is probably due to the elution of low molecular weight cofactors and substrates from the cells so that the cells can no longer maintain the integrity of their semi-permeable membrane. Le Page(7) has shown that 5 washes (centrifuging and resuspending the cells) of EAT cells will deplete all or almost all of the intracellular free glycine. Fig. 4 shows that repeated washings of EAT cells in buffered saline result in an increasing number of cells which take up the nigrosin dye indicating a change in membrane permeability. The effect of time on modification of the cells and the effect of repeated washing on the cells remain empirical factors in the modification procedure, since both are influenced by the amount of protein and salts present in the aliquot to be modified. Studies are in progress to attempt control of these factors through use of a relatively inert suspending medium.

Of particular interest with regard to previous studies and to the results herein obtained is the fact that all cells in a given population were not similarly nor simultaneously affected by the experimental conditions employed. Inspection of the figures shows that only a certain percentage of the cells were affected as measured by dye (nigrosin) uptake, although all cells were simultaneously subjected to the same environment. The change in membrane permeability or the reaction of these cells to a particular environment appears to be dependent upon the cell *per se* and seems to vary from cell to cell. It is not possible at this time to definitely ascribe this phenomenon to any particular biochemical or physiological characteristic or function of the cell.

*Summary.* A study was made of factors involved in modifying the semi-permeable nature of the cell membrane of Ehrlich ascites tumor cells. It was found that a minimum concentration of 0.1% nigrosin was necessary for accurate differential quantitation of modified and unmodified tumor cells. The cells were not visibly affected by hypotonic saline solutions as low as 3.0 mg NaCl/ml. Lower concentrations resulted in changes in membrane permeability, and lysis occurred at concentrations of 1.0 mg/ml, or less. Not all cells of a given population were affected similarly by experimental hypotonic shock.

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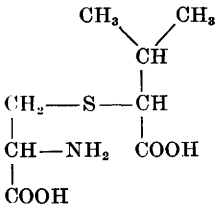
## A Convenient Preparation of Isovalthine.* (26635)

LEMUEL D. WRIGHT
*Graduate School of Nutrition, Cornell University, Ithaca, N. Y.*

Mizuhara *et al.* and Oomori and Mizuhara have recently described the discovery(1,2), isolation(1,2), identification(3), and synthesis(3) of a new amino acid, S-(isopropyl-carboxymethyl)-cysteine, to which they gave the name isovalthine. This amino acid was first observed in the urine of hypercholesterolemic individuals.



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Studies on the biogenesis of the compound in this laboratory were hampered by the inconvenience of using liquid ammonia required in the original synthesis. This communication describes an easier synthesis and details of an isolation procedure whereby the compound is easily made available in quantity.

*Methods and materials. Synthesis and isolation of isovalthine.* Five g of cysteine (0.041 M, free base, Nutritional Biochemicals Corp.) are dissolved in 100 ml of water. Eighteen g of α-bromoisovaleric acid (0.1 M, Eastman-Kodak Co., some other preparations have shown the presence of significant amounts of extraneous material) are added followed by 7.3 g solid sodium hydroxide (0.18 M, pellets) which are added with swirling over a period of a few minutes. The mixture is allowed to stand at room temperature for 2 hours and is then heated on a steam bath for one hour. The amount of α-bromoisovaleric acid used to react with 5 g cysteine (0.041 M) has been varied from 9 (0.05 M) to 18 (0.1 M) g and the time of heating varied from a minimum of 30 min of heating directly after mixing to a maximum of one hour of heating after standing at room temperature for 2 hours. The amount of α-bromoisovaleric acid used or the amount of heating employed, at least within the limits mentioned, does not influence the yield or identity of the final product obtained. In all instances where the quantities of reactants have been varied the sodium hydroxide has been varied correspondingly to maintain an amount equivalent to the carboxyl groups of cysteine and of α-bromoisovaleric acid plus the hydrobromic acid eliminated if all the cysteine used reacted with α-bromoisovaleric acid.

The reaction mixture is poured onto a column of 150 ml of Dowex-1, 50-100 mesh, freshly prepared in the $OH^-$ form by pretreatment in the column with several hundred ml of 10-20% potassium hydroxide and then with water. The reaction mixture is allowed to run through the column at a rate of about 10 ml/min and is followed with several hundred ml of water. The isovalthine, now free of cations, together with excess α-bromoisovaleric acid is eluted from the column with 0.1 N hydrochloric acid. 100 ml fractions of the run-through and eluates are collected. 0.01 ml amounts of the various fractions are spotted on a sheet of Whatman #1 paper and developed by ascending chromatography in butanol:acetic acid:water (160:40:40). The isovalthine is located with ninhydrin (RF of about 0.53) and those fractions containing the isovalthine combined.

The isovalthine fractions from the Dowex-1 column are poured onto a column of 200 ml of Dowex-50, 50-100 mesh, freshly prepared in the $H^+$ form by pretreatment in the column with several hundred ml of 5 N hydrochloric acid and then with water. The isovalthine fraction is allowed to run through the column at a rate of about 10 ml/min and is followed with several hundred ml of water. The isovalthine, now free of excess α-bromoisovaleric acid, is eluted from the column with 2 N ammonia. 100 ml fractions of the run-through and eluates are collected. 0.01 ml amounts of the various fractions are spotted on a strip of paper. The isovalthine is located with ninhydrin and those fractions containing isovalthine combined and concentrated to a small volume in a rotary evaporator at a temperature not exceeding 70°C. A white precipitate, identified as cystine, sometimes appears on concentrating the Dowex-50 eluates. This should be removed before proceeding to the next step.

The isovalthine concentrate from the Dowex-50 column is then poured onto a column of 150 ml of Dowex-1, 50-100 mesh, freshly prepared in the $Ac^-$ form by pretreatment in the column with several hundred ml of 5 N acetic acid and then with water. The concentrate is allowed to run through the column at a rate of about 10 ml per min and is followed with several hundred ml of water. The isovalthine from which essentially all of the contaminating cystine and/or cysteine has now been removed is eluted from the column with 2 N acetic acid. 100 ml fractions of the run-through and eluates are collected. 0.01 ml amounts of the various fractions are spotted on paper, developed in butanol:acetic acid:water and treated with ninhydrin. Those fractions containing the iso-

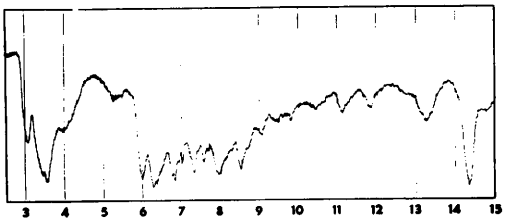


FIG. 1. Infrared absorption curve of isovalthine synthesized from L-cysteine and DL-α-bromoisovaleric acid. Figures on abscissa denote wavelengths in microns.

valthine are concentrated to a syrup *in vacuo* with the rotary evaporator and crystallized in a thin layer on the interior of the flask by constant motion of the flask until solidification of the product occurs. Excess acetic acid is removed with a stream of nitrogen.

The isovalthine is dissolved in a small volume of water and treated with norite. Ethanol to a concentration of about 50% is added to the hot filtrate. The mixture is cooled and crystallization occurs. The crystallization is apt to be slow and is facilitated in subsequent crystallizations by seeding with isovalthine. Several crops of crystals are obtained by repetition of the crystallization with successively smaller volumes. The yield of once-crystallized material obtained in several crops will amount to about 3-5 g or a yield of about 33-55%. An analysis of a typical batch of first crop material is as follows:

| | C | H | N | S |
|---|---|---|---|---|
| Calculated for $C_8H_{15}NO_4S$ | 43.42 | 6.83 | 6.33 | 14.49 |
| Found | 42.81 | 6.83 | 6.16 | 14.25 |

*Analysis by Scandinavian Microanalytical Laboratory*

The product is obtained in the form of clusters of needles. It melts (with decomposition) at 185°. Isovalthine prepared as described is free of extraneous ninhydrin-reacting material. The infrared absorption curve of a typical preparation is given in Fig. 1.

*Spacial configuration.* From a consideration of the structure of isovalthine it is apparent that a synthesis involving L-cysteine and DL-α-bromoisovaleric acid as moieties should yield 2 diastereoisomers (L-cysteine-D-α-bromoisovaleric acid and L-cysteine-L-α-bromoisovaleric acid). Attempts at separating the diastereoisomers by fractional crystallization have been unsuccessful. Thus successive crops of crystals from a preparation have, within experimental error, the same optical rotation. Specific rotations of isovalthine as obtained by the procedures described when determined at 21-24° at concentrations of 10 mg/ml in a 4 dec tube with a white light and simulated D line filter are as follows:

| Solvent | [α] |
|---|---|
| Water | − 7° |
| 2 N Hydrochloric acid | +18° |

*RF values.* Isovalthine prepared as described has RF values in the following systems as indicated; butanol:acetic acid:water (160:40:40), 0.53; butanol:ethanol:water (160:40:40), 0.12; butanol saturated with water, 0.04; pyridine:water (130:70), 0.35; buffered phenol(4), 0.28.

*Summary.* A convenient preparation of the new amino acid isovalthine is described. Some characteristics of the synthetic material are given.

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