liminary study, correlated approximately with an improvement in the symptoms of angina pectoris. The possible significance of this is discussed.

Addendum. Since completing these carbohydrate tolerance studies, we have tested the purity of several samples of the standard preparations of glucose and galactose, such as are routinely used in oral glucose and galactose tolerance tests. The galactose preparations had a non-galactose content of 4-14% (w/w), of which about half was copper reducing, and a small fraction glucose oxidase reducing: the glucose preparations had a non-glucose content of 2-10%, most of which was copper reducing, but not glucose oxidase reducing. However, since we used glucose and galactose from the same stock bottles throughout the tests, we believe that the changes of carbohydrate tolerance are genuine.

1. *Lancet*, 1961, v1, 150.
2. Weiss, J., Weiss, S., Weiss, B., *Ann. N. Y. Acad. Sc.*, 1959, v80, 854.
3. Sjoerdsma, A., Gillespie, L., Undenfriend, S., *ibid.*, 1959, v80, 969.
4. Laurence, D. R., Stacey, R. S., *Brit. J. Pharm.*, 1952, v7, 255.
5. Dormandy, T. L., Leak, D., Grant, M., *Lancet*, 1959, v2, 269.
6. Rowe, R. P., *Dis. Nerv. Syst.*, 1959, v20, Supple. No. 8, 5.
7. Marks, V., *Clin. Chim. Acta*, Amst., 1959, v4, 395.
8. Asatoor, A., King., E. J., *Biochem. J.*, 1959, v56, xliv.
9. Griffith, G. C., *Circulation*, 1960, v22, 1157.
10. Singh, I., Bardhan, P. N., *Lancet*, 1959, v2, 1141.
11. Fabrykant, M., Ashe, B. I., *N. Y. State J. Med.*, 1960, v60, 841.

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Urinary Metabolites of Mevalonic Acid: Evidence That Mevalonic Acid is not a Precursor of Isovalthine.* (27008)

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The discovery(1, 2), isolation(1, 2), identification(2), and synthesis(2) of a new amino acid, S-(isopropyl-carboxymethyl)-cysteine, termed isovalthine has been recently described by Japanese investigators. This amino acid was observed in the urine of hypercholesterolemic individuals. The structure of the amino acid is such as to suggest that it arises biogenetically by addition of cysteine to $\triangle^1$-isopentenyl pyrophosphate followed by or simultaneously with loss of pyrophosphate and oxidation of the primary alcohol to an acid. $\triangle^1$-Isopentenyl pyrophosphate may be visualized as a compound in equilibrium with $\triangle^2$-isopentenyl pyrophosphate which, in turn, is in equilibrium with $\triangle^3$-isopentenyl pyrophosphate. The latter 2 compounds are known intermediates in utilization of mevalonic acid (MVA) and are interconvertible by the enzyme isopentenyl pyrophosphate isomerase(3). If this or a related mechanism has any merit, it would suggest that isovalthine may be a significant "detoxification" product of intermediates in sterol biosynthesis whereby these intermediates are diverted from sterol biosynthesis and appear in the urine as an innocuous metabolite. The experiments described here involving recovery of added carrier isovalthine from the urine of hypercholesterolemic individuals that had received MVA-2-C^{14} demonstrate that either (a) MVA is not a precursor of isovalthine, or (b) MVA may be a precursor of isovalthine but the metabolite is not encountered in all cases of hypercholesterolemia. Evidence is also presented that MVA is the precursor of an amphoteric metabolite possibly related to

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isovalthine.

Materials and methods. DL MVA-2-C^{14} as the N, N'-dibenzylethylenediamine salt (2.48 mc per mM) was administered intravenously to 2 hypercholesterolemic individuals. One subject (HL, F, 58 yrs, hypertensive vascular disease) received 13μc. The other subject (ES, F, 47 yrs., coronary artery disease) received 20μc. At the time that the 2 subjects were studied one (HL) had a total cholesterol blood level of 301 mg per 100 ml while the other (ES) had a level of 371 mg per 100 ml. Neither subject was receiving antihypercholesterolemic therapy at the time these studies were done. Complete 24-hour urine collections from each subject were obtained following MVA administration and these collections were then frozen until studied in the laboratory.

Isovalthine at a level of 1 mg per ml was added to each urine sample immediately after unfreezing. In the case of HL this amounted to 1420 mg; in the case of ES, to 1200 mg. The isovalthine used was synthesized by condensation of L-cysteine with DL-α-bromoisovaleric acid in aqueous alkali according to a previously published procedure(4). The product was, therefore, a diastereoisomeric mixture. It has not, as yet, however, been possible to separate the diastereoisomers by fractional crystallization. Each urine sample was then poured onto a column of 150 ml of Dowex-1 freshly prepared in the hydroxide form. The urine was allowed to run through the column at a rate of about 10 ml per min and was followed with several hundred ml of water. The column was then eluted with 0.1 N hydrochloric acid. One hundred ml fractions of the run through and eluates were collected, and 0.01 ml amounts of the various fractions were spotted on a sheet of Whatman #1 paper and developed by ascending chromatography in butanol: acetic acid: water (160: 40: 40). The added isovalthine was located with ninhydrin (RF of about 0.53). Two ml amounts of the various fractions were also pipetted into planchets (2 inch diameter, stainless steel, concentric rings), neutralized where necessary to phenolphthalein and evaporated to dryness in a stream of air at room temperature. The samples were counted in a thin window gas flow counter. Those eluate fractions containing the isovalthine which coincided with those fractions containing radioactivity were combined.

The combined fractions from the Dowex-1 column were poured onto a column of 200 ml of Dowex-50 freshly prepared in the acid form. The column was washed with several hundred ml of water and then eluted with 2 N ammonia. 0.01 ml amounts of the various fractions (100 ml) were spotted on a strip of Whatman #1 paper and the isovalthine located with ninhydrin. Two ml amounts of the various fractions were also counted as in the previous step. Those fractions containing the isovalthine were combined and concentrated *in vacuo* to a small volume in a rotary evaporator.

The concentrated fractions from the Dowex-50 column were poured on to a column of 150 ml of Dowex-1 freshly prepared in the acetate form. The column was washed with several hundred ml of water and then eluted with 2 N acetic acid. The isovalthine was located and radioactivity of the various fractions determined as described earlier. Those fractions containing the isovalthine were evaporated *in vacuo* to dryness in the rotary evaporator.

The isovalthine fractions from the previous step were dissolved in a small volume of water and treated at steam bath temperature with norite. The filtrate from the norite treatment was concentrated to a small volume and ethanol added to a concentration of about 50%. Isovalthine crystallized on cooling and was filtered off, washed with 50% ethanol, and dried at room temperature. Identity and homogeneity of the products was established by paper chromatography and infrared spectrum analysis.

The recovered isovalthine was examined for radioactivity by liquid scintillation counting in a Packard 'Tri-Carb' scintillation spectrometer. This method of counting is approximately 4 times more sensitive than the gas flow counting used with the various chromatographic fractions. The mixtures counted contained 20 mg of recovered isovalthine, 1 ml of 1 M 'Hyamine' solution in methanol and 10 ml of scintillation mixture (4 g PPO and 30 mg POPOP per liter of toluene.)

Results. The results obtained are summar-

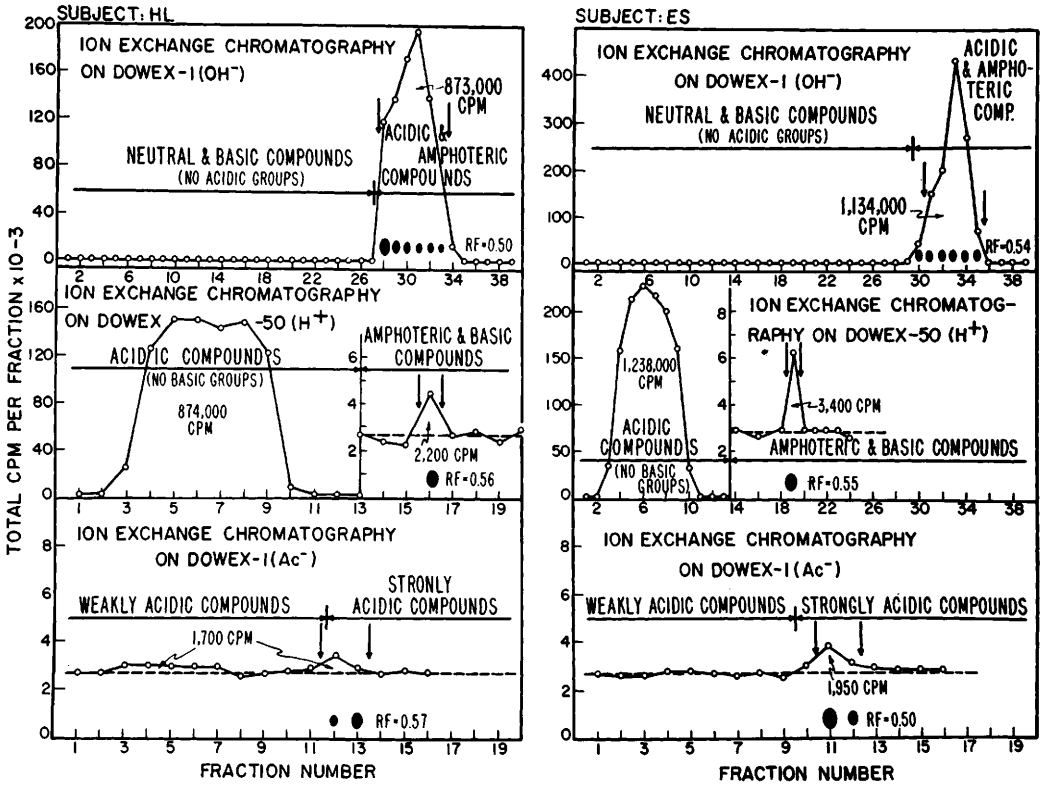

FIG. 1. Summary of chromatographic studies. Those fractions of a chromatogram that were combined and subjected to subsequent chromatography or crystallization are indicated between the vertical arrows. Counts summarized are *total* counts per fraction. Background where significant is indicated by a dashed line. The darkened elipses indicate location and approximate amount of added isovalthine as determined separately by ascending paper chromatography in butanol: acetic acid: water (160: 40: 40) and development with ninhydrin.

TABLE I. Summary of Information on Recovered Isovalthine.

Exp	Material studied	Radioactivity* counts in 30 min	RF value†
HL	Recovered isovalthine	577	.51
	Reference "	579	.49
ES	Recovered isovalthine	573	.51
	Reference "	572	.48

* Each sample counted contained 20 mg of recovered or reference isovalthine, 1 ml 'Hyamine,' and 10 ml of scintillation mixture.

† Ascending chromatography in butanol: acetic acid: water (160: 40: 40).

ized in Fig. 1 and in Table I. When urine from hypercholesterolemic individuals administered DL MVA-2-C^{14} and to which nonradioactive isovalthine had been added is chromatographed on the strong anionic exchange resin Dowex-1 in the hydroxide form radioactive material and isovalthine are retained by the column and are eluted with dilute hydrochloric acid (Fig. 1). The radioactive material is without doubt largely unnatural MVA (from the racemate administered) which is known to be inactive biochemically(5). When the hydrochloric acid eluates are chromatographed on the strong cationic exchange resin Dowex-50 most of the radioactivity of the urine is not retained by this resin as would be expected if the radioactivity is largely unnatural MVA (no basic groups). On the other hand, a small fraction of the radioactivity of the original urine is retained and since it had been previously retained by an anionic exchange resin it must represent amphoteric material. This radioactivity coincided with the isovalthine but it should be appreciated that no fractionation of amphoteric material is accomplished with the strong eluting agent (2 N ammonia) em-

ployed. When the ammonia eluates are chromatographed on the strong anionic exchange resin Dowex-1 in the acetate form most of the radioactivity is retained by this resin and is eluted by acetic acid (2 N). Under the conditions of the chromatography employed, monoamino monocarboxylic amino acids are not retained by the resin but monoamino dicarboxylic amino acids are held and eluted with the acetic acid. The results obtained furnish presumptive evidence that the radioactive, amphoteric metabolite in the urine of hypercholesterolemic individuals administered MVA-2-C^{14} is a monoamino dicarboxylic acid. On the other hand, isovalthine recovered from the radioactive fractions of the Dowex-1 acetate column is devoid of radioactivity (Table I). These results form the basis for the conclusion that either (a) MVA is not a precursor of isovalthine, or (b) MVA may be a precursor of isovalthine but the metabolite is not encountered in all cases of hypercholesterolemia.

Discussion. These studies have furnished evidence that MVA does not serve as a precursor of isovalthine in the hypercholesterolemic individual but instead is converted in small amounts into some other amphoteric metabolite presumably a monoamino dicarboxylic acid. The unqualified conclusion that MVA is not a precursor of isovalthine is not justified because of a possible alternative interpretation of the data. Obviously if isovalthine is derived from MVA but is only randomly encountered in the urine of hypercholesterolemics it is possible that the amino acid was not initially present in the urine samples examined. Under this condition carrier isovalthine would have been recovered without radioactivity following administration of radioactivity while in similarly treated hypercholesterolemics excreting isovalthine carrier isovalthine would have been recovered with radioactivity. If this is the explanation for the failure to recover radioactivity in carrier isovalthine then these studies have significance in that they show that isovalthine is not a ubiquitous, quantitatively significant metabolite of sterol intermediates.

If MVA is not a precursor of isovalthine, an explanation seems necessary for the existence in urine under conditions of what is probably increased sterol biosynthesis (hypercholesterolemia) of a metabolite that does not originate from sterol intermediates. Possibly isovalthine originates from a sterol precursor before MVA in the biosynthetic sequence. More likely under conditions of excess sterol biosynthesis relatively non-specific mechanisms for the diversion of sterol intermediates away from the "normal" course come into play and various compounds related in structure to sterol intermediates are indiscriminately metabolized.

Summary. Synthetic isovalthine (S-(isopropyl-carboxymethyl)-cysteine) was added to urine from hypercholesterolemic patients that had received mevalonic acid-2-C^{14} intravenously. Isovalthine recovered from the urine was found to be devoid of radioactivity thus indicating that either (a) mevalonic acid is not a precursor of isovalthine, or (b) mevalonic acid may be a precursor of isovalthine but the metabolite is not encountered in all cases of hypercholesterolemia. The existence in the urine samples studied of an amphoteric metabolite of mevalonic acid was demonstrated.

1. Mizuhara, S., Okuda, K., Kurahashi, K., Iwado, Y., *J. Biochem.*, 1959, v46, 101.

2. Oomori, S., Mizuhara, S., *Biochem. Biophys. Res. Comm.*, 1960, v3, 343.

3. Agranoff, B. W., Eggerer, H., Henning, U., Lynen, F., *J. Am. Chem. Soc.*, 1959, v81, 1254.

4. Wright, L. D., PROC. SOC. EXP. BIOL. MED., 1961, v107, 392.

5. Cornforth, R. H., Fletcher, K., Hellig, H., Popjak, G., *Nature*, 1960, v185, 923.

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