ON THE DETERMINATION OF CREATININE AND CREATINE IN BLOOD, MILK AND TISSUES.

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The determination of preformed creatinine in blood and milk.

The advantage of using known creatinine solutions instead of potassium bichromate as standards is nowhere else so decisive as in the determination of creatinine and creatine in such fluids as blood and milk where the preformed creatinine amounts to only 1–2 mgm. per 100 cc. By the help of such standard creatinine solutions the determinations become almost as simple as the corresponding determinations in urine. The method is as follows.

Ten cubic centimeters of blood or milk are measured into a 50-cc. volumetric flask, or better into a 50-cc. shaking cylinder which can be closed with a glass stopper. The flask or cylinder is then filled up to the 50-cc. mark with saturated picric acid solution and shaken a few times. About 1 gram of dry picric acid is then added to the mixture and the shaking is continued for five minutes. The mixture is then transferred to centrifuge tubes, the sediment and precipitate are shaken down and the supernatent fluid is poured through a filter. This is the most economical process where but little blood is available. When this is not the case the quantities taken may be doubled and the filtration can then be made without preliminary centrifuging process. By this treatment practically all the protein materials are removed and the creatine and creatinine are obtained in the picric acid filtrate. The filtrate is at the same time practically a saturated picric acid solution.

For the colorimetric determination of the preformed creatinine in the filtrate all that is necessary is to prepare a correspondingly dilute solution of creatinine in saturated picric acid solution. A
solution containing 0.2 mgm. of creatinine per 100 cc. is suitable for this purpose. This can be prepared in a few moments by transferring 1 mgm. of creatinine from the standard creatinine solution used for urine work to a 500-cc. volumetric flask and then making up to volume with saturated picric acid solution. This solution can be kept on hand as the creatinine is not precipitated on standing on account of the great dilution. On adding the same amount of alkali to equal volumes of filtrate from the blood or milk and of the known picric acid solution the color produced corresponds to the amount of creatinine present, provided that neither contains more than one and one-half times as much as the other. It is absolutely essential, however, that exactly the same amount of the 10 per cent alkali should be added to each solution, because in saturated solutions of picric acid the alkali deepens the color even when no creatinine is present. The amount of alkali which I have found to yield the most reliable results when making creatinine determinations in this manner is 5 cc. of 10 per cent sodium hydrate per 100 cc. of picric acid solution.

When measuring out the alkali with an ordinary burette the simplest way to get the same amount of alkali for the unknown and the standard is to determine how many drops, as obtained from the burette, correspond to 5 cc., and then to add one-fifth of that number to 20 cc. of the unknown filtrate and to 20 cc. of the known solution. Ten cc. of the filtrate from the blood or milk may be used for the color comparison, in which case the alkali added must, of course, be only as many drops as correspond to 0.5 cc.

Sometimes the filtrate obtained from blood becomes slightly turbid after the addition of the alkali. It must then be centrifuged or filtered before using it for the color comparison.

Ten minutes' standing after the addition of the alkali is adequate for the development of the color, and the solutions are then ready for the color comparison without any further dilution. They are accordingly transferred to the cylinders of the Duboscq colorimeter and compared in the usual manner. The standard creatinine solution in this case can advantageously be set at 20 mm. because the color of the solutions are not very deep, but it is not at all essential that this should be done.

The calculation of the creatinine in the blood or milk is the same whether the standard is set at 10, 15 or 20 mm. and whether 10 or
20 cc. of the filtrate were taken for the making of the color reaction. When 10 cc. of blood are diluted to 50 cc., or 20 cc. to 100 cc., and the standard contains 0.2 mgm. of creatinine per 100 cc., according to the directions described above, the reading of the standard divided by the reading of the unknown gives without any further calculations the creatinine in milligrams contained in 100 cc. of blood (or milk).

There is only one special precaution to be noted in connection with this determination, and this has to do with the collection of the blood. In collecting blood to be used for creatinine determinations some care should be taken not to add too much potassium oxalate for the prevention of clotting. Ten drops of a 20 per cent solution is enough for 30 cc. of blood, and it is better to measure it out in this way than to use unweighed amounts of the dry salt. The reason is that the potassium of the oxalate is precipitated by the picric acid and oxalic acid is set free. This reaction is probably not quantitative, but if too much oxalate has been used the amount of acid set free is large enough to introduce a considerable variation in the amount of color obtained. If a large excess of oxalate has been added a part of the filtrate obtained from the blood must be titrated to determine its acidity compared with that of the saturated picric acid solution, and a corresponding increase must be made in the alkali added for the development of the color.

It will doubtless occur to some that the difficulty arising from use of too much oxalate might be obviated by substituting sodium or some other oxalate for the potassium salt, but I have devoted considerable time to the study of this problem and have not been able to find any more satisfactory salt than the potassium oxalate.

The determination of creatine plus creatinine in blood and milk.

For the determination of the so-called total creatinine in blood, milk and exudates, the preliminary precipitation with picric acid is conducted in exactly the same manner as has been described above in connection with the determination of the preformed creatinine. The filtrate obtained from 10 cc. of blood diluted with picric acid solution to 50 cc. amounts usually to rather more than 30 cc., and 10 cc. of this filtrate are all that are needed for the
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total creatinine determination. In actual practice, embracing some two hundred determinations in many kinds of blood, we first of all measure out 10 cc. of the creatine-creatinine filtrate into a small Erlenmeyer flask (capacity 25 or 50 cc.) or large test tube and then we use 10, 15 or 20 cc. of filtrate, depending on how much remains, for the determination of the preformed creatinine.

For the conversion of the creatine into creatinine we have so far used only the autoclave method. The flask or test tube containing 10 cc. of the filtrate is covered with tinfoil, transferred to the autoclave and heated to about 120°C. for about twenty minutes. When using the autoclave it is important not to open it at the end of the heating until the temperature has fallen to below 100° so as to avoid all mechanical losses.

When cooled to room temperature the solution is then rinsed into a 25-cc. volumetric flask or to the 25 cc. mark in a measuring cylinder with saturated picric acid solution and 1.25 cc. of 10 per cent sodium hydrate solution are added for the development of the color reaction.

Two standard creatinine solutions in saturated picric acid are necessary in this determination because of the variations in the creatine contents of normal blood. When working on hospital patients the variations are greater still, and three standard creatinine determinations are desirable. These standard solutions contain 0.5, 1 and 2 mgm. of creatinine respectively per 100 cc. of saturated picric acid solution. To 20 cc. of each of these solutions in measuring cylinders is added 1 cc. of 10 per cent sodium hydrate. By inspection one can readily tell which standard comes nearest to having the same color as the unknown, and with this as a standard the color comparison is then made in the usual manner by the help of the Duboscq colorimeter. The colors of these solutions are much deeper than those obtained in the determination of the preformed creatinine, and the standard solution is consequently usually set at 10 mm.

The reading of the standard in mm. multiplied by 125 and by 0.5, 1 or 2, according to which standard is used, when divided by the reading of the unknown in mm. gives the amount of creatine + creatinine in milligrams per 100 cc. of blood or milk.

1 These solutions are made by putting 1, 2 and 4 cc. of the standard creatinine zinc chloride solution in 200-cc. volumetric flasks and making up to volume with saturated picric acid solution.
The determination of preformed creatinine in muscle and other tissues.

For the determination of preformed creatinine in muscles and other tissues 10 grams are desirable. The tissues must be fresh, and results obtained on human autopsy materials are therefore usually worthless. The weighed tissue is transferred to a mortar (inside diameter 10–15 cm.) and is then cut into small pieces with a pair of (preferably curved) scissors. About 20 grams of sand are added and the mixture is rubbed into a fairly uniform paste. 43 cc. of saturated picric acid solution are gradually added to the paste while the rubbing is continued and finally about 1 gram of solid picric acid. The rubbing and stirring is continued for five to ten minutes after the last addition of picric acid.

By this means the proteins of muscle are converted into insoluble picrates and the creatinine stays in solution. The volume of the picric acid solution added, 43 cc., is intended to give as nearly as practicable 50 cc. of solution making allowance for the fact that about 75 per cent of the muscle is water.

The mixture is poured on a filter, 20 cc. of the filtrate are transferred to a dry measuring cylinder, and 1 cc. of 10 per cent sodium hydrate solution is added to develop the color that is to serve as a measure of the creatinine present. The picric acid creatinine solution containing 0.5 milligram of creatinine per 100 cc. is used as a standard, and to 20 cc. of it is added 1 cc. of 10 per cent sodium hydrate at the same time that the alkali is added to the muscle extract. The standard is set usually at 20 mm. and this figure multiplied by 2.5 and divided by the reading of the muscle extract gives the creatine in milligrams per 100 grams of muscle.

In all such determinations the colored solution must be filtered or centrifuged before making the color comparison.

In the case of some tissues, notably the liver and the brain, it is sometimes impossible to obtain clear filtrates suitable for creatinine determinations. By the addition of a little formalin, 2 cc. of 40 per cent solution to 10 grams of tissue, and ten minutes' standing before the extraction with picric acid solution, clear extracts are obtained. The formalin does not interfere with the subsequent determinations in such extracts. Formalin cannot be used in connection with the determination of creatine and creatinine in urine.
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The determination of creatine in muscles and other tissues.

In all hitherto published procedures for the determination of creatine in muscles much time and labor is expended on the separation of the creatine from the protein materials. So far as I am aware there is nothing to show that such separation is necessary, and aside from the laboriousness of such a process it also undoubtedly diminishes the accuracy of the determination. For these reasons I prefer to dissolve the proteins at the same time that the creatine is being converted into creatinine, as does Baumann in the method recently published in this Journal.²

Baumann separates the creatinine from the protein materials before making the determination. It is, of course, possible that such a separation may be necessary, but from the character of the results which we have obtained in the course of a very large number of determinations it appears to me improbable that such is the case. Since we have found it convenient to use higher temperatures than is customary when converting creatine into creatinine by help of the autoclave, I have regarded the glycogen and the alleged decomposition of creatinine by acids as a greater source of danger than the protein materials. Creatinine is not destroyed, however, even when heated to 140°C., and glucose does not interfere at the temperature we use, 130–135°.

By the method described below the creatine in muscle can be determined in less than two hours. The method is as follows: Five grams of muscle or other tissue, cut up fine with scissors or a meatgrinder, are transferred to a 200-cc. Erlenmeyer flask and 100 cc. of ⁹/₁₀ sulphuric acid are added. The flask is covered with tinfoil and is then heated in the autoclave at 130–135° for thirty to forty minutes. The tissue is almost wholly dissolved by this treatment. After cooling to below 100°C. the autoclave is opened, the contents of the flask are cooled and transferred to a 200-cc. volumetric flask. After shaking a little to break up the flocculent skeletons of the tissues the contents are diluted to 200 cc. and well mixed.

² This Journal, xvii, p. 15. Baumann’s method was first presented at the annual meeting of the American Biochemical Society, December, 29, 1913. As I stated at that meeting I had worked out a somewhat similar process which had then been in use in my laboratory for about two months.
The solution is poured on a filter and 10 cc. of this filtrate are titrated with 10 per cent sodium hydrate with phenolphthalein as indicator. Another 10 cc. is then measured into a 100-cc. volumetric flask, and to it are added 20 cc. of saturated picric acid and enough 10 per cent sodium hydrate solution to give 1.5 cc. over and above that required for the neutralization of the sulphuric acid.

As standards for the determination of creatine in muscles we find it convenient to use solutions containing creatinine equivalent to 1 mgm. of creatine per cc. (1.389 grams of creatinine zinc chloride per liter) for striated muscle, and standards only half as strong for the determination of creatine in tissues other than striated muscles. In the former case the standard is set at 10 mm., in the latter case at 20 mm. when making the color comparisons. In either case 4000 divided by the reading of the unknown in mm. gives the creatine in milligrams per 100 grams of muscle.

In working with definite tissues of small animals it is in some cases not possible to obtain 5 grams of material nor is this necessary; 2 grams or even less can be used provided that the material is weighed out quickly so as to prevent loss of weight by the evaporation of water.
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