arginine remains unanswered.

Summary. The addition of lysine to a chick diet deficient in lysine, arginine, and methionine caused an abnormal feathering which was characterized by curved and degenerate feathers. A detailed description of the feathering abnormalities is given. When arginine was added, along with the lysine, the feathering of the birds was normal. The addition of the lysine without arginine apparently created a severe deficiency or imbalance of the latter amino acid which resulted in the abnormal feathering.

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Determination of Inulin by Means of Resorcinol. (17827)

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Despite numerous difficulties encountered in its use, inulin has remained the substance of choice for the determination of the glomerular filtration rate in man. The chief disadvantage has been the necessity of yeasting plasma and urine in the diphenylamine method(1), a step which is both cumbersome and a potential source of variable errors. Any method that avoids yeasting would be advantageous to renal physiologists and clinicians. Several investigators have examined the applicability of the reaction of resorcinol with fructose(2-4) to the determination of inulin(5-8) but differences in technic imply that methodological errors have not been reduced to a minimum. Accordingly it was decided to investigate the resorcinol method further and to compare it with the diphenylamine method under clinical conditions. Since this work was initiated, Roe(9) has reported an adaptation of the resorcinol method and we have included a comparison of our final method with this later procedure. Since completion of this work, Rolf *et al.*(10) have presented a modified diphenylamine procedure for fructose and inulin determinations. Their method advantageously eliminates the yeasting step but maintains the disadvantages of the tedious purification of diphenylamine, long heating period, and danger of crystallization of diphenylamine after cooling. Moreover, the method herein described yields a higher inulin/glucose color ratio and consequently lower blank values than the method of Rolf *et al.*

Conditions for the determination of inulin. Since the determination of inulin by the resorcinol reaction depends upon the hydrolysis of inulin to fructose, the strength of acid employed in the hydrolysis and the time and temperature of heating will all be critically important.

As judged by color development in a standard procedure using known amounts of inulin, we have found that 15 to 18% hydrochloric acid in the final reaction mixture gives maximal color development. It would appear that at this concentration maximal hydrolysis is achieved, and at the same time optimal conditions are set up for the reaction of fructose with resorcinol(3). As the concentration of acid is reduced color development becomes progressively less, while at higher concentrations slightly increased color

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development is attained but the effect of interfering substances in plasma becomes proportionately greater at concentrations of HCl above 18 per cent in the final mixture. Using a final concentration of 17% acid, maximum color development occurs when the hydrolysis is carried out at a water bath temperature of 80°C. Below this temperature color development is progressively less until at 40°C and below no color is developed, while at 100°C the reaction produces the pale yellow color described by Kruhoffer, instead of the red color characteristic of the reaction at lower temperatures. Color development increases as heating is prolonged from 5 to 20 minutes in a water bath at 80°C, but extending the period to 25 minutes does not alter the intensity, and heating for as long as 35 minutes gives color only slightly less intense than at 20 to 25 minutes. In our hands, therefore, the optimal conditions for the development of the red color comprise 17% hydrochloric acid in the reaction mixture, and heating for 25 minutes in a water bath at 80°C. These conditions differ from those of Roe's earlier method in that we heat longer, use slightly less acid and read in a spectrophotometer as described.

Although the red color follows the Beer-Lambert law rather closely in concentrations up to 2 mg/100 cc, the slope of the curve decreases somewhat variably with greater concentrations. We have therefore followed the usual practice of including with each batch of unknowns 3 standards in duplicate (1, 2 and 3 mg/100 cc) and establishing a standard curve for each set of determinations.

There are several locations on the absorption spectrum of this color which may be used for a spectrophotometric reading, a circumstance reflected in the fact that each of the previous investigators has chosen a different wave-length. There are two peaks on the absorption curve, one occurring at 410 μ and another 490 μ. We have chosen 490 μ as the reading point.

Methods. Based upon the above results we suggest the following procedure: A 2 cc aliquot of standard or unknown is pipetted into a 15 x 200 mm Pyrex ignition tube. Two cc of a 0.1% solution of resorcinol in 95% alcohol are added, this reagent being prepared daily. Finally, 5 cc of 30% hydrochloric acid are added. (This solution is prepared by adding 22.4 cc of water to 100 cc of concentrated acid.) The mixture is shaken and a glass tear placed in the mouth of the tube. The tubes are placed for 25 minutes in a water bath at 80°C,* after which they are cooled in tap water for 3 minutes and closed with a rubber stopper. Color intensity is read in a Coleman Junior Spectrophotometer at a wave length of 490 μ with an aqueous reagent blank set at 100% transmission. The color developed in this fashion is reasonably stable for periods up to 85 minutes, but routinely they should be read promptly. It is best to pour the reaction mixture in the cuvette at a distance from the colorimeter, and to keep the cuvettes stoppered, because the strong acid fumes may attack the colorimeter.

Two cc of standard solutions containing 1, 2 and 3 mg/100 cc of fructose-free inulin are treated in an identical manner for the determination of the standard curve.

Determination of inulin in plasma. In the precipitation of plasma, 2 cc of plasma are placed in a 250 cc narrow mouth Erlenmeyer flask, water for the appropriate precipitation dilution (for a given plasma inulin level) is added and mixed, followed by 6 cc of cadmium sulfate solution† with agitation and then 2 cc of 1.1 *N* NaOH solution which is added dropwise, again with agitation. The flasks are then stoppered, shaken well and allowed to stand for 10 minutes with some additional shaking. The precipitate is separated by centrifugation and the supernatant fluid is filtered through washed cotton.

Aqueous recoveries. Solutions of inulin containing 15, 30 and 45 mg/100 cc respectively were treated as described for plasma, to determine if the cadmium sulfate filtrate is suitable for use. Analysis of these aqueous filtrates was carried out as above. In 7 experiments at each inulin concentration, the

* The water bath we employed had a temperature variation of ± 1°C.

† 34.67 g $3CdSO_4 \cdot 8H_2O$ plus 169 cc 1.0 N H_2SO_4 (exact) are made up to 1000 cc and filtered if necessary.

TABLE I.
Comparison of Inulin Clearances by Diphenylamine and Resorcinol Methods.*

Conditions	No. of determinations	Avg ratio†	Standard deviation‡
During PAH clearance	30	1.006	.0822
During PAH Tm determination	16	1.004	.0942
During glucose Tm determination§	3	1.067	insufficient determinations

* Expressed as inulin clearance determined by the diphenylamine method(1) divided by the inulin clearance as determined in the same plasma and urine samples by the resorcinol method.
† Mean of the series.
‡ Square root of the average of the squares of the individual deviations.
§ Uncorrected for either plasma or urine glucose levels.

average aqueous recovery was 98% (range 94 to 101), with no consistent deviation from this figure at any particular concentration. To determine the plasma inulinoid blank blood samples were taken from 24 normal subjects. The plasma was precipitated 1 to 10 as described above. The inulinoid blank averaged 1.59 mg/100 cc (range 1.05 to 2.70) in inulin equivalent. Nine subjects in this group were undergoing alimentary glucose tolerance tests and were known to be in a post-absorptive state. In these the blank averaged 1.33 mg/100 cc. In those subjects undergoing an Exton-Rose glucose tolerance test, the inulinoid blank increased in proportion to the hyperglycemia. In 5 subjects who had ingested a high carbohydrate meal the blank increased far out of proportion to the hyperglycemia, reaching in one subject a value equivalent to 6.3 mg/100 cc. We interpret this to be due to a larger fraction of circulating fructose following the high sucrose meal. Since these data indicate that in a fasting state, most of the plasma inulinoid blank can be attributable to glucose, standard solutions of the latter containing 50, 100, 120, 150 and 200 mg/100 cc of glucose were analyzed. These solutions developed color equivalent to 0.55, 1.18, 1.34, 1.70 and 2.25 mg/100 cc of inulin. In one subject studied hourly from early morning to late evening, the blank varied from 0.75 to 1.90 mg/100 cc, the high value being reached after the evening meal.‡ Recovery of added inulin from plasma was examined by adding standard solutions of inulin to the plasma-water mixture during precipitation. The quantities of inulin so added corresponded to plasma inulin concentrations of 15, 30 and 45 mg/100 cc. The plasma inulinoid blank was determined directly on a separate sample. In 7 experiments at each concentration the average plasma recovery for all concentrations was 99% (range 95 to 104), with no consistent deviation from this average at any one concentration.

The resorcinol method was compared with the diphenylamine method under clinical conditions, using blood and urine samples from patients whose renal function was being routinely evaluated. Inulin was determined by the resorcinol method in our laboratory, while determinations by the diphenylamine method were carried out in an independent laboratory routinely employing this method. The results of the comparison are shown in Table I. Because the diphenylamine determinations were performed on a different spectrophotometer against a different standard curve the clearance values rather than the absolute plasma values are used as a basis for comparison.

Simultaneous determinations were also compared in 2 non-fasting subjects undergoing 24 hour clearance tests. The plasma ratios did not give satisfactory values, presumably because of the diurnal fluctuations in the plasma inulinoid blank. The ratio of the urinary inulin concentration as determined by the two methods in 12 periods was 1.005.

In his later method Roe(9) recommended a resorcinol reagent having glacial acetic acid as the solvent and containing thiourea as well

‡ In 3 post-prandial subjects the clearance of plasma inulinoid blank ranged from 1.89 to 3.42 cc per minute. Thus although plasma inulin values must be corrected for an inulinoid blank, the urine correction in normal subjects is relatively insignificant.

as resorcinol. Using a Somogyi filtrate, Roe reported recoveries of inulin from plasma ranging from 99 to 102%. Since many clinical investigations would be facilitated by the simultaneous determination of inulin and p-aminohippuric acid, we have examined the applicability of the Roe method to the cadmium sulfate filtrates routinely used in the determination of p-aminohippuric acid. Here plasma recoveries averaged 101% and aqueous recoveries averaged 99% when carried out at concentrations simulating 15, 30 and 45 mg/100 cc of inulin in plasma. Using 3 aqueous and 3 plasma filtrates and making 4 determinations by each method on each filtrate, the Roe method gave an average inulin recovery identical with the average recovery as determined by our method. The maximum discrepancy on any filtrate was 2.8%, while the average of 4 recoveries was 101.3% by the Roe method and 98.5% by our method.

The plasma inulinoid blank as determined by the Roe method appears to be slightly higher than that determined in our method. For example, the average inulinoid blank on 5 post-absorptive plasma samples was equivalent to 1.42 mg/100 cc inulin by the Roe method and 1.34 mg/100 cc of inulin by our method, and in no sample was the blank less by the Roe method. Roe's glacial acetic acid-urea method appears from these limited data to be equally as good as our modification of the resorcinol method. Our method has the advantage of somewhat greater simplicity.

Discussion. The resorcinol method described above, and the later method of Roe, offer several advantages over the diphenylamine method for the determination of inulin. They consume less time and are less tedious because yeasting is avoided. Resorcinol can be obtained commercially in a pure form, whereas diphenylamine must almost invariably be recrystallized. The fact that cadmium sulfate filtrates of plasma can be used to determine plasma inulin concentration is a distinct advantage where either the PAH clearance or Tm_{PAH} is determined simultaneously with inulin.

The plasma inulinoid blank in the post-absorptive state has the same order of magnitude in the diphenylamine and resorcinol methods. However, after the ingestion of food the blank may fluctuate in the resorcinol method because of the contribution of fructose. To reduce the significance of fluctuations under this circumstance it is recommended that high plasma levels of inulin (20 to 30 mg/100 cc) be employed.

With the urine dilutions used in routine clearance studies no correction is necessary for urinary chromogen. However, undiluted urine shows a significant absorption at 490 μ and this absorption is intensified when HCl is added, even in the absence of resorcinol. In the study of undiluted urines this chromogen may be removed(3) or a correction made by reading urine samples against a urine pigment blank composed of urine and acid without resorcinol.

In using any inulin method which does not involve the use of yeast to remove fructose, it is imperative that fructose-free inulin be used for renal clearance tests.

Summary. A simplified method, based on Roe's resorcinol method and avoiding the use of yeast, is described for the determination of inulin in plasma and urine. The method has been applied under clinical conditions to clearance determinations in patients with renal disease, and shown to yield results in good agreement with the diphenylamine method.

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