Our attempts to isolate the conjugated oestrogens in mare's urine have not so far been successful, but since we have obtained evidence on their probable chemical nature, the opportunity is taken to present a preliminary report on the work.

Distribution between various immiscible solvents of an alkali-washed butanol extract of urine from a mare at the seventh month of pregnancy, yielded a white amorphous solid containing about 40% of chromogenic oestrogen (calculated as oestrone). This material was insoluble in ether but easily soluble in water. It gave a negative naphthoresorcinol test indicating the absence of glucuronic acid. Sulphur was present however, and since after hydrolysis with dilute HCl a positive test for inorganic SO_4 was obtained it is possible that the oestrogens are conjugated with sulphuric acid. Millon's test was negative, indicating the blocking of the phenolic hydroxyl of the oestrogen by the conjugating group. Since the preparations obtained were obviously impure, a final decision concerning the chemical nature of these conjugated oestrogens must be deferred.

We wish to acknowledge with thanks the helpful cooperation of Professor N. E. McKinnon and Dr. R. D. H. Heard of the Connaught Laboratories in supplying us with samples of mares' urine. We are also indebted to the Banting Research Foundation for a personal grant to one of us (B.S.).

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Determination of Reduced Ascorbic Acid in Blood.

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Since the appearance of the clinical methods for determining blood ascorbic acid by Farmer and Abt[1, 2] considerable interest has been aroused in the study of vitamin C in various diseased conditions. The determination depends on the following proceduure: Deproteinization of the plasma by 10% metaphoric acid[3] and titra-

1 Farmer, C. J., and Abt, A. F., PROC. SOC. EXP. BIOL. AND MED., 1935, **32**, 1625.

2 Farmer, C. J., and Abt, A. F., PROC. SOC. EXP. BIOL. AND MED., 1936, **34**, 146.

3 Fujita, A., and Iwatake, D., *Biochem. Z.*, 1935, **277**, 293.

tion of the reduced ascorbic acid present in the filtrate with 2:6 dichlorophenol indophenol.[4]

By the use of this method we have obtained high and low ascorbic acid values in presumably normal subjects, or at least in individuals in whom there was no reason to suspect vitamin C deficiency. Furthermore, determinations on the same subject within 2 or 3 hours yielded various blood ascorbic acid values. In all these determinations great care was exercised to avoid the presence of disturbing catalysts.[5]

Duplicate and triplicate readings on the sample of plasma by the same investigator or by another, yielded diverse amounts of vitamin C present. Naturally, this difficulty aroused our suspicion as to the accuracy of the method for clinical purposes. Our first thought was that the error lay in observing the true endpoint of the titration due to the oxidation of the dye. Titrations were then carried out by the use of a photometer and the pink endpoint brought to the same intensity in all cases. Even this failed to yield consistent figures where the plasma had been collected some time previous to the experiment. We particularly noticed that samples of blood plasma which gave certain readings at one time, failed to give the same readings 2 or 3 hours later. The common practice of keeping blood, either in the ice-chest or at room-temperature, for various lengths of time was investigated. Two hundred cubic centimeters of blood were withdrawn from a normal individual, oxalated, and plasma separated from the cells. The plasma was divided into 2 portions, one maintained at 26°C. and the other at 0°-5°C. Titrations were carried out hourly, and on a subsequent occasion the experiment was carried out at 10- to 15-minute intervals for 2 hours. In each instance a fresh solution of 10% metaphosphoric acid was carefully made up. The dye-values were repeatedly checked with distilled water and acetic acid for the blank readings. The actual titrations were started within one-half hour from the time the blood was drawn from the patient.

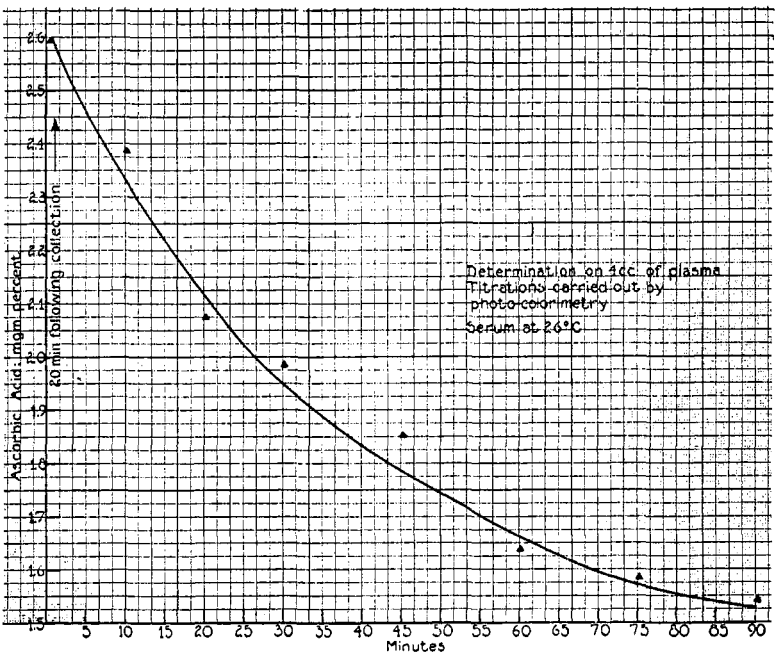
Four cc.* of plasma were diluted with 8 cc. of double-distilled water; to this was added 8 cc. of 10% metaphosphoric acid. Thorough mixing was effected by the use of a clean, dry stirring rod. The resulting precipitate was filtered through a Whatman

[4] Cohen, B., Gibbs, H. D., and Clark, W. M., *Public Health Rep. U. S. P. H. S.*, 1924, **39**, 804.

[5] Barron, E. S. G., Demeio, R. H., and Klemperer, F., *J. Biol. Chem.*, 1936, **112**, 625.

* 2 cc. of plasma were used, also, with 4 cc. of distilled water and 4 cc. of 10% metaphosphoric acid.

No. 42 filter paper. 8 cc. of the clean filtrate was used for titration with 2:6 dichlorophenol indophenol, which had been standardized so that 20 mg. of dye were equivalent to 20 mg. of vitamin C. A solution of 29 mg. of 2:6 dichlorophenol indophenol in 100 cc. of double distilled water is suitable for such purposes. The titrations were carried out by actual visual perception of the pink endpoint, and also by the use of the Evelyn colorimeter.[6] In no instance were metal parts, rubber, or cork material allowed to come in contact with the solution.



Plasma ascorbic acid values, in relation to the time of standing at 26°C. are shown in Chart 1. The loss of titratable ascorbic acid, presumably due to auto-oxidation, is even greater if the serum is kept standing at 0-5°C.

Summary. The ascorbic acid value of blood is materially affected by standing either in the ice-box or at room temperature. Determinations should be carried out within one-half hour after the collection of the blood.

[6] Evelyn, K. A., *J. Biol. Chem.*, 1936, **115**, No. 1.