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POSTSCRIPT

While this paper was in the press there have come to our notice two further references to similar work. Cohen & Thompson (1947) describe experiments on the electrophoresis of sera before and after treatment with the thymol reagent, which they interpret as indicating that the β -globulin fraction is the active agent in the thymol test. They report only slightly higher concentrations of β -globulin in positive sera and therefore suggest the possibility of a qualitative change in this fraction. There are two important differences in technique which may account for the discrepancy between their conclusions and ours. First, Cohen & Thompson's (1947) electrophoresis was conducted at pH 8.6 and ours at pH 8.0. The analytical classification is thus not necessarily identical, for as has been noted above γ -globulin separated at pH 8.6 is devoid

of the flocculating activity associated with γ -globulin separated at a lower pH (Moore *et al.* 1945). Secondly, the conditions of precipitation were not identical, as both the initial ionic strength and the final protein concentration would be much higher in Cohen & Thompson's (1947) experiments. They added only 2 vol. of thymol reagent to one of serum, and then dialyzed against a further 20–25 vol. of reagent, whereas the thymol test is performed by adding 60 vol. of reagent to one of serum. Their results would thus not necessarily apply to the thymol turbidity test as actually performed on serum.

Hanger (1946) has made a brief report on work which appears to be entirely in agreement with our own, in that he finds γ -globulin to be the active precipitating agent in the thymol test with phospholipids as an essential adjuvant.

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A Method for the Estimation of Creatinine

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It has always been realized that the alkaline picrate method (Folin, 1905) for the estimation of creatinine suffers from the defect of being non-specific (Hunter, 1928). A wide variety of substances react, among them glucose, formaldehyde, acetaldehyde and many creatinine-like nitrogenous compounds, some of which are likely to be present in biological fluids. 3:5-Dinitrobenzoic

acid (Langley & Evans, 1936) appears to offer greater specificity but only at the expense of sensitivity. Barrett (1936) described a qualitative test for creatinine using a reagent based on Nessler's solution. Further study has shown that such a reagent could be made the basis of a quantitative method, the estimation being carried out nephelometrically.

METHODS

The preparation of the reagent. Barrett's solution is prepared by adding KI to a Nessler's solution; the effect of varying the amount of KI added was first investigated (Fig. 1). It will be seen that the addition of KI diminishes

Fig. 1. The effect of added KI on the reaction of a Nessler's solution with creatinine and some biologically related substances. +—+ Creatinine, 1 mg./100 ml. solution; •—• creatinine, 100 mg./100 ml. solution; ●—● hydan-toin, 100 mg./100 ml. solution; ○—○ creatinine 1 mg./100 ml. + glucose 100 mg./100 ml. solution; ●---● guanidine 100 mg./100 ml. solution; ammonia 1 mg./100 ml. solution; ∞ denotes the appearance of a dense precipitate.

the reaction of the Nessler's solution with ammonia, guanidine and hydan-toin, and reduces the promoting effect of glucose on the creatinine reaction. The creatine and creatinine reactions reach a peak as KI is added and there-after the reaction is reduced. Either the point of greatest sensitivity to creatinine or the point where the reaction with interfering substances is at a minimum may be taken for analysis of biological fluids. A compromise must be reached, and the addition of 7.5 g. of KI/100 ml. of Nessler's solution would appear to give a satisfactory reagent.

It was found, however, that Nessler's solution differed from batch to batch and that each required an individual adjustment with KI. It was found possible, however, to

Fig. 2. The effect of alkalinity on the reaction of the artificial reagent containing 5% (w/v) KI. ○—○ hydan-toin, 100 mg./100 ml.; glucose 100 mg./100 ml. + creatinine, 1 mg./100 ml.; — creatinine, 1 mg./100 ml.; creatine 100 mg./100 ml. Reaction with ammonia (1% solution) ranging from + + + dense red ppt. to very slight ppt. (±).

Fig. 3. Effect of varying KI concentration on the sensi-tivity of the 'artificial' reagent containing 18% NaOH: symbols as in Fig. 2.

prepare an 'artificial' solution which would react in the same fashion by the addition of KI to an alkaline solution of potassium mercuric iodide. In Fig. 2 is shown the effect of varying the alkalinity of such a solution, the KI concn-

Table 1. *Creatinine content of autoclaved rat tissues: comparison of Folin and nephelometric methods*

(All values expressed as mg./100 g. wet tissue.)

Tissue	Folin method				Nephelometric method			
	Before treatment with creatinine-destroying bacilli (A)	After treatment (B)	True creatinine (A - B)	Ratio $\frac{A}{(A - B)}$	Before treatment with creatinine-destroying bacilli (A)	After treatment (B)	True creatinine (A - B)	Ratio $\frac{A}{(A - B)}$
Liver	12.5	7.5	5.0	2.5	5.9	0.9	5.0	1.18
Kidney	60	31	29	2.07	34	6	28	1.21
Spleen	60	41	19	3.16	20	3	17	1.18
Muscle	580	200	380	1.53	400	60	340	1.17
Average	—	—	—	2.31	—	—	—	1.184

tration being fixed at 5% (w/v). It will be seen that increasing the strength of the alkali markedly increases the reaction towards ammonia, hydantoin and, to a lesser extent, to creatinine. At an NaOH concentration of 18% (w/v), glucose has no promoting effect on the reaction and so a solution of this alkalinity was chosen as the most promising for further study. In Fig. 3 is shown the effect of varying the KI concentration of a solution containing 18% (w/v) of NaOH. When the KI concentration is increased to 7.5% (w/v) creatine and ammonia cease to react and the hydantoin reaction is very reduced. This then was chosen as the optimal concentration of KI and the composition of the reagent becomes: 1 g. potassium mercuric iodide, 18 g. NaOH and 7.5 g. KI, all dissolved in water, made to 100 ml. and mixed.

In addition to creatine and ammonia, substances giving no reaction include guanidine, methyl guanidine, sarcosine and uric acid; the hydantoin reaction is so slight as to make this reagent almost specific for creatinine in biological fluids.

PROCEDURE

Protein-free solutions. The sample is diluted to contain less than 3 mg. creatinine/100 ml. and 3 ml. of this solution are added to 1 ml. of the reagent. The solutions are mixed and allowed to stand for 5 min. The precipitate which appears is very stable and remains dispersed for at least 30 min. It is estimated nephelometrically in a photoelectric absorptiometer, using a Chance O.B. 2 glass light filter. Standard solutions covering the range required (not exceeding 3 mg. creatinine/100 ml. of solution) are treated with each set of samples.

Blood and plasma samples. The sample is deproteinized by the zinc hydroxide method (Somogyi, 1930) or the tungstic acid method (Folin & Wu, 1919). The filtrate is then estimated as above, using standard solutions treated in the same manner as the samples.

Tissues. The tissue is finely minced and mixed with a known volume of water. The suspension is mixed by shaking, filtered and the resulting clear solution treated in the same manner as a plasma

sample, the solution being adjusted by dilution to contain less than 3 mg. creatinine/100 ml. before estimation. For the estimation of total creatinine of a tissue the suspension of the material in water is autoclaved at 30 lb. pressure for 1 hr. and then treated as above.

RESULTS

Recovery of creatinine added to human plasma

In Fig. 4 are illustrated the results of a typical recovery experiment performed on a sample of human plasma to which creatinine has been added.

Fig. 4. Recovery of creatinine added to normal human plasma. • Samples deproteinized by the zinc hydroxide method (Somogyi, 1930); o samples deproteinized by the tungstic acid method (Folin & Wu, 1919).

The recovery obtained on the tungstic acid filtrates is slightly less satisfactory than that obtained on the zinc hydroxide filtrates and the former method gives a slightly lower value for the normal plasma creatinine level (zinc hydroxide filtrate 0.95 mg. creatinine/100 ml.; tungstic acid filtrate 0.87 mg./100 ml.). Similar results have been obtained on dog plasma.

The specificity of the reagent was compared with that of the Folin method by estimating the apparent creatinine content of autoclaved rat tissues before and after treatment with the specific creatinine-destroying bacillus N.C. of Miller & Dubos (1937). The analyses were made in both cases on zinc hydroxide filtrates of the tissue and performed against standards treated in the same fashion. The results show that the ratio of apparent to true creatinine is consistently smaller in the case of this method than in the Folin method. The results obtained for true creatinine agree well with those of Miller, Allison & Baker (1939).

Fig. 5. Histograms of glomerular filtration rates in a group of normal subjects. Solid line, values determined by inulin method; dotted line, values determined by creatinine method.

Simultaneous estimations of creatinine in blood and urine have been performed and the renal clearance of endogenous creatinine compared with that obtained by the inulin method (Smith, 1943). In Fig. 5 are plotted histograms of creatinine and inulin clearance values determined on normal adult subjects. The mode of the creatinine values lies at a value of 136 ml./min. Whilst higher by 30 ml./min. than our own inulin clearances the value is identical with that obtained by Goldring & Chasis (1944).

DISCUSSION

We have approached the problem of the preparation of a suitable reagent for creatinine by two methods. We have first found the best conditions for specificity

and sensitivity of the reagent and then compared the reagent so prepared with the Folin reagent. It can be seen from the figures that by suitable adjustment of NaOH and KI concentration, the reaction with substances which interfere with the Folin method can be eliminated or so diminished as to render negligible the error involved in applying this reaction to biological fluids. The very much reduced reaction with the chromogenic material of rat tissues confirms this observation.

We have then applied this reagent to the estimation of glomerular filtration rate since it was felt that if the reagent were sensitive to chromogenic material of either blood or urine, the clearance value so obtained would show a marked deviation from the normal figure, it being unlikely that the chromogenic substances would have the same renal clearance as creatinine. Two points can be seen from these results. The clearances obtained are very much lower than those obtained by workers (Rehberg, 1926) who estimated their creatinine by the Folin method. Secondly, the close agreement between the clearances herein quoted and those obtained by the inulin method suggests that we are dealing with a single substance and one which is treated in a very special way by the kidney. This offers support for the postulate of Ekehorn (1944) that creatinine provides a true measure of filtration rate. It will be seen that by the simultaneous use of two reagents prepared along these lines it is possible to estimate, for example, hydantoin or creatine in the presence of creatinine.

SUMMARY

1. A rapid method for the estimation of creatinine is described which has a greater specificity than the Folin method.
2. The extent of chromogenic interference of non-creatinine substances is illustrated by the estimation of apparent and true creatinine of autoclaved rat tissues.
3. When creatinine is estimated in blood and urine by this method the creatinine clearance gives a true measure of filtration rate.

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