in amounts ranging from 1.0-3.4 mg/hr.

The considerable variability in the normal excretion of any one amino acid which we have noted has also been observed by other workers(12-14). It illustrates the difficulties likely to be encountered in interpreting or ascribing significance to amino acid levels in urine.

Summary. The plasma and urine levels of 19 free amino acids as determined microbiologically in fasting, healthy, young males are reported.

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An Anthrone Procedure for Determination of Inulin in Biological Fluids. (19758)

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The use of inulin for extracellular space estimation, as well as clearance measurements, has increased the need for a simpler and more accurate method for the determination of this substance in biological fluids. At present, resorcinol(1,2) and diphenylamine(3) are most frequently used for this determination. The former has a greater specificity for fructose, but is somewhat less reproducible. While greater accuracy may be obtained with diphenylamine, this method is more tedious.

Recently, Morris(4) has described the quantitative determination of carbohydrates with Dreywood's anthrone reagent. The present report describes the application of this method to the measurement of inulin in plasma and urine. A modification of Dreywood's anthrone reagent makes possible the measurement of fructose under controlled temperature conditions and gives highly reproducible results. Glucose and other alkali-labile chromogens are destroyed by sodium hydroxide digestion(5) so that inulin can be determined in the presence of high concentrations of these materials. It has been found that commercial inulin contains a considerable amount of alkali-labile carbohydrate material that can be completely removed by further purification (6).

Methods. Reagent: To prepare one liter of anthrone reagent, 500 ml of concentrated sulfuric acid are added to 250 ml of water, and the mixture is cooled to room temperature. Four grams of anthrone (Eastman Organic

Chemicals) are dissolved in 250 ml of concentrated sulfuric acid and the solution is added to the diluted acid. Over-heating destroys the reagent and must be avoided. The reagent is stable up to 4 months if kept in brown bottles at room temperature. Color Development: Ten ml of anthrone reagent are added to two ml of standard or unknown solutions containing from 15 to 45 μg of inulin (William R. Warner Co. inulin ampules, Lot No. 016091, are used throughout unless otherwise stated). During the addition of the acid solution, the tube is shaken in a cool water bath to minimize heat development. After thorough mixing the samples are heated in a constant temperature water bath at 75°C ($\pm$0.5°) for 5 minutes. The tubes are cooled in tap water, and after 15 minutes the color is read at 630 mμ in a Coleman Junior Spectrophotometer.

Plasma: Plasma proteins are precipitated by Somogyi's method(7).* For plasma inulin concentrations of 15 to 45 mg %, 13 ml of water, one ml of 10% $ZnSO_4 \cdot 7H_2O$ and one ml of 0.5 N NaOH are added to one ml of plasma. The mixture is placed on an automatic shaker for 30 minutes. By this means 98% of added inulin can be recovered from plasma whereas only 92% is recoverable after brief manual shaking. One ml of 4 N NaOH is added to a 4 ml aliquot of the plasma filtrate making a final dilution of 1:20. Each tube is covered with a glass marble and placed in boiling water for 15 minutes. The tubes are cooled, and duplicate 2 ml aliquots are used for the development of color.

Urine: Protein-free urine is diluted so that inulin concentrations approximate that of the plasma filtrate: 4 ml aliquots are digested with sodium hydroxide and analyzed in the same manner as plasma filtrates.

Results. I. Conditions for optimal color development: The absorption maximum was found to be 630 mμ in both the Coleman Junior and Beckman Model B Spectrophotometers. Fig. 1 illustrates the effect of temperature on the color developed after 5 minutes heating between 60 and 90°C.† Maximal color is obtained at 75°C, but as seen in Fig. 1, small variations in bath temperature between 70 and 80°C do not greatly influence color development. The color is stable at room temperature for 4 hours.

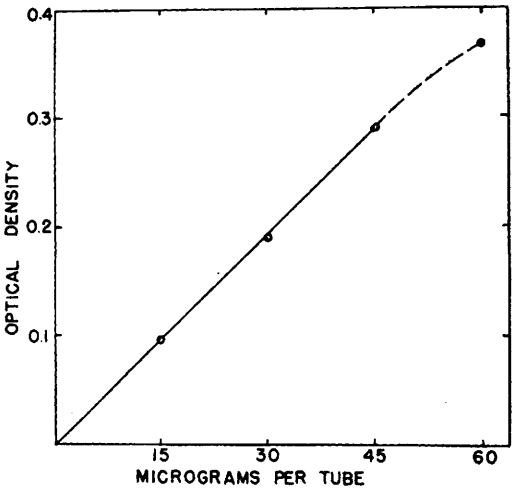

FIG. 1. Effect of temperature on color development. All tubes contained 60 μg of undigested inulin and were heated for 5 min.

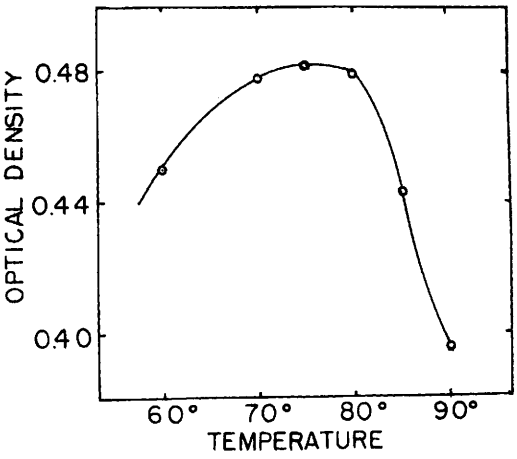

FIG. 2. Inulin standard curve.

II. Sensitivity and reproducibility. Fig. 2 shows the standard curve obtained with 15, 30, 45 and 60 μg of inulin per tube (equivalent to 15 to 60 mg % in plasma). This curve obeys the Beer-Lambert law up to 45 μg per tube. Curves prepared from determinations

* Cadmium sulfate and trichloroacetic acid give incomplete recoveries, and sodium tungstate is found to give a color with anthrone.

† Heating for longer than 5 min. serves only to reduce the color; *e.g.*, heating for 10 min. yields only 97% of the color developed by 5 min. heating.

TABLE I. Reproducibility of Optical Densities with Standard Inulin Solutions.

Concentration, μg/tube	No. of determinations	Optical densities	Range O.D.	Stand. dev. O.D.	Stand. dev., μg
15	14	.097	± .002 .003	± .002	± .30
30	23	.189	± .002 .007	± .002	± .38
45	10	.289	± .001 .004	± .002	± .31
60	14	.367	± .009 .007	± .005	± .82

TABLE II. Recoveries of Inulin (30 mg %) Added to Plasma and Urine and Urinary Recoveries of Infused Inulin.*

	Plasma		Urine		% urinary recovery of infused inulin
	mg % recovered	% recovery	mg % recovered	% recovered	
Avg	29.3	97.7	29.9	99.7	99.6†
Range	+.8–1.2	+2.5–4	+1–1.3	+3.1–4.5	+2.9–1.6

* 10 experiments were performed.
† One of these recoveries represented a recovery of repurified inulin (see text).

with fresh standards over a period of 2 months show a high degree of reproducibility (Table I). Between 15 and 45 μg per tube the results give a standard deviation of only 0.3 to 0.4 μg or 0.7 to 2.0%.

III. Sodium hydroxide digestion. Sodium hydroxide digestion effectively destroys interfering chromogens in plasma and diluted urine. The optical densities of plasma and urine blanks were essentially zero except for one of 14 plasma blanks in which the optical density was 0.005 (equivalent to 0.8 mg % inulin). A comparison of the effects of added glucose on blank chromogen values determined by Roe's(1) resorcinol method and our method was made. The blank values (expressed as inulin equivalent), obtained by Roe's method from the addition of 250, 500 and 1,000 mg % glucose, ranged from 2.4 to 3.0, 4.5 to 6.1, and 8.3 to 11.3 mg %, respectively. However, using sodium hydroxide digestion and the anthrone reagent, the values only ranged from 0.0 to 0.4, 0.1 to 1.0, and 1.3 to 2.5 mg % respectively.

Sodium hydroxide digestion destroys about 25% of the chromogenic material in both ampuled and dry Warner's inulin and about 10% of another dry preparation (Fisher Scientific Co. C.P.). This destruction is complete in 15 minutes and there is no further loss after 25 minutes digestion. The loss also appears when resorcinol is used to measure chromogen. Treatment of Fisher's inulin by sodium hydroxide digestion followed by repeated recrystallization yields an apparently pure material that is alkali-stable and gives the same color per unit weight as fructose (Fisher Scientific Co. C.P.). This repurified inulin was tested by infusion in a normal dog and gives the same recovery and volume of distribution as the material in ampules.

IV. Recoveries from plasma and urine. The recovery of inulin added to plasma and urine is shown in Table II. The average of 10 recoveries from plasma is 97.7% with a standard deviation of ±2.53% and from urine is 99.7% ± 2.0. Using the recovery method (8) for inulin space determinations in normal dogs we obtained urinary recoveries of 99.6% (standard deviation of ±1.5%) of the infused inulin when urine collections were continued for 5 hours after infusion.

Discussion. The anthrone method has the advantage that the reagent is stable, simple to prepare, and easier to handle than resorcinol or diphenylamine, a highly accurate constant temperature bath is not required, and the final color is stable. The method appears to have as great a sensitivity and somewhat greater reproducibility than other procedures. Sodium

hydroxide digestion appears to be more effective with anthrone than with diphenylamine in that the problem of interference from glucose and other blank chromogens is solved without the addition of glucose suggested by Little(5).

Cotlove(9) has found that commercial inulin contains 3 fractions: about 1% is a reducing material which diffuses like fructose, 15 to 40% is non-reducing but alkali-labile and diffuses like a much larger molecule, while the remainder is non-reducing and alkali-stable and diffuses even slower. He could detect no difference in the renal clearance and distribution in muscle between the total non-reducing and the alkali-stable fractions. Weil(6) has reported that 34% of commercial inulin is alkali-labile and feels that purified material by recrystallization after alkali digestion is more suitable for analysis of inulin in muscle. We have compared inulin and creatinine clearance in 4 experiments in normal dogs. The average inulin creatinine clearance ratios for 3 to 4 consecutive clearance periods were 0.97, 1.00, 1.02, and 1.03.

The application of the anthrone reaction to other carbohydrates can be considerably extended, especially when controlled heating is possible. We have measured sucrose at 75°C, though at this temperature the standard curve is not as reproducible as it is for inulin. Simultaneous measurements of sucrose and inulin are possible since sucrose is yeast-labile. Applications of the anthrone method to the measurement of glycogen(10), glucose(11), dextran(12), and polyglucose(13) in biological materials have been reported. The presence of any of these materials except glucose would interfere with the inulin determination.

In our preliminary experiments using less anthrone (0.2%) we noted that digestion of high concentrations of glucose yields a product that diminishes inulin color development. This interference has been resolved by the use of higher concentrations of anthrone (0.4%).

The incomplete recovery of inulin from plasma apparent not only in our data, but also in other reports(14), probably represents loss during the precipitation of plasma protein. This loss is diminished by prolonged shaking of the precipitate. We have observed that sucrose recovery is higher than inulin recovery, suggesting that substances of higher molecular weight are more liable to be lost in plasma precipitates.

Summary. 1. A method for the determination of inulin in biological fluids using a modified anthrone reagent is described. 2. This reagent gives a sensitive and reproducible test for inulin in amounts of 15 to 45 μg. 3. Interfering substances are effectively removed from plasma filtrates and diluted urine by sodium hydroxide digestion. Ten to 25% of commercial inulin is found to be alkali-labile. 4. Recoveries from plasma and urine average 97.7 ± 2.5% and 99.7 ± 2.0%, respectively.

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