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The Oxalate Content of Blood

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The quantity of oxalate present in blood is still in dispute. Japanese workers give the normal value as 2–4 mg./100 ml. (method of Izumi [1933], Suzuki [1934], Kamiya [1937] and Sato [1941]). On the other hand, Leulier & Dorche [1939] found 0.2–0.5 mg./100 ml. in good agreement with Flaschen-träger & Müller [1938]. Voinar & Babkin [1940] failed to isolate oxalic acid from the blood or tissues (as CaC_2O_4), and expressed doubts as to the reliability of the methods then available. Barber & Gallimore [1940] applied the ester method of Dodds & Gallimore [1932] and reported 0.4–0.6 mg./100 ml. of human blood.

The method of Izumi [1933], which depends on the precipitation of cerous oxalate ($\text{Ce}_2(\text{C}_2\text{O}_4)_3$) at pH 2.3 from a trichloroacetic acid filtrate, received some support from the previous work of Merz & Maugeri [1931]. These authors found 3–4 mg./100 ml. by direct precipitation from blood filtrate by means of lime water, but Thomsen [1935] and Voinar & Babkin [1940] have claimed that the precipitate obtained does not consist of oxalate. Thomsen [1935] also criticized the Ce procedure used by Suzuki [1934], but this criticism was answered [Suzuki, 1936] and the method has been in use in a modified form ever since. The purpose of the present paper has been to investigate the Ce procedure used by Kamiya, Noye & Sato [1937] and the Ca procedure of Merz & Maugeri [1931], since these two methods agree in yielding high and unconfirmed results. The ester procedure of Barber & Gallimore [1940] has also been examined.

METHODS

(1) *Investigation of the cerous oxalate method* [Suzuki, 1934; Kamiya *et al.* 1937]. Thomsen [1935], following the procedure of Suzuki [1934], could obtain no precipitate from blood filtrates, and pointed out that $\text{Ce}_2(\text{C}_2\text{O}_4)_3$ is

soluble in excess of CeCl_3 . Suzuki [1936] suggested that Thomsen had overlooked or lost the small precipitate of $\text{Ce}_2(\text{C}_2\text{O}_4)_3$ and insisted that special centrifuge tubes were required. Subsequently, Kamiya *et al.* [1937] suggested $\text{Ce}_2(\text{SO}_4)_3$ as precipitant since an excess of this substance has less tendency to redissolve $\text{Ce}_2(\text{C}_2\text{O}_4)_3$.

In the present experiments the special centrifuge tubes recommended by Suzuki [1936] were employed. With 2 drops of 0.1% $\text{Ce}_2(\text{SO}_4)_3$ and 3 ml. of a solution of $\text{Na}_2\text{C}_2\text{O}_4$ equivalent to 1.33 mg. $\text{H}_2\text{C}_2\text{O}_4$ /100 ml., no difficulty was encountered in obtaining a crystalline precipitate of $\text{Ce}_2(\text{C}_2\text{O}_4)_3$ at pH 6. In 2 hr. 98% of the oxalate was precipitated. This result indicates that an aqueous solution of oxalate of the strength which Suzuki [1934] supposed to be present in blood filtrates yields an easily visible crystalline precipitate, suitable for analysis. However, when the precipitation was attempted under the conditions set out by Izumi [1933], viz. at pH 2.3 (colorimetrically, thymol blue) and in the presence of CCl_3COOH , no precipitate could be obtained. In these experiments the number of drops of $\text{Ce}_2(\text{SO}_4)_3$ was varied from 1 to 4 (0.04–0.16 ml.) and the solutions were left overnight. Since oxalate is not precipitated from aqueous solution under these conditions it is improbable that a precipitate of $\text{Ce}_2(\text{C}_2\text{O}_4)_3$ would separate from a blood filtrate. Experiments showed that in some cases a small amorphous precipitate did appear, but in no case could oxalate be recovered when the precipitate from 10 ml. of blood was subjected to the process of Dodds & Gallimore [1932]. This result therefore supports the contention of Thomsen [1935].

(2) *Investigation of the precipitate obtained with lime water* [Merz & Maugeri, 1931]. If the precipitate consists of CaC_2O_4 , it should be possible to convert it into $\text{Et}_2\text{C}_2\text{O}_4$ and to estimate the quantity so esterified by the procedure of Dodds & Gallimore [1932]. The 'oxalate' content of a sample of heparinized human blood was determined, and 4.55 mg./100 ml. were found. The precipitate from 20 ml. of blood (0.91 mg. 'oxalate') was dissolved in a small quantity of HCl and transferred to the Claisen flask of the apparatus used for the distillation of the ester. Only 0.092 mg. oxalate was recovered. It is probable that at least a part of this oxalate arose from the decomposition of ascorbic acid of

the blood filtrate when the latter was made alkaline with lime water. The whole procedure was verified by carrying out a 'blank' experiment in which 1 mg. $\text{H}_2\text{C}_2\text{O}_4$ was added and 1.06 mg. recovered. These results confirm the findings of Thomsen [1935], and of Voinar & Babkin [1940].

(3) *Investigation of the ethyl oxalate procedure* [Barber & Gallimore, 1940]. This method entails a preliminary deproteinization with phosphotungstic acid, evaporation of the filtrate, and precipitation of the oxalate as CaC_2O_4 . 10 ml. samples of blood were employed in order to insure the presence of sufficient oxalate to give a clear-cut precipitate. The volume of fluid prior to the addition of lime water was not allowed to exceed 2 ml. The only other modification adopted was the use of an ice-bath in the distillation of the ester, as suggested by Athanasios & Reinwein [1934].

The most likely sources of error in this method are: (a) Loss of $\text{H}_2\text{C}_2\text{O}_4$ by volatilization during the evaporation of the blood filtrate *in vacuo*. (b) Incomplete extraction of the $\text{H}_2\text{C}_2\text{O}_4$ with ether. (c) Incomplete precipitation by means of lime water. (d) Production of $\text{H}_2\text{C}_2\text{O}_4$ from other constituents of the blood during the prolonged and somewhat drastic processes involved in the determination.

The first three possibilities were investigated by carrying out a blank test on the reagents with and without the addition of a quantity of $\text{H}_2\text{C}_2\text{O}_4$ likely to occur in blood. In these tests the amount of phosphotungstic acid was reduced from 4 to 0.2 g., since with blood nearly all the phosphotungstic acid is removed by the proteins. When 0.1 mg. $\text{H}_2\text{C}_2\text{O}_4$ was added to the 'blank' 82% of it was recovered. This recovery is satisfactory. It is probably an advantage to have a small quantity of oxalate present in the reagents, as although the absolute value of the 'blank' is unlikely to be obtained owing to unavoidable losses, such losses occur in both 'blank' and 'test' and the difference, which is the value required, is not likely to be affected.

Nine specimens of normal heparinized human blood were analysed. The values obtained were 0.23, 0.31, 0.40, 0.42, 0.47, 0.51, 0.57, 0.60, 0.77 mg. oxalate/100 ml. with an average result of 0.48 mg./100 ml. (In two cases 0.06 mg. $\text{H}_2\text{C}_2\text{O}_4$ was added to two 10 ml. samples of blood; the recoveries were 84 and 85%.) These figures are in close agreement with those reported by Barber & Gallimore [1940] although the range for normal blood is wider than that (0.4–0.6 mg./100 ml.) given by them.

l-Ascorbic acid was selected as the most probable normal constituent of blood which might yield $\text{H}_2\text{C}_2\text{O}_4$ when subjected to the procedure of Barber & Gallimore [1940]. Its easy conversion into $\text{H}_2\text{C}_2\text{O}_4$ has already been noted by Flaschenträger & Müller [1938] and Jurist & Christiansen [1939], while Scheinkman [1940] found an increased output of oxalate in guinea-pigs after administration of *l*-ascorbic acid. The *l*-ascorbic acid used in the following experiments was supplied by B.D.H., and gave no precipitate when treated with Ca salts at pH 5. In three cases 4 mg. of ascorbic acid were added to 10 ml. samples of human blood and the oxalate content was determined. In each case an increase was obtained. The average value of the three specimens prior to addition of *l*-ascorbic acid was 0.50 mg./100 ml. and after the addition rose to 1.5 mg./100 ml. An addition of 40 mg. *l*-ascorbic acid to 100 ml. blood therefore increased the apparent oxalate content by 1.0 mg. If this oxalogenic effect is directly proportional to the quantity of *l*-ascorbic acid present, the quantity of oxalate formed by the 1 mg. of *l*-ascorbic acid present in 100 ml. of normal

blood would be 0.025 mg. This value is not of great significance when compared with the normal figure of 0.5 mg./100 ml. but it does indicate that all the $\text{H}_2\text{C}_2\text{O}_4$ found by this method is not necessarily present in the original blood.

DISCUSSION

No evidence could be found in support of the high level of blood oxalate reported by the Japanese group of workers. A negative result of this type is not entirely satisfactory, and it may be argued that the reagents employed in this investigation were not pure. However, only three reagents were used in the $\text{Ce}_2(\text{C}_2\text{O}_4)_3$ experiments. Two of these substances, viz. $\text{CCl}_3\cdot\text{COOH}$ and NaOH , were of Analar quality, while the $\text{Ce}_2(\text{SO}_4)_3$ was specially prepared from $\text{Ce}(\text{SO}_4)_2$. The latter was purified by the procedure of Bowles & Partridge [1937]. Izumi [1933], the originator of the cerium procedure, conducted the precipitation at pH 2.3. He investigated the effect of pH on the precipitation by mixing 5 ml. buffer solution (acetic acid + acetate or glycine + HCl), 0.5 ml. $N/10 \text{ Na}_2\text{C}_2\text{O}_4$ and 0.2 ml. 2% CeCl_3 , and found complete precipitation of oxalate at pH 2.3 and upwards. It is evident that the conditions in this experiment have little connexion with those existing in a blood filtrate. Here the concentration of oxalate is many times lower, and the Ce salt is employed in 0.1% solution.

It is unfortunate that our present knowledge of the variation of blood oxalate in health and disease has been acquired mainly by the methods of Merz & Maugeri [1931], of Suzuki [1934], and of Kamiya *et al.* [1937]. The defects inherent in these methods appear to be such that any published work in which they have been employed must be regarded with suspicion.

There appears to be little doubt that the procedure of Barber & Gallimore [1940] gives results of the correct order. However, their figures should be regarded as representing the total oxalate derived from the blood, since the demonstration that their final precipitate consists of CaC_2O_4 does not necessarily imply that this quantity of oxalate is present in the original blood.

SUMMARY

1. No evidence could be obtained to support the contention of Kamiya *et al.* [1937] and Merz & Maugeri [1931] that the oxalate content of blood is 2–4 mg./100 ml.

2. The method of Barber & Gallimore [1940] for the determination of oxalate in blood is confirmed, but the quantity of oxalate involved is too small to permit great accuracy of determination. It is not certain that all the oxalate found by this method pre-exists in the blood.

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A New Technique for the Determination of Phosphorus by the Molybdenum Blue Method

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Numerous methods are available for the colorimetric determination of phosphorus by molybdenum blue. A number of reducing agents have been applied, but no method can be regarded as entirely satisfactory. The methods using SnCl_2 have the advantage of high sensitivity, but in general do not obey Beer's Law over a wide range, and the variation in tint at different intensities is sufficient to cause difficulty in matching. Bodansky [1932] corrected for the deviation from Beer's Law in the method of Kuttner & Cohen [1927]. When absolute colour measurement is adopted difficulty is experienced in controlling the colour development and in eliminating variation caused by instability of the colour with time and with changes in room temperature. In methods using other reducing agents these disadvantages are reduced, but the sensitivity is also diminished. The method of Fiske & Subbarow [1925] is probably the most popular and, even though the colour is not quite stable, Allen [1940] has found that a Pulfrich photometer can be used for matching. Probably the only method which gives a stable colour under conditions which are easy to control is that of Zinzadze [1935] using metallic Mo as the reducing agent. A recent review of methods is given by Woods & Mellon [1941].

Arising from an observation that KI in acid solution is a suitable reducing agent for phosphomolybdic acid, a new method has been developed which has none of the above disadvantages and is

simple to operate. The range is 1–100 μg . P and, where applicable, it gives a higher degree of accuracy than existing methods. The sensitivity is almost three times that of the Fiske-Subbarow method. Wu [1920] suggested the use of HI as a reducing agent for phosphomolybdic acid and devised a rough test. Berenblum & Chain [1938] used HI in a study of the mechanism of the reduction to Mo blue.

Suitable conditions for reduction to Mo blue and for removal of liberated I_2 were determined. A temperature of 100° was found to be suitable for the development of the colour. The colour intensity increases with the amount of molybdate used, but the 'blank' colour increases in the same manner. The 'blank' can be reduced by increasing the acidity or by using less reducing agent, but if the acidity is too high the colour fades after reaching its maximum intensity at 100°. If too little reducing agent is present the colour is not stable. Suitable concentrations of reagents were ascertained. $\text{Na}_2\text{S}_2\text{O}_3$ cannot be used to remove the liberated I_2 owing to precipitation of S, but Na_2SO_3 was satisfactory. Only a small excess is permissible, otherwise it imparts a yellow colour to the solution. The H_2SO_4 and $(\text{NH}_4)_2\text{MoO}_4$ must be added separately since a combined reagent gives a high and variable 'blank'. The solution of KI keeps satisfactorily if it contains a small amount of Na_2CO_3 to prevent liberation of free HI.