, Lipid and Protein Chemistry Branch, Chemistry Division
B. M. Tolbert, Ph.D., Professor of Chemistry, University of Colorado and Consultant to USAMRNL

and

Fitzsimons General Hospital
Denver, Colorado

C. A. Moore, M.D., Lt. Col., MC, Chief, Urology Service
O. G. Stonington, M.D., Professor of Urology, University of Colorado School of Medicine and Consultant to Fitzsimons General Hospital

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Section I. General Introduction

1. Purpose of request

a. Par. 3b(3), AR 40-37, "Radioisotope License Program (Human Use)," dated 12 August 1963, requires that written approval be obtained from the Secretary of the Army prior to the submission of license application (through channels to AEC) for human use (of radioisotopes), when volunteers are to be used as experimental research subjects. This paragraph (Par. 3b(3) AR 40-37) cites AR 70-25, "Research and Development: Use of Volunteers as Subjects of Research," dated 26 March 1962 as the basis for the requirement.

b. AR 70-25 prescribes policies and procedures governing the use of volunteers as subjects, including research in nuclear, biological and chemical warfare, wherein human beings are deliberately exposed to unusual or potentially hazardous conditions. Par. 6 of this AR requires approval of the Chief of Research and Development prior to the research and, in the case of nuclear, biological or chemical agents, approval of the Secretary of the Army is required.

c. Radioisotopes in tracer amounts as used in metabolic and physiological experiments are not nuclear agents (in the context and intent of AR 70-25 these words undoubtedly mean nuclear warfare agents). Furthermore, tracer quantities of radioisotopes as licensed by AEC and used by competent medical research scientists constitute a health hazard so minimal as to permit debate re the applicability of AR 70-25.

d. Nevertheless, to comply with the administrative technicality imposed by Par. 3b(3), AR 40-37 and Par. 6, AR 70-25, this request is submitted for approval for human use of stated radioisotopes in tracer amounts in volunteer experimental research subjects at U. S. Army Medical Research and Nutrition Laboratory and Fitzsimons General Hospital and in field studies conducted by USAMRNL.

2. Scope of request

a. Experiments included in this request are not, in and of themselves, unusual or potentially hazardous under the definitions of AR 70-25. They would be considered potentially hazardous (and minimally so) only to the extent that radioactive isotopes in tracer quantities are used.

b. Therefore, this request seeks approval only for use of the specified radioisotopes, the experiments otherwise not requiring individual approval under AR 70-25. However, sufficient description is furnished to indicate importance of the studies in warranting use of radioisotopes.

c. For any studies later contemplated under the general description given in this request which would, in and of themselves (apart from the use of radioisotope tracers), constitute unusual or hazardous experiments, specific approval (directed to the non-isotope aspects) would then be requested per AR 70-25.

d. This request will present the health physics aspects of the radioisotope tracers required (Section II); will present in detail the research methods and plans for C-14 and H-3 usage in vitamin C studies, and outline other

proposed studies referring to the vitamin C studies as a model (Section III, Par. 11); and will describe other metabolic and nutritional studies requiring other radioisotope tracers in addition to C-14 and H-3 (Section III, Pars. 12-15).

3. General guidelines for requested studies

a. The administered radioactive material would in no case exceed a radiation dose high enough to approach the permissible dose indicated in CFR Title 10, Part 20, RC-12, "The Medical Use of Radioisotopes--Recommendations and Requirements by the Atomic Energy Commission." In fact, in no case will the dose exceed one-half that of the permissible dose and every attempt will be made to use even lesser amounts of isotope when compatible with obtaining reliable data.

b. All policies, procedures and regulations prescribed in AR 70-25 and AR 40-37 will be rigidly adhered to in all investigations.

c. The person in charge of each specific phase of the studies proposed herein will be formally designated prior to the research by the Commanding Officer, USAMRNL, from the Government scientists listed in this application, and the attending physician will similarly be designated from the Medical Officers among them.

4. History of USAMRNL isotope usage

a. This laboratory has employed radioactive labeled compounds in studies with human subjects under AEC License Number 5-46-6 since 17 December 1957. Authorization was given initially to use Iodine ^{131} labeled

human serum albumin to measure the turnover rate of albumin of 10 normal young men in various nutritional states.

b. USAMRNL staff members have had experience in use of various radioisotopes in a number of chemical forms in collaborative clinical investigations with Fitzsimons General Hospital involving the basic disease process or new treatment procedures. Such work has been carried out under the Fitzsimons General Hospital's AEC License 5-46-9 which includes authorization for use of the following:

(1) Iodine^{131} for diagnosis of thyroid function and thyroid scanning.

(2) Iodine^{131} labeled human serum for the determination of blood volumes and plasma volumes.

(3) Iodine^{131} labeled Rose Bengal dye for determination of liver function and liver scans.

(4) Iodine^{131} labeled fats and fatty acids for determination of fat absorption.

(5) Iodine^{131} labeled renal function compounds.

(6) Phosphorus^{32} for the treatment of polycythemia vera, leukemia and bone metastasis.

(7) Chromium^{51} for the determination of red cell volume and red cell survival time.

(8) Cobalt^{60} labeled vitamin B_{12} for the diagnosis of pernicious anemia.

(9) Iron^{59} for iron metabolism studies.

c. On 11 December 1959, authorization was granted in License No. 5-46-12(L 61) for the use of Carbon-14 labeled glucose, glucuronic acid, glucuronolactone and ascorbic acid to measure the pool size and turnover rate of body ascorbic acid in normal human subjects and for investigation of the possibility that humans may be able to synthesize small amounts of ascorbic acid.

d. License No. 5-46-12(L 61) was renewed on 24 October 1961 and expanded to include Carbon-14 labeled glycine, cholesterol, mevalonic acid acetate and carbon monoxide, in addition to the compounds previously authorized, for use in metabolism and physiological tracer studies in humans.

e. A recent amendment to License No. 5-46-12 (including prior approval by the Secretary of the Army) permitted the use of tritiated water for the determination of total body water in 112 human volunteers at Ft. Carson, Colorado.

f. Current status of AEC radioisotope licenses of USAMRNL and Fitzsimons General Hospital:

(1) This laboratory is currently licensed by AEC (License No. 5-46-12 (L 63) for human use of the isotopes listed above in Par. 4c and d; the specifically authorized study in Par. 4e having been completed. All human usage not explicitly covered by Par. 4c of AR 70-25 has been discontinued pending authorizations (requested by this document) required by AR 40-37. This AEC license will expire 31 December 1963.

(2) This laboratory also currently operates under the general (animal usage) AEC radioisotope License No. 5-46-11 (H 63). This was originally scheduled to expire 31 August 1963, but has been extended indefinitely by AEC (who are holding our renewal application dated 21 May 1963) pending consolidation of the separate FGH and USAMRNL licenses into one broad license for the entire post (a joint FGH and USAMRNL license).

(3) Consolidation of the heretofore separate licenses of FGH and USAMRNL into one broad license has been recommended by the Preventive Medicine Division of Office of The Surgeon General, and sanctioned by AEC because of the favorable record of radioisotope handling by both FGH and USAMRNL. Application for the new joint license (omitting the radioisotopes requested herein for volunteer research use) will be forwarded to AEC (through The Surgeon General) by FGH-USAMRNL within approximately 10 days.

(4) Upon approval of the radioisotopes requested herein per Par. 3b(3) AR 40-37, application will be made to AEC (through The Surgeon General) for addition to the joint AEC license by amendment.

5. Specific radioisotopes to be used

a. Use of the following radioisotopes in volunteer human research in tracer dosages is requested:

By-product Material	Chemical and/or Physical Form
Carbon-14	Vitamins
	Amino acids
	Lipids (as glycerides, cholesterol and free fatty acids)
	Carbohydrates
	Acetate
	Mevalonic acid
	Bicarbonate or CO_2

By-product Material	Chemical and/or Physicai Form
Hydrogen-3	Vitamins
Magnesium-28	MgO, $MgCl_2$, Mg citrate
Calcium-47	$CaCl_2$
Calcium-45	$CaCl_2$

b. All the labeled compounds to be employed are naturally occurring nutrients or metabolites for the human.

Section II. General Health Physics for Requested Isotopes

6. Carbon-14

Carbon-14 has a soft beta emission that lends itself to tracer studies. Fat in the body is usually considered the critical organ. The biological half life for Carbon-14 in fat is given as 35 days. The National Bureau of Standards Handbook No. 69 lists the maximum permissible burden in fat as 300 μc. Constants for calculating maximum permissible internal concentration of radioisotopes assumes that 50% of the Carbon-14 that is present in the blood is transferred to the critical organ, fat. However, based on animals, it can also be assumed that few of the Carbon-14 labeled compounds proposed to be used would approach this retention in the critical organ. The majority of the compounds proposed are readily metabolized and removed from the body as expired CO_2 or metabolites in the urine, and would reduce even further the body burden of irradiation. Flushing procedures could also be employed in the case of the labeled vitamins to hasten their removal from the body upon completion of the studies. In all investigations, balance will be performed that will permit careful knowledge of the extent of retention and turnover of the labeled compound administered.

7. Hydrogen-3

Hydrogen-3 emits only a very soft beta particle, but with present counting instruments is a very useful isotope for tracer studies. The entire body is generally considered the critical organ and the isotope has a biological half life of approximately 12 days. The maximum permissible body burden is 1-2 mc.

This approximate amount has been used routinely in numerous laboratories for the determination of total body water in the human. Permission has been granted this laboratory to use this technique utilizing one millicurie of tritiated water on volunteers at Ft. Carson.

The use of tritiated vitamins is proposed since several vitamins are available only as the tritiated compounds. Because of the considerably smaller pool size, the dosage of tritium employed as a vitamin will be much less than that employed in the measurement of total body water. Amounts less than 0.1 mc are anticipated. Tritiated folic acid and pyridoxine are presently employed at a number of laboratories for studying malabsorption syndromes in humans such as may be encountered in tropical sprue.

8. Magnesium-28

This isotope is available as a cyclotron produced element. It has a very short half life of only 21 hours. Magnesium-28 has been used in a number of laboratories with humans. Dr. J. K. Aikawa, Department of Medicine, University of Colorado School of Medicine, Denver, has administered 90 μc of Mg-28 to normal subjects and patients and found essentially no activity in the urine or plasma after 40 hours. By this time, approximately 90% of the Mg-28 was accounted for in the feces and urine. (Peaceful Uses of Atomic Energy, Vol. 24, p. 148, 1958; The Role of Magnesium in Biological Process, J. K. Aikawa, 1963, C. C. Thomas, Publishers, Springfield, Ill.)

9. Calcium-47

This relatively recently available isotoped with a half life of only 4.9 days has seen use in a number of studies with human subjects. The maximum permissible burden when the total body is considered the critical organ is approximately 10 μc; with bone the critical organ a permissible burden of 5 μc is allowed. For the proposed studies, a dose not to exceed 5 μc would be used, with an anticipation that a dose of only 2 μc may be sufficient.

10. Calcium-45

If the use of Calcium-47 should prove not feasible because of the short half life and transportation or delivery difficulties, Calcium-45 would be employed instead. Calcium-45, with a soft beta emission and a half life of 163 days, has a maximum permissible burden in bone of 30 μc or 200 μc for the total body. The dosage proposed for the studies outlined would not exceed 15 μc.

All use of radioisotopes in humans would be in accordance with the following:

1. Use will be confined to metabolic and physiological tracer studies.

2. The licensee shall comply with the provisions of Title 10, Part 20, Code of Federal Regulations, Chapter 1, "Standards for Protection Against Radiation," and RC-12 "The Medical Use of Radioisotopes--Recommendations and Requirements by the Atomic Energy Commission."

3. Radioisotopes for use in humans shall be acquired from a supplier other than an Atomic Energy Commission facility, who certifies the pharmaceutical quality and assay of such material.

4. The licensee, except as otherwise specifically provided for in the license, shall possess and use the material as described in this license in accordance with statements, representations and procedures contained in supplementary sheets attached to the application.

5. All rules, regulations and limitations set forth by Army, AEC, and local authorities (including those set forth in AR 70-25, AR 40-37 and Handbook 69 of the National Bureau of Standards) will be complied with.

Section III. Proposed Nutrition and Metabolism Tracer Studies

11. Vitamins: Investigations on the vitamin requirement of the human with the use of Carbon-14 or Hydrogen-3 labeled vitamins or related compounds (References cited in the paragraph (11) are listed in subparagraph 11h)

a. Background for vitamin C studies

Past 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 ascorbic acid, it was decided to study the metabolism of the lactone in two ways. One was to give the 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 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.

Results of studies (2) 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. In addition, it was found that one-half of the urinary oxalate arises from the breakdown of ascorbic acid and is excreted at a constant rate. 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.

One of the more interesting findings of these experiments was that no $C^{14}O_2$ activity could be detected in the expired air of the subjects receiving L-ascorbic-1-C^{14} acid even with the use of a 15-liter ionization chamber for greater sensitivity. A fraction of 1% oxidation to CO_2 during the first 8 hours could have been easily detected by this technique. This finding was in agreement with the earlier work of Hellman and Burns (3). Recently, Abt et al. (4) reported that man excretes approximately 25% of the total activity of L-ascorbic-1-C^{14} acid via the lung. Because doubt had been caused as to whether or not man could decarboxylate C^{14} labeled ascorbic acid to $C^{14}O_2$, a series of experiments were performed in this laboratory which resulted in a publication (5) showing: 1. Chromatographic and radioautographic evidence was presented showing that progressive degradative changes occur in L-ascorbic acid dissolved in water and kept at 25° C. for a 72-hour period; 2. When a human subject received 20 μc of freshly dissolved L-ascorbic-1-C^{14} acid solution, little or no C^{14} appears in his respiratory CO_2; 3. Men who were given similar samples of L-ascorbic-1-C^{14} acid aged for 36 and 72 hours, respectively, excreted 30.6% of the ingested C^{14} as respiratory CO_2. The true nature of the compounds undergoing decarboxylation in man in these studies cannot be defined from the work presented here except that they are not reduced ascorbic acid.

b. Request for use of Carbon-14 to label vitamin C and related compounds

Therefore, because of demonstrated usefulness and necessity of using tracer techniques to study metabolic pathways, the proposal is being made that tracer amounts of Carbon-14, as glucose-6-C^{14}, glucuronolactone-6-C^{14}, glucuronic-6-C^{14} acid and ascorbic-1-C^{14} acid be administered by mouth to humans in further studies for the purpose of measuring the pool size and the rate of utilization of body ascorbic acid under varying conditions. The subjects to be used will be military personnel (volunteering for the specific study) or laboratory personnel, both male and female, or Fitzsimons General Hospital personnel (as well as Fitzsimons General Hospital patients who volunteer). The possible hazards of the experiments will be explained in advance to all subjects. Although multiple experiments may be performed on individuals, in no case will the total body radiation dose from this experiment, other experiments, or from x-rays, exceed the maximum permissible limits for normals of 5 rem per year (lower below age 25).

c. Experimental methods (using labeled vitamin C and related compounds

The L-ascorbic-1-C^{14} acid will be obtained from the California Corporation for Biochemical Research, Los Angeles. All C^{14} labeled compounds will be checked for purity prior to use by melting point measurement and by paper chromatography. The activity of all C^{14} labeled compounds will be checked by radioassay. The L-ascorbic-1-C^{14} acid will be freshly dissolved in

distilled water and immediately swallowed by the experimental subject. No cold carrier will be given to these subjects.

Total daily urine will be collected and measured from all subjects; these samples will be refrigerated and 2.0 ml of each will be taken for radioassay.

Immediately after receiving the tracer quantity of L-ascorbic acid, the subjects will be 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 is recorded automatically on a 6-channel recorder.

The total activity of each urine sample is determined by use of a liquid scintillation counter using P-dioxane-toluene. Oxalate in selected samples is isolated as calcium oxalate, recrystallized 4 times and dissolved in 1 N hydrochloric acid for counting in the liquid scintillation counter. Quantitative determination of the total oxalate is done by the Archer method (6). Efficiencies for all liquid scintillation counting of samples are determined individually by use of added standard C^{14} samples.

Urinary ascorbic acid levels are chemically determined by the Schaffert method (7). Urinary ascorbic acid is then isolated by the method described by Jackel et al. (8). After the dinitrophenylhydrazone (DNPH) derivatives are recrystallized, they are dissolved in P-dioxane and applied to weighed planchets and counted in a gas flow counter. All DNPH derivatives are recrystallized to constant activity which usually requires 4 to 6 recrystallizations.

Total body tissue volume (V) is estimated in duplicate tests using a body volumeter based on displacement of water (9). From body weight (M) and V, fat (F) in kg is calculated according to an equation developed in this laboratory: $F = 4.834\ V - V.336\ M$.

d. Experimental plan; studies on factors that may influence the vitamin C metabolism and requirements in man:

(1) Recapitulation of the method

Studies of body composition and the use of C^{14} isotopes have resulted in a method for stating the actual utilization of ascorbic acid by healthy men.

In human 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.

Ingestion of a single, comparatively large 0.5 gm quantity of unlabeled ascorbic acid or its precursors by subjects whose body ascorbic acid pools had 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 8 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 kg of fat-free body weight. Rarely, if ever, do adult males exceed 90 kg in lean body mass. Therefore, 28 mg per day intake would match the greatest quantity of ascorbate metabolized by the largest healthy man. Further, it is of interest to note that despite repeated reports in the literature of loss of ascorbic acid in sweat, when one of the subjects discussed above was sweated for a 6-hour period in a hot room after being labeled with 20 μc of ascorbic-1-C^{14} acid, no C^{14} activity could be detected in the collected total body sweat. The chemical analysis of the sweat indicated the presence of a small amount of ascorbate. However, when the sweat was lyophilized to dryness and then applied to a chromatographic sheet and run in a standard solvent system, no reduced ascorbic acid could be demonstrated. These results are not surprising in view of the fact that it is well known that all the chemical determinations for ascorbic acid are not absolutely specific for ascorbic acid in biological fluids.

The method as employed consists of giving an individual a single oral dose of 20-50 μc of L-ascorbic-1-C^{14} acid and then collecting a single 24-hour urine sample. The ascorbic acid contained in the urine is isolated as the dinitrophenylhydrazine derivative and counted to obtain specific activity as μc/mg of ascorbate excreted. The oxalate that is derived from the labeled accorbate and excreted is also isolated and counted. Then, by simply dividing the specific activity of the excreted ascorbate by the total C^{14} activity of the formed and excreted oxalate, one can obtain an estimate of the number of milligrams of ascorbate utilized during the 24-hour period.

This method could be used in human studies to determine whether or not there is an increased utilization or an increased need for ascorbic acid in the following conditions:

Cold
Heat
Acclimatization to heat, cold, stress and altitude
Stress
Trauma and burn patients
Infections

Moreover, this method could be used in human studies to determine whether or not adaptation occurs in peop! who have been on a chronic low dietary intake of vitamin C.

(2) Need for more data on vitamin C metabolism

According to the text "World Review of Nutrition and Dietetics" (Vol. III, 1963) published by G. H. Bourne, p. 187, the following conclusions regarding vitamin C are stated:

"1. The most frequently quoted recommended allowance of vitamin C for adult man under the prevailing conditions of civilization and climate varies around 80 mg L-ascorbic acid/day.

"2. High doses, though not toxic are not recommended and may, according to some findings, lead even to a negative adaption of the organism.

"3. Medium doses (up to 200 mg) are probably reasonable under some special conditions-certain types of work, rehabilitation and therapeutic allowances."

At a recent meeting held by the Federation of American Societies for Experimental Biology in Washington, D.C. on 14-15 March 1963, the Ad Hoc Committee on Military Applicability of Research on Ascorbic Acid made the following recommendation: That this laboratory, i.e. USAMRNL, attempt to study the utilization of vitamin C in humans in the following conditions:

Cold
Heat
Acclimatization
Stress
Interrelationship of vitamin C with other vitamins
Wounds and burns

(3) Extension of the methods to problems stated in preceding paragraph

In view of the above recommendations and the lack of information on the above problems, it is requested that vitamin C utilization studies in the above-named conditions, using the method previously described, in human volunteer subjects be considered for authorization.

To accomplish this request, it would require that a team of 2 or more investigators from this laboratory be sent to several geographical areas

with differing climatic conditions. The areas under consideration are (1) Camp Hale, Leadville, Climax or Mt. Evans areas in Colorado for studies on ascorbic acid in acclimatization to cold, and (3) a tropical area within the Caribbean Command for studies on acclimatization to tropical conditions. In addition to this general approval for use of isotopes, the approval and concurrence of the local commander or appropriate local health authorities would be obtained for each location and experiment. At each location, comparisons would be made between subjects who had recently arrived and those who had resided at the location for an extended period of time. These results would in turn be compared with findings obtained at this laboratory on subjects residing in Denver, Colorado. At each location, not more than 10 subjects would be studied. Normal, healthy volunteers, preferably military, would be selected to receive the C^{14} labeled ascorbic acid as previously outlined; body composition data would be obtained by skinfold or other appropriate measurements. With the data obtained from these studies, one could then assign what would be the ideal vitamin C pool size and utilization under these climatic conditions in comparison with the data previously obtained at this laboratory on normal healthy subjects.

Upon evaluation of the data, considerations would be made as to recommended allowances for vitamin C under conditions of cold, heat, altitude and acclimatization.

Studies on the interrelationship of vitamin C with other vitamins would be performed at this laboratory with normal volunteers, employing C^{14}

labeled ascorbic acid in the amounts and manner as previously outlined. Evidence of a relationship between vitamin C and vitamin B_6 in the human has been recently obtained at this laboratory in non-isotopic studies. The use of C^{14} ascorbic acid in these investigations would permit a better understanding of the apparent interrelationship. The possible increased needs for vitamin C in situations of stress (surgery or radiation therapy, as examples), wounds or burns would be performed in conjunction with Fitzsimons General Hospital should suitable patients become available.

e. Proposal to use C-14 and Hydrogen-3 labeled vitamins other than vitamin C, using the outlined vitamin C studies as a general model

Other Carbon-14 labeled vitamins would be studied in essentially the same manner and employing the same techniques and procedures as those indicated for ascorbic acid. Excretion rates, pool size, turnover rates, absorption and metabolic products will be measured for each. The influence of various nutritional states on the above parameters will be investigated in an attempt to evaluate dietary requirements for vitamins. The body pool size of ascorbic acid is considered greater than that of any other vitamin and the turnover rate is as slow or slower than other vitamins; therefore, the amount of radioactive label used for the other vitamins will be less, and with the greater turnover rate, will produce less of a body burden than the vitamin C.

When Carbon-14 labeled vitamins are not available, the above studies will be performed with the use of tritium labeled vitamins. The most commonly

employed tritiated vitamins are pyridoxine and folacin. These two vitamins have been employed at various laboratories in malabsorption studies with humans. The same parameters and procedures as outlined for Carbon-14 labeled vitamin C and the above will be employed, except that electrometer measurements of expired air will be omitted. The dosage employed will in no case exceed 100 μc of tritium labeled vitamin. This dosage of tritium represents only 10% of that routinely employed in total body water measurements and is indicative of the low radiation dose received.

f. Health physics

The dose of any of the C^{14} labeled vitamins will not exceed 50 microcuries. The following maximal radiation dosages are calculated with the aid of the ICRP Handbook (Appendix I, reference 1). The physical half time of C^{14} is considered infinite. The following is an approximation of the biologic half time. All the vitamins to be used are highly reactive; however, for ascorbic acid the turnover rate is probably slower, possibly much slower than for the other vitamins. From nutritional data, the total body ascorbic acid is almost certainly less than 6 gm, and the daily turnover greater than 10 mg. The half time of body vitamin C under these conditions would be 400 days. This estimate is obviously too long, perhaps by as much as an order of magnitude. The only data in the literature on man (L. Hellman and J. J. Burns, J. Biol. Chem. 230: 923, 1958) (E. M. Baker, H. E. Sauberlich, S. J. Wolfskill, W. T. Wallace and E. E. Dean, Proc. Soc. Exp. Biol. and Med. 109: 737, 1962) shows a pool size

of about 1.4 gm in a 70 kg man, with a half time of about 16 days. With the maximum estimate of a 400-day half time, a dose of 50 microcuries of C^{14} ascorbic acid evenly distributed in a 70-kg man will give a total radiation dose of only 1.13 rem (0.164 rem the first 13 weeks). With a half time of 16 days, the same dose will give a total radiation dose of 0.045 rem (0.044 rem the first 13 weeks). In the case of tritiated vitamins, the dosage will in no case exceed 100 μc of tritium. This dosage of tritium represents only 10% of that routinely employed in total body water measurements; consequently, the body burden is very low.

g. Personnel

For each specific phase of the studies, one of the following (other than the consultant) will be designated as project leader, and one of the Medical Officers named below will be designated as attending physician per Par. 6, AR 70-25.

Maj. E. M. Baker, Ph.D., MSC
H. E. Sauberlich, Ph.D. (PL-313)
Lt. Col. M. E. McDowell, M.D., MC
Lt. Col. J. E. Canham, M.D., MC
Lt. Col. J. E. Hansen, M.D., MC
Capt. J. R. Handy, M.D., MC
B. M. Tolbert, Ph.D. (Consultant, University of Colorado)

h. References

(1) Baker, E. M., E. L. Bierman, [illegible] C. Plough. Metabolism 9: 478, 1960 (reprint attached, Appendix IV).

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(5) Baker, E. M., N. G. Levandoski and H. E. Sauberlich. Proc. Soc. Exp. Biol. and Med. 113: 379, 1963 (reprint attached, Appendix IV).

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(7) Schaffert, R. R. and G. R. Kingsley, J. Biol. Chem. 212: 59, 1955.

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(9) Allen, T. H., H. J. Krzywicki, W. S. Worth and R. M. Nims, U. S. Army Med. Rsch. Nutrition Lab. Rpt. No. 250, 24 Sept. 1960.

(10) A study of the military applicability of research on ascorbic acid. Life Sciences Research Office, Federation of American Societies for Experimental Biology, Washington, D. C., August 1963.

12. Amino Acids: Investigations on the metabolism of amino acids in the human with the use of Carbon-14 labeled compounds (references cited in this paragraph are listed in Par. 12f)

a. Background

The recent work of Crawhall et al. (1) using $\left[1\text{-}C^{13}\right]$-glycine has demonstrated that this isotope was diluted about $2\frac{1}{2}$ times during the conversion of glycine from the first metabolic pool (i.e. the pool of glycine with which a dose of glycine mixes immediately after absorption and distribution, and which can be sampled by means of the uncombined urinary glycine (2)) to oxalate, indicating that about 40% of the urinary oxalate was derived from glycine during this period.

Berlin et al. (3) did not measure the C^{14} activity in the urinary oxalate in their glycine-2-C^{14} studies. However, they did show with the use of the methyl labeled glycine that 90% of the C^{14} activity was accounted for in the expired $C^{14}O_2$ and that only 5% of the C^{14} activity was excreted in the urine. This would tend to indicate that the catabolism of glycine-2-C^{14}, insofar as oxalate formation is concerned, is far different than that of the carboxyl labeled glycine-1-C^{14}.

When C^{14} labeled ascorbic acid was orally administered to humans, Hillman and Burns (4) reported that an average of 44% of the total radiocarbon excreted in urine was recovered as oxalate. It was demonstrated in this laboratory that 50% of the urinary oxalate was derived from L-ascorbic acid-1-C^{14} and was excreted at a constant rate per day (5, 6). Therefore, it is of interest to study both glycine-1-C^{14} and glycine-2-C^{14} metabolism in humans to determine (1)

whether or not the glycine C^{14} is partially converted to and excreted as oxalate at a constant rate per day as well as (2) the amount per day excreted as urinary oxalate.

Further, it would be desirable to measure the expired $C^{14}O_2$ in a vibrating reed electrometer to determine the amount and extent of decarboxylation of the C^{14} labeled glycine in man. It should be noted that this has been done in humans using glycine-2-C^{14} (3), but not with the glycine-1-C^{14}.

b. Experimental plan (Part 1)

A total of no more than 10 human subjects would be involved in these experiments. Further, the subjects would be staff members of this laboratory, 20-46-year-old males. There would be no dietary restriction placed on these subjects.

Each subject would have to receive orally 20 μc of glycine-1-C^{14} as well as a further 20 μc of glycine-2-C^{14} at a much later date (40-80 day interval). No cold carrier glycine will be given to the subject at the time the labeled material is administered. Immediately after taking the C^{14} glycine, the subject will be made to breathe through a drying train directly into the ionization chamber of the vibrating reed electrometer. The subject will continue to breathe at intervals through the system until he reaches his background trace signal. This is done by having the subject breathe into the system for 20-30 minutes, then allowing a 30-minute rest. This process is continued until the electrometer tracing returns to background signal.

Further, 24-hour urine collections will commence with the ingestion of the C^{14} glycine label and will continue on every other 3rd day for a period of 2 weeks. The urine samples will then be analyzed for the total urinary oxalate content. The oxalate of each sample will then be isolated and counted in the liquid scintillator. Also, certain selected samples of the urine will be analyzed on the amino acid analyzer which has a flow-through scintillation detector attached. This will enable us to obtain both the specific and total activity of the urinary free glycine of other radioactive metabolites. Each urine sample will be counted for total activity. Thus, if one knows the total dose given as glycine C^{14} as well as the dose remaining in the body at any given time, as well as the specific activity of the excreted urinary glycine, one should be able to approximate the total body pool size and turnover rate of the free glycine pool.

c. Health physics pertaining to Par. 12b

The normal A.E.C. procedures shall be adhered to insofar as the administration, handling of the isotope and the disposal of the urine samples obtained from the subjects.

The dose of the C^{14} glycine will not exceed 20 μc for either the glycine-1-C^{14} or the glycine-2-C^{14}. The following maximal radiation dosages are calculated with the aid of the ICRP Handbook (Appendix I, reference 1).

The physical half life of C^{14} is considered infinite. The following is an approximation of the biologic half time of glycine-2-C^{14}. According to N. I. Berlin, B. M. Tolbert and C. Lotz (J. Clin. Investigation 31, No. 3:

335-337, 1952), the longest "half time of glycine-2-C^{14} elimination from the tissues of man is approximately 50 days." With the estimate of a 50-day half time, a dose of 20 μc of glycine-2-C^{14} evenly distributed in a 70 kg man will give a total radiation dose of only 0.056 rem (0.0478 rem the first 13 weeks). Another body compartment of considerably less importance and a half time of about 100 days was described at a later date by Berlin et al. (Proc. Soc. Exp. Biol. and Med. 88: 386, 1955). However, since the majority of the dose is not retained, but lost within the first 24-hour period as expired CO_2 as urinary excretory products, the body irradiation burden is considerably less than this value.

In the case of glycine-1-C^{14}, we have only the data of R. W. E. Watts and J. C. Crawhall (Biochem. J. 73: 277-86, 1959) using the stable C^{13} isotope to estimate the glycine metabolic pool in man. According to these authors, the pool size of glycine in a 70 kg man is 406 gm or 5.8 gm/kg. Further, they state that the turnover rate in a 70 kg man is 3.2 gm/hr. or 76.8 gm/day. Thus, $\frac{76.8}{406} = 0.189$ or 18.9% turnover. The biological t $\frac{1}{2}$ would then be equal to $\frac{0.693}{0.189} = 3.7$ days. Assuming then a 4-day half life for the glycine-1-C^{14}, a dose of 20 μc evenly distributed in a 70 kg man will give a total radiation dose of only 0.005 rem (0.005 rem the first 13 weeks).

d. Experimental plan (Part II)

Other Carbon-14 labeled amino acids would be studied in essentially the same manner as that employed with glycine. Similarly, pool size, turnover rates and metabolites would be measured in an attempt to study the protein and

amino acid requirements of the human. These studies should also provide additional knowledge as to the metabolic pathways and interrelationships of amino acids in the human. It is anticipated that not more than 3 subjects will be required for each amino acid investigated. The dosage of Carbon-14 employed would not exceed that indicated for Carbon-14 glycine, and with the half time estimated not to exceed that for glycine. The radiation burden, therefore, would be low and would not in any instance approach the maximum permissible dose.

e. Personnel

Maj. E. M. Baker, Ph.D., MSC, Project Leader
H. E. Sauberlich, Ph.D. (PL-313) Co-Project Leader
G. A. Leveille, Ph.D.
Lt. Col. M. E. McDowell, M.D., MC (Serving also as attending physician)
Lt. Col. J. E. Canham, M.D., MC (Serving also as attending physician)

f. References

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13. Lipids: Studies on lipid metabolism in the human with the use of Carbon-14 labeled compounds (references cited in this paragraph are listed in Par. 12c)

a. Experiment 1: Carbon-14 tracer studies on cholesterol metabolism in the human

(1) Background and procedures

Tracer amounts of C^{14}, as cholesterol-4-C^{14}, will be administered orally to study the influence of neomycin and various fats on cholesterol absorption in order to determine whether the hypocholesteremic effect of these materials is the result of an impaired absorption. In order to ascertain whether these materials influence cholesterol synthesis, acetate-1-2-C^{14} and mevalonic acid-2-C^{14} will be administered intravenously.

The work of Samuel and Steiner (Proc. Soc. Exp. Biol. Med. 100: 193, 1959) has demonstrated a hypocholesteremic effect for neomycin. Unsaturated fat has also been shown to have a cholesterol lowering effect. The mechanisms by which neomycin or unsaturated fat depress plasma cholesterol remain obscure. Unpublished data from this laboratory indicate that neomycin functions by interfering with cholesterol and/or bile acid absorption. The effect of unsaturated fats appears to be twofold: a) an interference with cholesterol absorption mediated by the sterol fraction in vegetable fats and b) a particular systemic effect (Bronte-Stewart, Fed. Proc. 20: No. 1, Part III, p. 127, 1961).

In order to further elucidate the mechanism of action of these compounds, human volunteers fed neomycin, different fats or a control diet

will be given an oral dose of cholesterol-4-C^{14} (20-50 μc) and its absorption determined. In other subjects, similarly treated, acetate-1-2-C^{14} or mevalonic acid-2-C^{14} will be administered intravenously (50 μc) and incorporation into cholesterol will be ascertained by determining the specific activity of plasma cholesterol.

(2) Health physics

The maximum dosage to be employed is 50 μc for acetate, mevalonic acid and cholesterol. The maximal radiation dosage, calculated with the aid of the ICRP Handbook (Appendix I, reference 1) for these levels of administered radioactivity, will not exceed the permissible limits for normal subjects of 5 rem/yr. with no more than 3 rem in any 13 consecutive week period (above age 18). The half life of cholesterol is approximately 20 days (Cook, R. P.: Cholesterol, 1958, Academic Press, N. Y.) and if that of other compounds synthesized from acetate or mevalonic acid is assumed to be similar and, further, the physical half life of C^{14} is considered infinite, a dose of 50 μc of C^{14} labeled cholesterol, mevalonic acid or acetate evenly distributed in a 70 kg individual will give a total radiation dose of 0.056 rem (0.054 rem the first 13 weeks).

(3) Personnel

Gilbert A. Leveille, Ph.D., Project Leader
Howerde E. Sauberlich, Ph.D., Project Leader
Lt. Col. M. E. McDowell, M.D., MC (Serving also as attending physician)
Lt. Col. J. E. Canham, M.D., MC (Serving also as attending physician)

b. Experiment 2: Suppressibility of cholesterol synthesis by exogenous cholesterol loading in man

(1) Background

The relative stability of serum cholesterol levels, despite marked variation in dietary intake of cholesterol, has been attributed to compensatory changes in hepatic synthesis of cholesterol (1-5). Recently, Sipperstein and Guest have suggested, on the basis of in vitro studies, that the mechanism of this homeostatic effect is a sensitive negative feedback system whereby cholesterol inhibits the conversion of β-hydroxy-β methyl glutaryl Co A to mevalonic acid (6). These authors speculate that insensitivity of this feedback might be involved in disorders of cholesterol metabolism.

In order to test this hypothesis, it is planned to ascertain quantitatively the response of cholesterol synthesis to an exogenous cholesterol load. After data on normal subjects have been obtained, these will be compared with groups demonstrating abnormalities of cholesterol metabolism, i.e. idiopathic hypercholesterolemia-proven atherosclerosis, diabetes, hypo- and hyperthyroidosis, nephrotic syndrome.

(2) Method

Patients will be given 100 microcuries 1-C^{14} acetate intravenously or orally. Timed serum samples will be analyzed for total and C^{14} cholesterol. In certain patients, C^{14} of other serum lipids and C^{14} as $C^{14}O_2$ will also be measured.

These procedures will then be repeated after a standard cholesterol load sufficient to elevate serum cholesterol in normal subjects (7). Differences in total and specific activity of serum cholesterol before and after cholesterol loading will be used as an index of the sensitivity of the hepatic response to exogenous cholesterol. Methods of analysis will be similar to those described by Gould et al. (8).

(3) Health physics

With regard to radiation safety, reference is made to the work of Gould et al. (8):

> "The dose of 100 μc was chosen so that repeated doses could be given to the same subject without exceeding accepted values for the maximum permissible dose for man. Our studies of $C^{14}O_2$ in expired air after the administration of 1-C^{14} acetate demonstrated that approximately 56 per cent of the radiocarbon was eliminated during the first 24 hours.* On this basis, we have made the assumption that a single 100 μc dose will result in the 'retention' of not more than 25 μc of C^{14} in the slowly exchanging 'fat compartments' of the body. The maximum permissible dose for C^{14} compounds retained in the body fats is estimated to be 250 μc, according to calculations in Handbook 52 of the National Bureau of Standards.[7] Thus, we believe we are justified in administering, over a period of several months, a maximum of five such doses to human subjects without regard to their life expectancy.
>
> "Ref. 7. Maximum Permissible Amounts of Radioisotopes in the Human Body, etc., Nat. Bur. Standards Handbook, 52, pp. 12 and 18, G.P.O., Washington, D.C., March 20, 1953.

"*Shreeve also reported that 56 per cent of the C^{14} in acetate was eliminated as $C^{14}O_2$ by man at the end of 24 hours. Hellman reported that 60 per cent of the radiocarbon was retained at the end of 24 hours, and 35 per cent at the end of the first week after administration of acetate. It should be noted that he used the methyl-labeled acetate (2-C^{14}-acetate)."

In the present study, only 2 doses of 100 microcuries each will be given instead of 5 doses of 100 microcuries as above. The dose will therefore be well below the maximal permissible dose quoted.

(4) Personnel

Gilbert A. Leveille, Ph.D., Project Leader
Howerde E. Sauberlich, Ph.D., Project Leader
Lt. Col. M. E. McDowell, M.D., MC (Serving also as attending physician)
Lt. Col. J. E. Canham, M.D., MC (Serving also as attending physician)

c. References

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14. Carbohydrates: Investigations on the digestibility and metabolism of carbohydrates in the human with the use of Carbon-14 labeled compounds (references cited in this paragraph are listed in Par. 14e)

a. Background

Experiments have been in progress for some time at this laboratory investigating the digestibility of Carbon-14 labeled cellulose, hemicellulose and various uncommon sugars in laboratory animals as the rat, hamster and guinea pig (1, 2). Balance studies employing a vibrating reed electrometer to measure the expired carbon dioxide, together with urine and fecal measurements, have demonstrated that the rat may digest as much as 25% of the ingested cellulose (1).

In the case of the human, it is considered that cellulose passes through the digestive tract without being attacked by any of the digestive enzymes, though some bacterial decomposition probably takes place in the large intestine. Whether or not the bacterial actions are of value to the human are unclear. Various studies at this laboratory as well as elsewhere would indicate that at times cellulose is digested to a limited extent by the human (3-5). However, it must be recognized that in human balance studies, the methods for the measurement of cellulose and hemicellulose are less than satisfactory for critical evaluation. Furthermore, the possibility of bacterial decomposition of cellulose or hemicellulose in the lower intestinal tract may give rise to an "apparent" digestibility without any "true" digestibility in terms of nutrient benefit to the human. The use of Carbon-14 labeled cellulose with human subjects would give more definitive results with regard to this problem. Very exacting balance studies could be conducted.

Furthermore, the presence or lack of presence of radioactivity in the expired carbon dioxide, or in the urine and blood, would be rather conclusive evidence, which could be quantitated, that cellulose is or is not utilized. If utilization is indicated, the question of whether or not it is mediated through the intestinal flora could be readily investigated with the use of oral antibiotics (3, 4).

b. Procedures

The procedures employed would be very similar to those previously described for use with Carbon-14 labeled vitamin C. Normal, healthy volunteer subjects (minimum number) would receive orally specially prepared Carbon-14 labeled cellulose in an amount not to exceed [illegible] μc. The subjects will have previously received controlled levels of cellulose in the diet to investigate the influence of this dietary component on the digestibility of the C^{14} cellulose. Balance studies will be conducted with the aid of markers for the stools. The expired air will be monitored with the aid of a 5 or 15-liter chamber with a Cary-Tolbert vibrating reed electrometer and automatic carbon dioxide measurements. The expired air will be monitored until no radioactivity is detectable. Urine collections will also be made throughout the period and radioactivity measurements performed with a scintillation counter. If significant amounts of radioactivity are found to be present, attempts will be made to determine the nature of the radioactive compounds.

If evidence of cellulose digestion is noted, additional subjects will receive prior to receipt of the C^{14} cellulose oral supplements of antibiotics in

an effort to study the possible role of the intestinal flora in the digestion process. The antibiotics, neomycin and bacitracin would be employed, using the amounts and procedure previously employed in recent studies by the Metabolic Division of this laboratory to reduce or eliminate the intestinal flora of human subjects (3, 4). If C^{14} labeled hemicellulose or pectins can be made available, similar digestibility studies would be conducted with these materials.

c. Health physics

As indicated above, the dosage of the Carbon-14 labeled cellulose, hemicellulose or pectin would not exceed 100 μc. Although the digestibility of these compounds is not fully known, it is exceedingly doubtful that any are completely absorbed. If cellulose is digested to any extent, it would be most likely converted to glucose which the body readily metabolizes with a major portion being removed quickly through the lungs as carbon dioxide. Even if it is assumed that 100% digestion and assimilation takes place, this dosage of radioactivity would be considerably less than the maximum permissible dose. Glucose, the unit component of cellulose, is metabolized in large quantities each day by the human body. The radioactivity would, therefore, be readily diluted throughout the body and not concentrated or localized in a small amount of tissue. The eventual critical organ for what Carbon-14 that would be retained would be fat; but in consideration of the amount of Carbon-14 retained and deposited in the fat and with a biological half life of 35 days for Carbon-14 in fat, the radiation body burden produced by 100 μc of cellulose-C^{14} would be considerably less than the maximum permissible dose.

With the aid of the ICRP Handbook (Appendix I, reference 1), the calculated total dose would be 0.690 rem (0.576 rem the first 13 weeks). Assuming no absorption, the greatest dose received by the intestinal tract would be 0.691 rem.

d. Personnel

H. E. Sauberlich, Ph.D. (PL-313), Project Leader
Lt. Col. M. E. McDowell, M.D. (MC) (Also attending physician)
Lt. Col. J. E. Canham, M.D. (MC) (Also attending physician)
Maj. E. M. Baker, Ph.D. (MSC), Co-Project Leader
B. M. Tolbert, Ph.D. (Consultant, University of Colorado)

e. References

(1) Johnson, R. B., D. A. Peterson and B. M. Tolbert. Cellulose metabolism in the rat. J. Nutrition 72: 353, 1960.

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15. Minerals: Studies on mineral metabolism and interactions in the human with the use of radioisotopes (references cited in the paragraph are listed in Par. 15f)

Initial studies would seek the role of magnesium and calcium in human kidney stone disease:

a. Background

Urinary calculi are among the most ancient afflictions of man. This painful and often fatal disease is known to have occurred as long as 8,000 years ago (1), and no race or geographical area has been entirely free of a calculus problem. There appear to be "stone belts" of high incidence in regions such as southern China, northern Thailand, the Punjab district of India, Arabia and Iraq. In addition, there have been reports of "stone waves," one such occurring in Europe during this century (2).

Despite the antiquity and frequency of this disease, the basic mechanisms of calculus formation remain unknown, and fully 85% of all patients who form urinary calculi have no recognized local or systemic disease (3). Most authorities agree that a nutritional deficiency or imbalance is a probable factor, but few reliable studies have been conducted to relate specific nutrients to calculus formation in human populations (4).

Studies at this laboratory (5) and elsewhere have established that nephrocalcinosis and urolithiasis (principally phosphates and carbonates of calcium) are frequently associated with magnesium deficiency in rats and other species. Of particular interest is the observation that about 20% of apparently otherwise

normal rats consuming a semipurified diet containing 400 ppm magnesium (minimum requirement for growth is 120-150 ppm) will develop uroliths similar to those found in the markedly deficient rat and that elevation of the dietary Mg to 4,000 ppm will prevent this occurrence.

Furthermore, Selye has shown that intraperitoneal administration of magnesium will prevent the formation of uroliths which normally follows experimental hyperparathyroidism in the rat. An increase in dietary magnesium will also markedly lessen the accumulation of calcium in the kidney which results from a high phosphorus intake. Despite these indications of an important role for magnesium in calculus formation and calcium metabolism, practically no published information exists on the metabolism of this nutrient in human urinary calculi disease (4).

Preliminary studies at this laboratory (6) have indicated that some populations in areas with a reported high incidence of stone formation (e.g., Burma investigations) may, indeed, consume relatively low amounts of magnesium. In addition, magnesium supplements have brought at least a temporary (6 months) halt to the formation of phosphatic type stones in a patient with no demonstrable infection or metabolic disorder and a previous rate of stone formation of 2 per month for a period of 3 years (7).

Based on this evidence, it is felt that considerable justification exists for the study of the role of magnesium as well as other factors in human renal lithiasis. Certainly, a primary objective is a determination of the value

of magnesium supplements in a large number of patients and a study of any changes in urinary constituents associated with a favorable response to this therapy.

b. Basic experimental plan (regardless of use of radioisotope tracers)

(1) Patients will be obtained through the Departments of Urology at Colorado General Hospital and Fitzsimons General Hospital. Only those subjects will be chosen who form stones at a relatively rapid rate (at least one every 2 months) and who are free of renal infections.

A subject so chosen will be kept on a metabolic ward for a 2-week period so that 2 complete 3-day fecal and urine collections can be made. Total Ca, P, Mg and vitamin B_6 intake during this period can be estimated from tables of composition or by actual analysis. The total fecal collection for 3 days will be pooled, homogenized and ashed for a determination of its content of calcium, phosphorus and magnesium. A routine urinalysis (pH, sp. gravity, crystals, etc.) will be performed on each 24-hour urine collection and at least 500 ml will be saved for subsequent analysis. Proposed urinary constituents to be analyzed for are magnesium, calcium, phosphorus, oxalate, citrate, uromucoid, vitamin B_6 and xanthurenic acid. After the specimens are received from the 2 balance periods, 420 mg of MgO (250 mg Mg) will be given daily in a single capsule to be taken after supper. Therapy should continue for at least 6 months, during which time the patient's rate of stone formation will be noted. A 24-hour collection of urine will be made every 30 days and the above-mentioned tests will be performed. Patients may be asked to repeat the balance study after 6 months to ascertain

any changes in balance or retention of calcium, magnesium or phosphorus as a result of this treatment.

(2) Progress to date

To date, one patient has been studied completely in terms outlined above, that is, this man has been on magnesium supplements as treatment for his recurrent urolithiasis. He has been on these supplements for 6 months without a recurrence in stone formation; he was then brought back into the hospital and denied the supplements for a period of a month. During both periods, his urinary excretion of calcium, phosphorus, magnesium, oxalate and mucoprotein was determined.

A second patient has been given magnesium for 6 months and has shown a favorable response in that he has not formed stones during this period. He recently returned to the hospital for follow-up studies. The findings on magnesium therapy with these two stone-forming patients will be submitted as a manuscript to the Journal of Urology.

Additional patients are understudy with the cooperation of Lt. Col. C. A. Moore, M.D. (MC) of Fitzsimons General Hospital, Dr. O. G. Stonington of Colorado General Hospital, and Lt. Col. J. E. Canham, M.D. (MC) of the Metabolic Division of this laboratory. As additional cooperative patients become available, expanded clinical trials as to the effectiveness of this treatment for chronic lithiasis will be undertaken.

c. Experimental plan incorporating use of radioactive tracers (Par. 12b(2))

The mineral balance studies thus far conducted on the above subjects appear to indicate abnormalities in calcium and magnesium absorption and excretion. However, the balance techniques leave much to be desired from the standpoint of a precise and exacting procedure to give the definitive information necessary for an unequivocal evaluation of small changes that may occur in absorption or excretion. In addition, the method gives little information on turnover rates or retention of the dietary calcium and magnesium and of the magnesium supplements.

In order to obtain the desired information that may permit a better understanding of the cause of uroliths in humans and the effect of magnesium in their treatment, the use of Magnesium-28 and Calcium-45 or 47 is proposed. Accurate information on the absorption and turnover of the elements in the stone-forming subject could be readily obtained with the use of these isotopes. The information could be obtained on the patient both before treatment and after a period of magnesium treatment to determine changes or interactions that may have occurred in calcium or magnesium metabolism. Such data may give an insight into the mechanism of action involved. Of equal importance, comparative studies with the use of several salts or oxides of Magnesium-28 could be readily performed in an attempt to explain the reason for the apparent success obtained with MgO at this laboratory, while other salts of magnesium have been of no value (4, 8). Similar studies in a minimum number of normal volunteer subjects would be carried out as necessary to evaluate the findings in the patients.

It is hoped that a better understanding of the problem would result from the isotope studies which would lead to a screening test that would identify which stone-forming patients that could be expected to receive beneficial effects from magnesium therapy. The performance of the isotopic studies indicated appear highly necessary before recommendations or large scale treatment with magnesium be initiated with stone-forming subjects.

d. Procedures and health physics

The patients would be handled in a manner similar to that employed at present for non-radioactive mineral balance studies of calcium, magnesium and phosphorus. Selected stone-forming patients or normal volunteers would be placed on the Metabolic Ward at this laboratory. The subjects would receive a controlled diet without magnesium therapy. After a period of 7-10 days on these diets, with balance studies conducted, the patients would receive a tracer dose of either Mg-28 (not exceeding 20 μc) or Calcium-47 (not exceeding 5 μc) or Calcium-45 (9) (not exceeding 15 μc) orally. Markers would be employed to assist in the stool collections. Urine, stools and blood samples would be collected and analyzed until essentially no activity could be detected. It is hoped that dosages of radioisotope may be reduced further to one-half the amounts indicated and still permit satisfactory measurements. This would then permit double labeling of selected patients or repeat labeling of a patient or normal volunteer following 6 months of magnesium therapy without approaching the maximum permissible body burden of radiation. If the dosage cannot be reduced sufficiently, then other

patients or subjects would receive the second isotope or the isotope after 6 months of magnesium therapy. Balance studies with known diets and intakes and maintenance on the Metabolic Ward for periods of 5-6 days (i.e. before and after the 6-months' therapy period--not maintenance on the Metabolic Ward throughout the 6-months' period) would be associated with all subjects. If the oral studies indicate further evaluation of the retention and turnover of magnesium or calcium in the body, a limited number of select volunteer patients or normal volunteers would receive intravenously administered isotopes. In all instances, the intravenous dose would not exceed 35 μc. For comparative purposes, a limited number (3-5) of normal, healthy volunteer subjects would be placed on the same diets and balance studies performed with the use of the same isotopes in the same dosage as employed with the volunteer patients.

The healthy physics of Mg-28, Ca-47 and Ca-45 has been considered briefly before. It should be emphasized that the maximum dosages obtained with the amount of isotopes used in the proposed studies will at no time equal the maximum permissible dosage.

With the aid of the ICRP Handbook (Appendix I, reference 1), the calculations below were made. In each case "1" is the intinite dose received by the critical organ and "2" is the dose received during the first 13 weeks by the critical organ. The critical organ is given in parentheses.

A. 5 μc of Ca-47 administered orally (bone)

1. 0.359 rem
2. 0.359 rem

B. 2.5 μc of Ca-47 administered intravenously (bone)

1. 0.150 rem
2. 0.150 rem

C. 15 μc of Ca-45 administered orally (bone)

1. 5.883 rem
2. 1.897 rem

D. 7.5 μc of Ca-45 administered intravenously (bone)

1. 0.245 rem
2. 0.079 rem

*E. 20 μc Mg-28 administered orally (bone)

1. 0.465 rem
2. 0.465 rem

*F. 35 μc of Mg-28 administered intravenously (bone)

1. 1.992 rem
2. 1.992 rem

*G. 35 μc of Mg-28 administered intravenously (whole body)

1. 0.044 rem
2. 0.044 rem

*H. 20 μc of Mg-28 administered orally (stomach)

1. 0.116 rem
2. 0.116 rem (i.e. residence time of 1 hour)

*I. 20 μc of Mg-28 administered orally (small intestine)

1. 0.079 rem
2. Same (i.e. residence time = 4 hours)

*J. 20 μc of Mg-28 administered orally (upper large intestine)

1. 1.284 rem
2. Same (i.e. residence = 8 hours)

*K. 20 μc of Mg-28 administered orally (lower large intestine)

1. 1.720 rem
2. Same (i.e. residence time = 18 hours)

*Radiation burden for Mg-28 were obtained by calculations and the use of:

1. ICRP Handbook (Appendix I, reference 1).

2. Peaceful Uses of Atomic Energy, Vol. 24, Part 1, "Isotopes in Biochemistry and Physiology," 1958, United Nations Publication.

3. Radioactive Isotopes in Medicine and Biology: Medicine, S. Silver, 1962, Lea and Febiger, Publishers.

4. The Role of Magnesium in Biologic Processes, J. K. Aikawa, 1963, C. C. Thomas, Publisher.

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6. Radiological Health Handbook, PB 121784R, U. S. Dept. of Health, Education and Welfare, Public Health Service, U. S. Dept. of Commerce, 1960.

According to information supplied from the above sources, absorption of magnesium from the G.I. tract is very low. However, for the purpose of these calculations, 60% absorption was assumed when the isotope was administered orally and the critical organ considered bone. However, when segments of the G.I. tract were considered the critical organ, no absorption was assumed. As previously stated, elimination after absorption is very rapid. Again, however, in order not to underestimate the dosage, an intake and retention of 90% of the isotope was assumed to be removed from the blood by the critical organ (bone).

All collected excreta would be disposed of in an acceptable manner under the supervision of the Radioisotope Branch of this laboratory.

e. Personnel

H. E. Sauberlich, Ph.D. (PL-313), USAMRNL, Project Leader
Lt. Col. C. A. Moore, M.D. (MC), Fitzsimons General Hospital, Project Leader (Also attending physician)
Lt. Col. J. E. Canham, M.D. (MC), USAMRNL (Also attending physician)
Dr. O. G. Stonington, M.D., Colorado General Hospital (Also attending physician)
Capt. G. E. Bunce, Ph.D. (Consultant, Tripler General Hospital, Hawaii)
Lt. Col. M. E. McDowell, M.D. (MC), USAMRNL (Also attending physician)

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Appendix I. References on General Health Physics

1. Recommendations of the International Commission on Radiological Protection, ICRP Publication 2, Report of Committee II on Permissible Dose for Internal Radiation, 1959, Pergamon Press.

2. Radiological Health Handbook, U. S. Department of Health, Education and Welfare, Sept. 1960.

3. Radioactive Isotopes in Medicine and Biology: Medicine, S. Silver, 1962, Lea and Febiger, Publishers.

4. Radioactive Isotopes in Medicine and Biology: Basic Physics and Instrumentation, E. Quimby and S. Feitelberg, 1963, Lea and Febiger, Publishers.

5. Use of Radioisotopes in Animal Biology and the Medical Sciences, Vol. 1 and 2, 1962, Academic Press.

6. Maximum Permissible Amounts of Radioisotopes in the Human Body and Maximum Permissible Concentrations in Air and Water. Handbook 52, U. S. Dept. of Commerce.

7. Progress in Nuclear Energy: Series VI, Biological Sciences, J. G. Bugher, J. Coursaget and J. F. Loutit, Editors, 1959, Pergamon Press.

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VOLUNTARY CONSENT STATEMENT

Military _____ Military Patient _____ Civilian _____ Civilian Patient _____

I, _____, having the capacity to consent, voluntarily and without force or duress consent to participate in research involving the use of tracer amounts of radioisotopes. I have been informed of, and understand, the nature, duration, and purpose of the experiment, the method and means by which it is to be conducted, the inconveniences and hazards to be expected, and the effects upon my health and person which may possibly come from participation in the experiment.

Specifically, I agree to receive (intravenously / orally) a small quantity of _____ containing _____ microcuries of _____. I also agree to furnish urine and stool samples for the period following until no detectable radioactivity is present and submit to measurements of expired gases if Carbon-14 has been received.

I understand that I may at any time during the course of the experiment revoke my consent and withdraw from the experiment without prejudice.

I do not at this time have any physical diseases, except for the following _____ _____, or mental disease, to the best of my knowledge.

_____ _____

DATE SIGNATURE

_____

SIGNATURE OF WITNESS

APPROVAL

I have personally ascertained that the quality of the foregoing consent is sufficient to permit the volunteer to participate in the experiment.

_____ _____

ATTENDING PHYSICIAN PROJECT LEADER

Form 3

UNITED STATES ATOMIC ENERGY COMMISSION

APPLICATION FOR BYPRODUCT MATERIAL

SUPPLEMENT A—HUMAN USE

Form approved.
Budget Bureau No. 38-R0220.1

This page may be completed by the physician's preceptor (if any) in the medical use of radioisotopes. When the information is not furnished by the preceptor, the name and present address of the preceptor (if any) should be shown in item 12 below.

9. (a) USING PHYSICIAN'S NAME

DiBELLA,
[illegible]

(b) NAME AND ADDRESS OF APPLICANT (If different from 9(a))

[illegible]

File
Lic. No. 5-46-13

10. CLINICAL TRAINING AND EXPERIENCE OF PHYSICIAN WHO WILL USE BYPRODUCT MATERIAL

(A) ISOTOPE	(B) CONDITION(S) DIAGNOSED OR TREATED	(C) NUMBER OF CASES	(D) TYPE OF PARTICIPATION FOR ALL CASES IN COLUMN D (circle applicable numbers of items in accordance with key as set forth below)
I-131	Diagnosis of thyroid function	200	(1) (2) (3) (4)
	Treatment of hyperthyroidism	4	(1) (2) 3 4
	Treatment of thyroid cancer	1	(1) (2) (3) (4)
	Treatment of cardiac conditions		1 2 3 4
	Brain tumor localization		1 2 3 4
	Blood determinations		1 2 3 4
	Others:		(1) 2 3 4
			(1) 2 3 4
P-32 Soluble	Treatment of polycythemia and leukemia		1 2 3 4
	Brain tumor localization		1 2 3 4
	Treatment of bone metastases		1 2 3 4
	Others:		1 2 3 4
			1 2 3 4
P-32 $CrPO_4$	Treatment of prostatic cancer		1 2 3 4
	Treatment of cervical cancer		1 2 3 4
	Treatment of pleural effusions and/or ascites		1 2 3 4
	Others:		1 2 3 4
			1 2 3 4
Au-198 Colloid	Treatment of prostatic cancer		1 2 3 4
	Treatment of cervical cancer		1 2 3 4
	Treatment of pleural effusions and/or ascites		1 2 3 4
	Others:	32	(1) (2) (3) 4
			1 2 3 4
Cr-51	Blood determinations	2	(1) (2) (3) 4
	Others:	1	(1) 2 (3) 4
		2	(1) 2 3 4
Other Isotopes	[illegible]	41	(1) 2 3 4
	[illegible]	57	(1) 2 3 4
	[illegible]	23	(1) 2 3 4
	[illegible]	60	(1) (2) (3) (4)

Key to above numbers (column D) Active Participation and Discussion

1. Examination of patients to determine suitability for radioisotope diagnosis and/or treatment and recommendations on dosage to be prescribed.
2. Collaboration in calibration and administration of dosages including related measurements and plotting of data.
3. Active period of training and experience of sufficient duration to permit followup of patients through treatment and posttreatment period including reevaluation as to effectiveness and complications.
4. Study and discussion of case histories to establish most efficacious diagnostic and/or therapeutic techniques for this radioisotope use.

11. TOTAL NUMBER OF HOURS OF PARTICIPATION IN CLINICAL TRAINING_____ hours

12. THE TRAINING AND EXPERIENCE INDICATED ABOVE WAS OBTAINED UNDER THE SUPERVISION OR GUIDANCE OF

[illegible]

_____ AT _____

(Name of physician (preceptor)) (Institution) (Signature)

APPENDIX IV