three runs, with following respective sets of constants corresponding to successive time intervals—3.1, 2.9, 3.0, 3.1, 3.2, 3.1, 3.0, 3.1; 2.9, 3.0, 2.8, 3.0) and $3.2 \times 10^{-6}$ sec.$^{-1}$ (average from two runs, with following respective sets of constants—3.3, 3.4, 3.2, 3.1; 3.1, 3.0, 3.2, 3.1). The corresponding values found by Moelwyn-Hughes for the reaction in aqueous solution are $4.47 \times 10^{-4}$ and $4.9 \times 10^{-6}$ sec.$^{-1}$. The effect of replacing a portion of the solvent water by methanol is thus to diminish the rate of methyl iodide hydrolysis. Comparison of the figures at the respective temperatures indicates that the activation energy is but slightly affected, if at all. These results are entirely similar to the effects of solvent change on the rates of hydrolysis of secondary and tertiary alkyl halides in mixtures of ethanol and water.[2] It would appear that the hydrolyses by *neutral water* of methyl halides are kinetically similar to the corresponding reactions of secondary and tertiary halides, and that an identical intimate mechanism obtains for all of these reactions.

(2) See E. D. Hughes, *Trans. Faraday Soc.*, **34**, 185 (1938), for a review of this field.

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## A New and Direct Method for the Determination of Creatine. I. Preliminary Report

BY CHARLES F. SCHAFFER

Investigations on the origin and significance of the excretion of creatine in the urine have received no general acceptance. The reason for this uncertainty is that creatine is not determined directly, but by difference. The preformed creatinine is determined by Folin's colorimetric method, and then the urine is heated with acid which converts the creatine to creatinine. The creatinine is then redetermined. The difference between this total creatinine value and that of the preformed creatinine is called creatine. The fallacies of such an analytical procedure are obvious; especially when considering that the quantitative determination of creatinine itself is made by measuring its reducing action. In fact it has been pointed out independently by Arnold,[1] Emden,[2] Hurtley,[3] Greenwald[4] and confirmed by Graham and Poulter,[5] that acetoacetic acid is capable of showing a false presence of creatine.

In attempting to formulate a copper reagent that would eliminate entirely the effect of reducing nitrogenous substances, but still retain its maximum ability to be reduced by sugars, Folin and Svedberg[6] developed one which is reduced by nitrogenous substances such as uric acid, creatine, creatinine and allantoin, but is not affected by sugar.

It was the use of this reagent which enabled Larson[7] to promulgate his colorimetric method for the determination of allantoin.

The subsequent use of this procedure for the determination of added allantoin in urine of man resulted in the recovery of excessive amounts. On investigation it was found that the substance responsible for this greatly increased reducing action was creatine.

Aware of the negligible amounts of allantoin present in human urine, it was decided that an earnest endeavor to utilize this procedure for the determination of creatine warranted investigation. It was soon discovered that by increasing the alkalinity of the copper solution, creatine demonstrated reducing properties which varied directly as its concentration and could be measured quantitatively.

Larson's procedure, slightly modified for its adaptation to creatine, is used. The determination is made by treating a portion of the filtrate of the test sample in a Folin–Wu[8] sugar tube with the ammoniacal copper solution; the alkalinity is increased by the addition of sodium hydroxide solution, and immediately it is placed in a rapidly boiling water-bath for ten minutes, cooled and the blue color, developed after the addition of acid molybdate, compared with a 1-mg. creatine standard similarly treated.

### Reagents Required

**Phospho-24-tungstic Acid.**—It is essential that the phosphotungstic acid used be pure. Larson obtained inconsistent results in the precipitating power of eight different lots of C. P. phosphotungstic acid procured from four leading manufacturers and the results have been substantiated in this Laboratory. Basic lead acetate;[9] 5% (by weight) sulfuric acid solution; Folin ammoniacal cop-

(1) Arnold, *Zentr. inn. Med.*, **21**, 417 (1900).
(2) Emden, *Zentr. Stoffenechael, Verdauungs-Krankheiten, N. F.*, **2**, 250–289 (1907).
(3) Hurtley, *Lancet*, **184**, 1160 (1913).
(4) Greenwald, *J. Biol. Chem.*, **14**, 87 (1913).
(5) Graham and Poulter, *Proc. Roy. Soc.* (London), **B87**, 205 (1913).
(6) Folin and Svedberg, *J. Biol. Chem.*, **70**, 418 (1926).
(7) Larson, *ibid.*, **94**, 3 (1932).
(8) Wu, *ibid.*, **43**, 197 (1920).
(9) Hawk and Bergeim, "Physiological Chemistry," Blakiston, Philadelphia, Pa., 11th ed., 1937.

per reagent;[10] 20% sodium hydroxide solution; acid molybdate reagent;[11] creatine standard: dissolve 100 mg. of creatine in water and dilute to 100 cc.

**Technique.**—Place 1.5 g. of phosphotungstic acid in a large Pyrex tube, 30- to 50-cc. capacity, and dissolve in 6 cc. of water. With the aid of a pipet add 4 cc. of urine sample and centrifuge until clear. Add 4 cc. of the basic lead acetate solution and centrifuge again.[12] When clear, add 6 cc. of 5% sulfuric acid and recentrifuge. Transfer 2 cc. of the resultant, clear filtrate to a Folin–Wu sugar tube. Add 2 cc. of ammoniacal copper solution and 1 cc. of 20% sodium hydroxide. Place in a vigorously boiling water-bath for ten minutes, cool, add 2 cc. of acid molybdate reagent, dilute to mark and compare immediately with a 1-mg. creatine standard similarly treated.

## Observations

The phosphotungstic acid precipitates the proteins and some of the other interfering substances while the addition of lead acetate removes the excess phosphotungstic acid and the remaining interfering substances. The subsequent use of sulfuric acid precipitates the excess lead completely.

The optimum alkalinity of the medium required for the creatine to exert its most efficient reducing action necessarily detracts from the non-sugar reducing properties of the copper solution.

Normally, urine contains traces of glucose which are so minute that the error introduced by such amounts is negligible. However, when sugar is present in any appreciable amounts it must either be removed by the brief fermentation process described by Folin and Svedberg[6] and designed for use on filtrates, or the quantitative sugar determinations of Folin–Wu[11] run in conjunction with the determination of creatine. This latter method is preferred as the fermentation procedure must be regulated rather too exactly, otherwise the reducing powers are increased rather than decreased.

**Recovery of Creatine Added to Urine.**—Urine that had no reducing action on the copper solution or at best produced only a slight coloration which was impossible to read colorimetrically was selected and varying amounts of a standard creatine solution added.

Filtrates of these various specimens were then prepared as described above, and the determination proper performed on the filtrates.

From a series of twenty-eight different determinations recoveries ranging from 90 to 110% were obtained in contrast to recoveries of 82 to 92% employing the method of Folin.

The accompanying protocol shows a comparison of the values for creatine recovery, obtained by both the Folin and the suggested colorimetric method, when added to urine.

PROTOCOL

COMPARISON OF CREATINE RECOVERIES AFTER THE ADDITION OF A DEFINITE AMOUNT TO URINE

| Urine no. | Creatine present, mg. per cc. | Recovery Folin method, mg. per cc. | Percentage error | Recovery Colorimetric method, mg. per cc. | Percentage error |
|---|---|---|---|---|---|
| 1 | 2.6 | 2.5 | − 3.9 | 2.6 | 0 |
| 2 | 3.6 | 3.2 | −11.2 | 3.4 | −5 |
| 3 | 4.6 | 3.8 | −17.4 | 4.45 | −3 |
| 4 | 1.0 | 0.92 | − 8.0 | 0.97 | −3 |
| 5 | 2.0 | 1.83 | − 8.4 | 2.1 | +5 |
| 6 | 3.0 | 2.57 | −14.3 | 3.12 | +4 |
| 7 | 1.0 | 0.88 | −12.0 | 0.97 | −3 |
| 8 | 2.0 | 1.74 | −13.1 | 1.92 | −4.2 |
| 9 | 3.0 | 2.38 | −20.7 | 3.13 | +4.5 |
| 10 | 0.6 | 0.49 | −17.7 | 0.6 | 0 |

## Conclusion

A preliminary report on a direct method for the determination of creatine in urine is presented. The full report is to be submitted on completion of the work.

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(10) Folin, *J. Biol. Chem.*, **82**, 88 (1929).

(11) Folin and Wu, *ibid.*, **41**, 367 (1920).

(12) Prior to the addition of the basic lead acetate, Larson recommends refrigerator incubation of the tube for one-half hour to minimize the time required for complete phosphotungstate precipitation. This procedure may be omitted as the results obtained, on final analysis of the filtrate, show very little variation.

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# The Common Basis of Intramolecular Rearrangements. IV.[1] A Correction: The Benzilic Acid Rearrangement

BY FRANK C. WHITMORE

Since the formulation of the working hypothesis used in this Laboratory, no facts have been found contrary to the assumption of the basis of molecular rearrangement as due to a carbon with an open sextet of electrons. However, the extreme extension of the hypothesis to include the benzilic acid rearrangement involving the addition of a proton to benzil to form a positive ion, cannot be supported. Ingold[2] has proposed the existence of an intermediate negative ion produced by the addition of hydroxyl ion to benzil and indi-

(1) Whitmore, THIS JOURNAL, **54**, 3274 (1932); Whitmore and Stahly, *ibid.*, **55**, 4153 (1933); Whitmore and Fleming, *J. Chem. Soc.*, 1269 (1934); Wallis and Whitmore, THIS JOURNAL, **56**, 1427 (1934).

(2) Ingold, "Ann. Repts. Chem. Soc. (London)," Vol. XXV, 1928, pp 124–134.