the absorption spectra.

Discussion and Summary. As previously stated, the administration of testosterone to many animals including man and the Rhesus monkey, results in an increased urinary excretion of androgens and 17-ketosteroids. In contradistinction to this we noted no increase in either of these substances in the urine of treated Cebus monkeys. This finding parallels those of Dingemanse and Tyslowitz,[10] Paschkis *et al.*,[11] who found no increase in the urine of dogs similarly treated. In dogs[11] and man[12] increased urinary estrogenic activity has been shown after testosterone treatment. Our results would suggest that in the Cebus monkey as in man and the dog, testosterone can be converted to estrogens, but further work is necessary to elucidate this point.

[10] Dingemanse, E., and Tyslowitz, R., *Endocrinology*, 1941, **28**, 450.

[11] Paschkis, K. E., Cantarow, A., Rakoff, A. E., Hansen, L. P., and Walking, A. A., PROC. SOC. EXP. BIOL. AND MED., 1943, **53**, 213.

[12] Dorfman, R. I., and Hamilton, J. B., *Endocrinology*, 1939, **25**, 33.

16687

Determination of Creatine, Creatinine, and Related Compounds in Urine by Means of Paper Chromatography.*

STANLEY R. AMES AND HUGH A. RISLEY. (Introduced by Philip L. Harris.)

From the Research Laboratories of Distillation Products, Inc., Rochester, N. Y.

A partition chromatographic procedure for the identification and estimation of creatine and creatinine and their demethylated analogues, glycocyamidine and glycocyamine (guanidoacetic acid) was devised during the course of an investigation of aminoaciduria in dystrophic humans.[1] Two different color-reactions were investigated to indicate the location of the various substances on the developed chromatogram. One was based on the Jaffe reaction, the formation of a reddish-orange product when alkaline picrate is added to solutions of creatinine and related compounds. This reaction has been used by Dent[2,3] to identify creatinine on a paper strip following partition chromatography. The other was a modification of the Voges-Proskauer reaction in which diacetyl in alkaline solution forms a pink product with creatine and similar compounds.

Method Using Jaffe Reaction. The Jaffe reaction is the conventional method for the detection of creatinine. It is dependent on a cyclic lactam structure such as is found in creatinine and glycocyamidine and is not given by the hydrated straight chain analogues, creatine and glycocyamine. It is necessary, therefore, to dehydrate the latter compounds *in situ* before applying the color reaction. It was found that heating the dry paper strips for one hour at 100-110°C transformed creatine to creatinine. Glycocyamine was dehydrated to glycocyamidine by heating for one hour at 100-110°C but the conversion was not quantitative. Further heating did not appear to increase the amount of glycocyamidine formed as evidenced by the depth of the color reaction. Therefore, in actual practice a constant heating period at a constant temperature was carefully followed in order to make valid comparisons between the depth of color formed with standard solutions and with urines of unknown composition.

Details of the experimental procedure are as follows: A 0.025 ml aliquot of urine or

* Communication No. 144.

[1] Ames, S. R., and Risley, H. A., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 131.

[2] Dent, C. E., *Biochem. J.*, 1947, **41**, 240.

[3] Dent, C. E., in Reifenstein, E. C., Conference on Metabolic Aspects of Convalescence, Josiah Macy, Jr. Foundation, New York, 1946, p. 126.

standard solution is placed dropwise at a starting point on a filter-paper strip. The strips are then developed for 15-20 hours using water-saturated solutions of butanol, phenol or lutidine-collidine (50-50) as solvents. The strips are air-dried, heated to 100°C for 1 hour and cooled to room temperature. The Jaffe reaction is obtained on spraying the strips with an alkaline picrate solution prepared by adding 10.0 ml of 10% NaOH to 50.0 ml saturated picric acid. The presence of creatinine or related compounds is evidenced by the appearance of an orange spot against a light yellow background. The color appears immediately and is comparatively stable. The strips are roughly evaluated in a manner similar to that suggested for amino acids.[1] The color intensity is estimated from "1" to "5" where "1" represents the faintest band observable and "5" represents an intense color. The *dw* value for each band is calculated by multiplying its width in cm by the color intensity. The procedure is quantitized by modification of any one of a number of published procedures: Extraction of the material and analysis by micro-Kjeldahl,[4] extraction of the colored product followed by conventional colorimetric analysis,[2,5] or less precisely by spot dilution technics[4] or by spot area technics.[6]

In order to test the accuracy and sensitivity of this procedure, the various compounds under consideration were analyzed individually, as well as in mixtures, and when added to urines. In Table I the average R_F values obtained in the various solvents are given. Water-saturated solutions of butanol and lutidine-collidine give a better separation of creatine and creatinine than a water-saturated phenol solution. However, phenol gives an excellent resolution between glycocyamine and the others. Differentiation of creatinine and glycocyamidine by this method is not feasible because of the close correspondence of their R_F values. The smallest amount of creatinine or creatine (converted to creatinine by heating) which could be detected was 11 μg. One hundred micrograms of glycocyamine heated to convert it to glycocyamidine yielded a barely distinguishable spot.

TABLE I.
Average R_F Values for Creatine and Related Compounds.

Substance	Solvent (H_2O-saturated) Butanol	Phenol	Lutidine-collidine
Creatine	.08	.85	.24
Creatinine	.33	.91	.56
Glycocyamine	.07	.66	.28
Glycocyamidine	.30	.89	.52

Creatine and creatinine were Eastman Kodak Co. products. Appreciation is expressed to Dr. F. Smith of the University of Rochester Medical School for a generous sample of glycocyamine. Glycocyamidine in all cases was obtained *in situ* by dehydration of glycocyamine by heat. The solvents were used as water-saturated solutions at about 23°C and the chromatograms were developed in a thermostated room at the same temperature. The R_F values are the averages of a large number of determinations using pure solutions, mixtures and samples of urine, and are reproducible to ± 2 R_F units.

The method for the determination of creatine and creatinine is therefore quite sensitive and precise. It is also easy to perform and it is possible to determine creatinine and related compounds in pure solutions or in urines with equal facility. Urines from a number of species, including rats, rabbits and humans, have been analyzed successfully. In studying urines it is possible to test for both amino acids and creatinine and related compounds on the same strip or sheet by spraying the Jaffe reagent onto the paper which has previously been treated with ninhydrin. The sensitivity and resolution of the creatinine determination is not impaired in the above procedure.

Following the completion of this investigation, a paper appeared by Maw[7] concerned with the determination of creatine by partition chromatography. In this procedure developed strips, after being airdried and heated in an

[4] Polson, A., Mosley, V. M., and Wyckoff, R. W. G., *Science*, 1947, **105**, 603.

[5] Naftalin, J., *Nature*, 1948, **161**, 763.

[6] Fisher, R. B., Parsons, D. S., and Morrison, G. A., *Nature*, 1948, **161**, 764.

[7] Maw, G. A., *Nature*, 1947, **160**, 261.

oven at 100°C, were sprayed with 0.5 N sulfuric acid and heated at 100°C for 1 hour. This was stated to result in the dehydration of creatine to creatinine, the creatinine then being tested for by the Jaffe reaction. The method did not prove to be satisfactory in our laboratory, since the paper strips charred and were unusable. We have since learned[8] that Dr. Maw has been unable to satisfactorily reproduce this technic and is now following a procedure somewhat similar to ours with the exception that the strips are heated for 3 hours rather than 1 hour.

Method Using Diacetyl Reaction. The use of alkaline diacetyl for the determination of creatine proved to be applicable to paper chromatography. This method is specific for creatine and gives no color with creatinine. Therefore, the strips were not heated, but with this exception the procedure as previously outlined was followed. A 50-50 water-alcohol solution saturated with sodium carbonate was prepared by adding 15 ml of absolute alcohol to 20 ml of saturated sodium carbonate solution and removing the resulting precipitate by filtration. Two ml of diacetyl were added to 10 ml of the resulting filtrate. When this solution was sprayed onto the strips, the presence of creatine was indicated by pink spots against a yellow background. Too high a concentration of sodium carbonate caused the strip to turn brown obliterating the pink color of the reaction. Insufficient sodium carbonate reduced the sensitivity of the method. After drying at room temperature the strips were placed in the oven at 100°C for exactly 5 minutes to hasten the color development which at room temperature is very slow. The smallest detectable amount of creatine was 33 μg, a sensitivity of about $\frac{1}{3}$ that of the Jaffe reaction. Neither glycocyamine nor glycocyamidine in quantities up to 200 μg developed a color with the diacetyl reagent. The diacetyl reaction is given by several amino acids, and confusing results can be obtained on pathological urines. Its principal value is in the positive identification of creatine since it clearly differentiates it from creatinine.

Summary. A procedure for the identification and determination of creatine, creatinine, glycocyamidine and glycocyamine is presented. Following resolution by paper chromatography, the developed chromatograms are heated to 100°C for one hour to dehydrate creatine and glycocyamine *in situ* to creatinine and glycocyamidine respectively. The latter compounds and others of similar structure give an orange color on treatment with the Jaffe reagent (alkaline picrate).

The Voges-Proskauer reaction (alkaline diacetyl) for creatine was also investigated and found suitable but less precise.

R_F values for creatine, creatinine, glycocyamine, and glycocyamidine are given for water-saturated solutions of butanol, phenol, and lutidine-collidine (50-50).

The chromatographic method possesses the advantage that creatine and creatinine can be determined independently and directly rather than by a differential procedure as in the conventional methods.

[8] Maw, G. A., personal communication.