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CRITICAL REMARKS ON THE DETERMINATION OF URINARY EXCRETION OF ASCORBIC ACID

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Szent-Györgyi (1), Svirbely and Szent-Györgyi (2) and Tillmans (3) have demonstrated that ascorbic acid possesses reducing properties, and that it can be determined by titration with the redox-dye 2,6 dichlorophenol indophenol. It therefore became possible to measure this vitamin in tissues and body fluids. In urine, an indophenol reducing substance was demonstrated by

van Euler and Klussman (4), van Eekelen *et al.* (5), and Harris *et al.* (6). Harris *et al.* (6), van Eekelen *et al.* (7), and Johnson and Zilva (8) have shown that following the intake of large amounts of ascorbic acid the urinary excretion of reducing substances increases. Application of the reduction reaction to the quantitative determination of ascorbic acid requires that other reducing substances be removed or prevented from reacting by controlling the conditions. Titration in an acid medium excludes ferrosalts and gluta-

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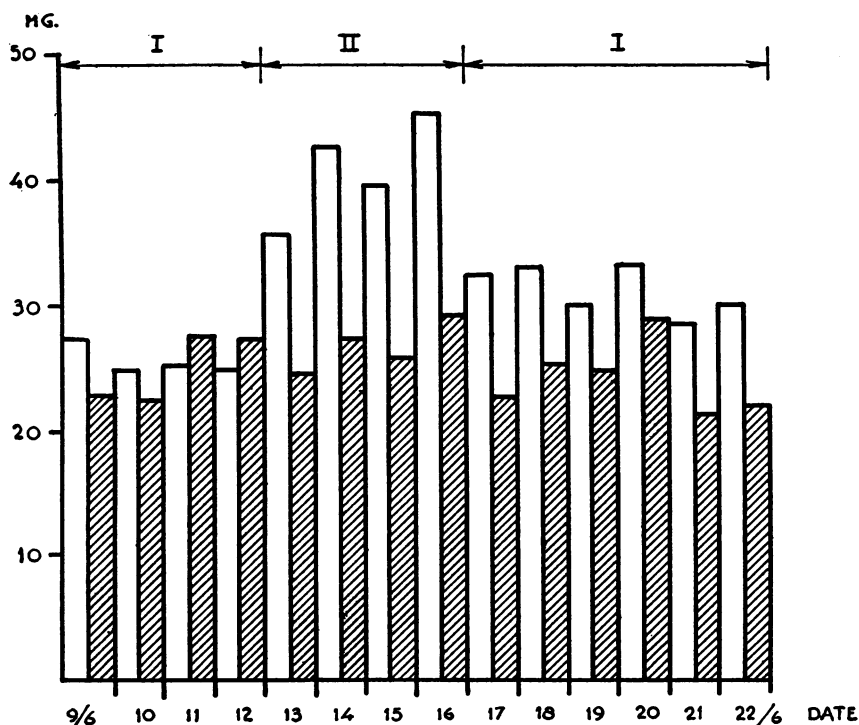


FIG. 1. THE INFLUENCE OF DIETARY PROTEIN ON THE EXCRETION OF NONSPECIFIC REDUCING SUBSTANCES

mgm. cevitamic acid in urine after precipitation with mercuric acetate, in 24 hours.

total reducing substances in 24 hours expressed as mgm. cevitamic acid.

I low protein diet.
II high protein diet.

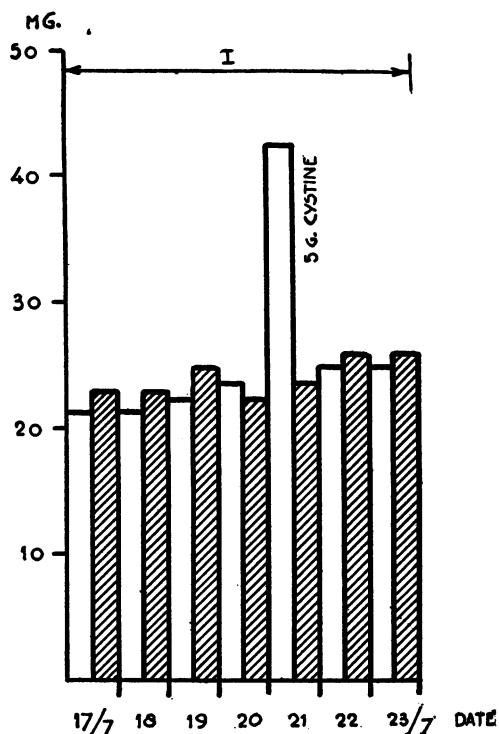




FIG. 2. THE INFLUENCE OF CYSTINE ON THE EXCRETION OF NONSPECIFIC REDUCING SUBSTANCES

 mgm. cevitamic acid in urine after precipitation with mercuric acetate, in 24 hours.
 total reducing substances in urine expressed as mgm. cevitamic acid, in 24 hours.
 I low protein diet.

thione (10, 11, 12). Other substances, *e.g.*, cysteine, ergothionine, thiosulphate, interfere even in an acid medium. These substances can be removed by precipitation with mercuric acetate (13, 14, 15) or with barium acetate (16). Some experiments will be presented demonstrating the magnitude and the variability of the errors which may be incurred if these interfering substances are not removed. Furthermore, the technique of the saturation test will be discussed.

Since our method has not been described in the American literature, it will be given in some detail.

METHOD

Reagents. One hundred mgm. of 2,6 dichlorophenol indophenol (Hoffman-LaRoche) are dissolved in 500 cc. of distilled water at 85° C., filtered, and approximately 0.2 gram of NaHCO_3

added. This solution is standardized against crystalline ascorbic acid (levo-ascorbic acid Roche) and is fairly stable for a period of a few weeks if kept in a cool dark place. It must, however, be standardized once a week. In the course of a month we have noted a decrease in concentration of not more than 5 per cent.

Mercuric acetate, 20 per cent solution, filtered, after standing 2 days, to remove $\text{Hg}(\text{OH})_2$ formed by hydrolysis.

Trichloroacetic acid 3 per cent and 10 per cent.
Procedure. Freshly voided urine² has been

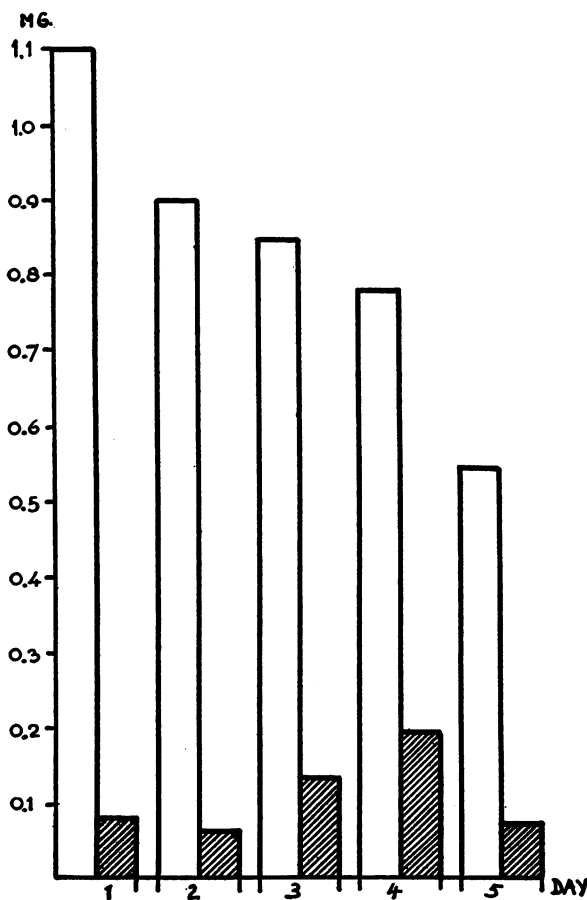




FIG. 3. THE EXCRETION OF NONSPECIFIC REDUCING SUBSTANCES BY A DIABETIC PATIENT

 mgm. cevitamic acid per ml. of urine after precipitation with mercuric acetate.
 total reducing substances per ml. urine expressed as mgm. cevitamic acid.

² No satisfactory method is available to prevent loss of ascorbic acid from urine on standing (8, 21, 22).

titrated with and without the removal of interfering substances in human subjects under various conditions. In the direct titration of urine without preliminary removal of nonspecific reducing substances, 1 or 2 cc. of urine are acidified with 5 cc. of 3 per cent trichloroacetic acid and titrated at once. Titration is carried out from a microburette containing the dye into a white porcelain evaporating dish, which contains the aliquot to be assayed.

To eliminate interfering substances, 20 cc. of the mercuric acetate solution are mixed with 10 cc. of freshly voided urine in a 40 cc. centrifuge tube and centrifuged for 2 minutes. H₂S is immediately bubbled through the decanted supernatant fluid. The time elapsing between addition of mercuric acetate and treatment with H₂S should not exceed 10 minutes in order to avoid irreversible oxidation of the vitamin. Treatment with H₂S is continued until no more mercuric

sulphide is formed from the surplus mercuric acetate in the solution. The solution is then filtered, resaturated with H₂S, stoppered with a cork and kept in a dark place for at least 6 hours. Thereafter, the H₂S is removed with a continuous stream of O₂-free nitrogen or carbon dioxide, until lead acetate paper does not darken. Five cc. of this filtrate is titrated after adding 1 cc. of 10 per cent trichloroacetic acid. With urines containing large amounts of ascorbic acid, a smaller aliquot is taken, e.g., 1 cc., in which case 5 cc. of 3 per cent trichloroacetic acid are added.

Solid barium acetate has been used to remove interfering reducing substances in place of mercuric acetate solution, thus eliminating the need for H₂S. In general, the values obtained by this method, if it is carried out immediately after the urine has been voided, agree closely with those found after mercuric acetate precipitation.

Saturation test. After depletion of ascorbic

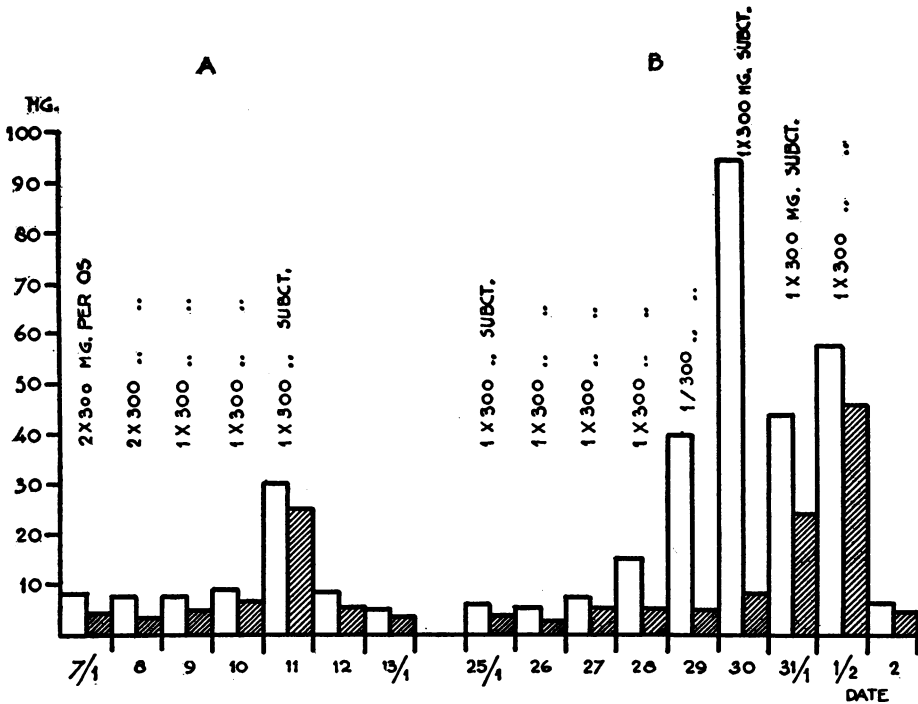


FIG. 4. THE EXCRETION OF NONSPECIFIC REDUCING SUBSTANCES BY A TUBERCULOUS PATIENT

A and B represent different saturation tests

- 6-hour urinary excretion of mgm. cevitamic acid, after precipitation with mercuric acetate.
- 6-hour urinary excretion of total reducing substances expressed as mgm. cevitamic acid.

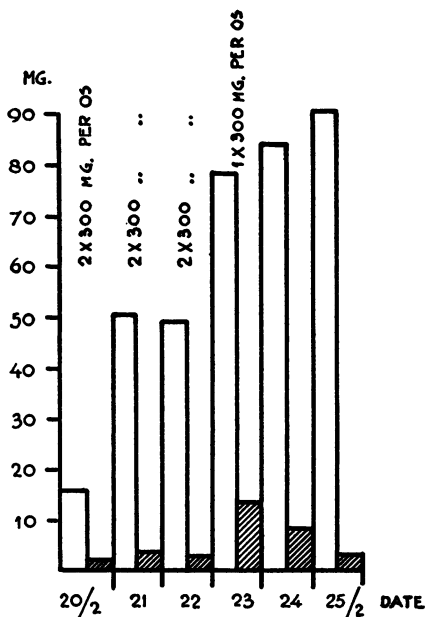




FIG. 5. THE EXCRETION OF NONSPECIFIC REDUCING SUBSTANCES BY A PATIENT WITH PEPTIC ULCER DURING A SATURATION TEST

-  6-hour urinary excretion of mgm. cevitamic acid, after precipitation with mercuric acetate.
 6-hour urinary excretion of total reducing substances expressed as mgm. cevitamic acid.

acid repeated doses of this vitamin can be given without any increase in urinary excretion until the deficiency has been completely corrected. The point at which urinary excretion increases under these circumstances is taken as the saturation point. At this point the ascorbic acid concentration in whole blood is quite regularly 14 mgm. per liter (17). The dose necessary to produce this saturation and a consequent increase in urinary output is inversely proportional to the amount of the vitamin stored in the body. Therefore, the saturation test has the practical importance of demonstrating various degrees of hypovitaminosis, the effects of administered ascorbic acid varying with the degree of saturation or depletion of the organism concerned.

These effects depend, too, on the rapidity of absorption. In an organism depleted of ascorbic acid, the capacity of the tissues to take up ascorbic acid from the blood stream may, following intake *per os*, be so great that moderate doses are absorbed completely without increase of urinary excretion. In the same subject, a similar dose

given by parenteral route might cause a transitory increase in urinary excretion, not because the tissues are truly saturated, but because even unsaturated tissues require time to take up the vitamin. This is especially true if the vitamin is given intravenously, since in this case the concentration in the blood is elevated so suddenly that a transitory overflow into the urine simulates a peak of saturation. That this is true is corroborated by the observation that the same dose given *per os* the next day causes no surplus excretion (17). Contrasting the various methods of administration, Hawley and Stephens (18) and Heinemann (19) have shown the *surplus* output (in a saturated subject) during the first 6 hours following intake *per os* to be about 50 per cent of the 24 hour excretion. After subcutaneous injection, approximately all of the *surplus* excretion occurs in 6 hours. Whereas, following intake *per os*, the rise in urinary excretion takes place in the second 3 hours of the 6-hour period, it is observed in the first 3 hours following subcutaneous injection (20). The quantity of ascorbic acid given also influences the rise of concentration in the blood. A very large dose, *e.g.*, 1000 mgm. as a single dose *per os*, may induce phenomena similar to those observed after intravenous administration. For this reason most investigators never use a dose greater than 300 mgm. at one time.

Saturation tests, therefore, should be carried out by giving ascorbic acid *per os* or, for special purposes, subcutaneously, and in modest daily doses. Intravenous administration of even modest doses or intake by mouth of an excessively large dose at one time can simulate saturation by causing a urinary surplus excretion before the depletion of the body has been overcome.

Examples demonstrating the independence between total reducing capacity of the urine and that resulting from ascorbic acid only are given below. In order to simplify the technique of saturation tests, the procedure previously followed, observation of urinary excretion for a period of 24 hours, has been altered in the following way (19): immediately after voiding at 9 a.m., ascorbic acid is given and its concentration in the urine is determined in samples voided at 12 p.m. and 3 p.m. respectively.

Subjects on a high protein diet or after intake

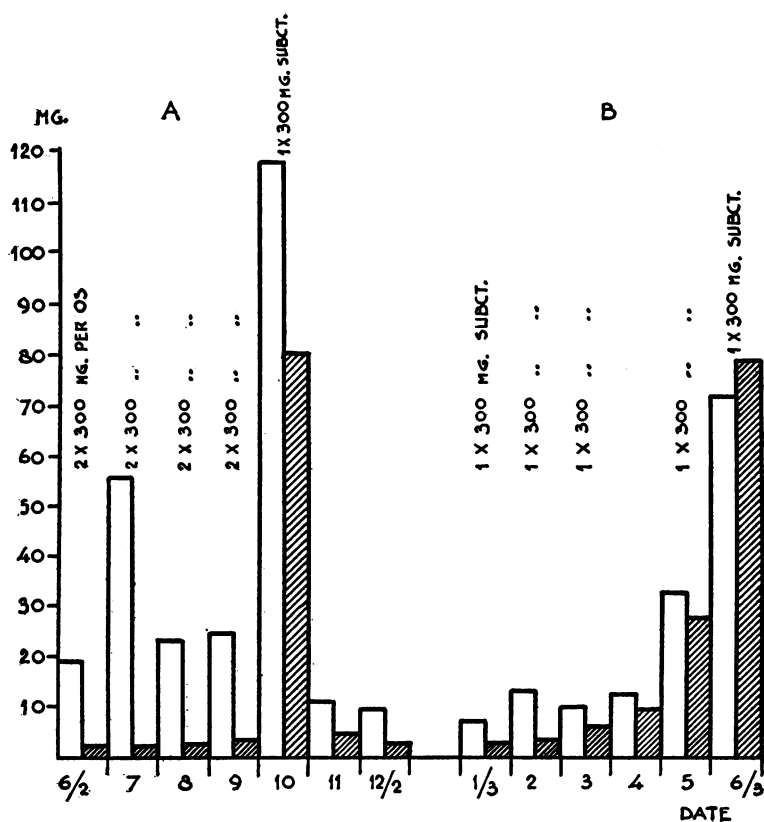




FIG. 6. THE EXCRETION OF NONSPECIFIC REDUCING SUBSTANCES BY A PATIENT WITH PEPTIC ULCER DURING TWO DIFFERENT SATURATION TESTS, A AND B

 6-hour urinary excretion of mgm. cevitamic acid, after precipitation with mercuric acetate.

 6-hour urinary excretion of total reducing substances expressed as mgm. cevitamic acid.

of cystine excrete a large quantity of reducing substances even though the ascorbic acid intake is maintained at a constant level (21). In Figures 1 and 2 the error of direct titration of urine is demonstrated, the tall blank columns indicating total reducing substances and the dark columns the total amount of ascorbic acid after mercuric acetate precipitation. In certain pathological conditions (diabetes (Figure 3), tuberculosis (Figure 4), peptic ulcers (Figures 5 and 6)) the excretion of large amounts of unspecific reducing substances other than ascorbic acid has been observed. Even in these diseases the excretion of reducing substances other than ascorbic acid has been observed to vary largely from patient to patient and in the same individual from day to day.

It so happens, that in Figure 4A the total reducing capacity parallels the amount of ascorbic acid excreted; in Figure 4B a second saturation of the same patient has been carried out and an increase in the output of total reducing substances is observed after a dose of 1500 mgm. of ascorbic acid, while surplus output of ascorbic acid occurs only after an intake of 2100 mgm. A similar observation is given in Figure 6A (increased total reducing capacity after 900 mgm., surplus excretion of ascorbic acid after 2700 mgm.) and Figure 6B (parallelism between results of direct titration and that after mercuric acetate precipitation).

From the foregoing examples it follows that the total reducing capacity of urine, estimated by direct titration, can be very high while ascorbic

acid is found in normal amounts. This high total reducing capacity is chiefly due to thiosulphate (16), a fact on which the method of its elimination with barium acetate is based.

In connection with these observations there are certain other problems that must be discussed. Harris *et al.* (23), Abbasy *et al.* (24), Youmans *et al.* (25) claim that healthy people, under normal nutritional conditions (with a sufficient supply of vitamin C, but not saturated), excrete daily urinary amounts which are nearly constant. Based on these observations, a normal level of urinary output is supposed; excretion below this level consequently is considered as demonstration of deficiency. These levels are based on direct titration, which, as has been shown above, cannot be regarded as reliable. In a number of observations we have noticed that the urinary output of ascorbic acid, estimated after precipitation by mercuric acetate, declined when no vitamin C was taken. On the other hand, when only 60 per cent of the daily dose required to maintain saturation was taken, no decrease in urinary output could be observed in spite of decreasing ascorbic acid content of the blood (22). Furthermore, the daily urinary output can differ under normal dietetic conditions, one of us having an average excretion of 12 mgm., the other of 23 mgm. daily (after precipitation with Hg—acetate or Ba—acetate). Cognizant of the fact that even in the same individual daily fluctuations can occur, it would be difficult, if not impossible, to distinguish a "normal level" of urinary excretion.

Biological assay indicates that the substance causing increased reducing power of the urine after massive doses of ascorbic acid is indeed ascorbic acid (9). Whether or not the small normal excretion actually represents ascorbic acid has not been established yet and seems of little importance for the purposes of our studies.

SUMMARY

Ascorbic acid in urine cannot be determined reliably by direct titration with 2,6 dichlorophenol indophenol. Reducing substances other than ascorbic acid, present in urine, also decolorize this indicator. They can be removed by precipitation with mercuric acetate. The excretion of these interfering substances can increase considerably,

under various conditions, and independently of that of the vitamin.

The technique and significance of saturation tests have been discussed.

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