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ISOLATION AND IDENTIFICATION OF VITAMIN C*

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The previous work reported from this laboratory on the chemical nature of vitamin C has been continued, particularly in the study of the solubility of the active material in organic solvents, leading towards its isolation by crystallization. This paper deals with (a) the precipitation of the active material as the lead salt, and *(b)* the isolation of a crystalline compound which is active in preventing scurvy in guinea pigs. The properties of this active crystalline substance correspond with those given for the "hexuronic acid" studied by Szent-Györgyi as an oxidation-reduction factor in adrenal cortex, oranges, and cabbage. We believe that the two substances are identical, as stated in a previous communication.

EXPERIMENTAL

The method used for testing the activity of our preparations was essentially that described by Sherman, La Mer, and Campbell, except that we have adopted a shorter time interval (56 days) to facilitate fractionation studies. The procedure employed for concentrating the antiscorbutic factor was essentially the same as that previously reported from this laboratory.

2.5 liters of lemon juice were decitrated and an absolute acetone solution of the active material was obtained according to the general method previously reported. This solution was evaporated to dryness and taken up in 10 cc. of *n*-propyl alcohol. To this was added an equal volume of petroleum ether. The solid, previously found to be of low activity, was centrifuged off and the clear liquid divided into two parts. A half saturated solution of lead acetate in absolute

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TABLE I

Precipitation by Lead and by Petroleum Ether

Test preparation	No. of animals	Fed daily, lemon juice equivalent	Average gain or loss in weight	Average survival (56 day test)	Average scurvy score
		cc.	*gm.*	*days*	
First lead precipitate	5	10-5*	171	56	2
Second lead precipitate	5	10-5*	−107	49	13
Solids not precipitated by lead	4	10-5*	−100	35	16
Combination of above three fractions	4	10-5*	149	56	1
Ethyl acetate-petroleum ether-soluble fraction	4	10-5*	−111	35	14
Ethyl acetate-petroleum ether-soluble and insoluble fractions	5	10-5*	80	56	3
Ethyl acetate before adding petroleum ether	5	10-5*	153	56	2
Positive controls	2	1-2†	103	56	1
Negative controls	3	0	−95	24	13

* The lemon juice equivalent was reduced from 10 cc. to 5 cc. on the 31st day.

† The lemon juice was increased from 1 cc. to 2 cc. on the 40th day.

methyl alcohol was added to one part of the solution until precipitation was somewhat over half completed. The yellow semicrystalline solid was centrifuged and dissolved in alcoholic hydrochloric acid to remove the lead. This was made up in aqueous solution, after evaporating off the hydrochloric acid, and stored in an ice box under carbon dioxide for feeding. Precipitation in the centrifuged liquid was then completed by the addition of more lead solution, and the second precipitate was treated in the same manner as the first. The results given in Table I indicated that the active material was precipitated by lead, with most of the activity in the first precipitate,

The remainder of the clear liquid from the petroleum ether-propyl alcohol precipitation was evaporated to dryness and extracted with anhydrous ethyl acetate overnight in an ice box. Purified quartz sand was mixed with the residue to facilitate extraction. The ethyl acetate extract was evaporated to approximately 5 cc. and an equal volume of petroleum ether added. A light yellow semicrystalline material was thrown down which, on standing at room temperature, formed a sirup. From Table I is it evident that the greater part of the active material was in the fraction which separated from solution. By carrying out the precipitation in dry ice, a pale yellow crystalline material was obtained. This was centrifuged and redissolved in ethyl acetate. It was

found that all of the material would not redissolve in a small volume of ethyl acetate, but, instead, a part remained as a semisolid which, when dried in a vacuum desiccator, formed needle-like crystals resembling the lactone form of the hexuronic acid described by Szent-Györgyi.

The vitamin can be recrystallized readily from butyl alcohol, acetone, ethyl acetate, ethyl alcohol, or methyl alcohol, by the addition of petroleum ether. The appearance of the crystals varies with different solvents.

The evidence from which we conclude that vitamin C as isolated in our laboratory is identical with the hexuronic acid studied by Szent-Györgyi and Kendall as a reducing factor in plant and animal tissues, is as follows *(i.e.* they correspond in): *(a)* natural occurrence so far as studied (the protective level of 0.5 mg. daily of our preparation corresponds with 0.5 mg. of hexuronic acid estimated by Szent-Györgyi (1928) in 2 cc. of orange juice, *(b)* oxidation by iodine and by Benedict's reagent (quantitative), (c) optical rotation ($[\alpha]^{20}_{D}$ = + 25° ± 1°), *(d)* acid titration equivalent (exact for the free acid), *(e) C* and H combustion, for CeHsQe, 0 reversible formation of a lactone and a free acid, (*g*) typical crystal forms, *(h)* solubility in a number of organic solvents, (*i*) precipitation as a lead salt, (*j*) instability toward alkalies and oxidizing agents, *(k)* diffusion rate and electrical transference (McKinnis and King), (*l*) melting-point (183-185°).

Additional studies on the chemical nature of Szent-Györgyi's nexuronic acid and our own crystalline vitamin C preparations are under way. In a preliminary paper which has just been received, it is evident that Szent-Györgyi and Svirbely have found the hexuronic acid (from adrenal glands) protective against scurvy on a 1 mg. level.

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