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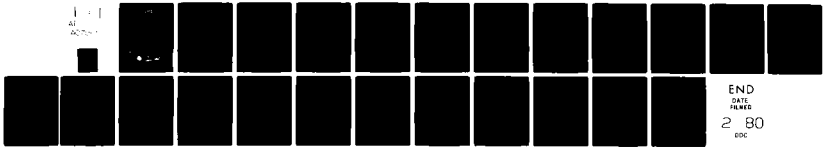

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6 MICRODETERMINATION OF SUCROSE IN PLASMA WITH THE ANTHRONE REAGENT

10 JOHN P. HANNON PhD

DIVISION OF RESEARCH SUPPORT

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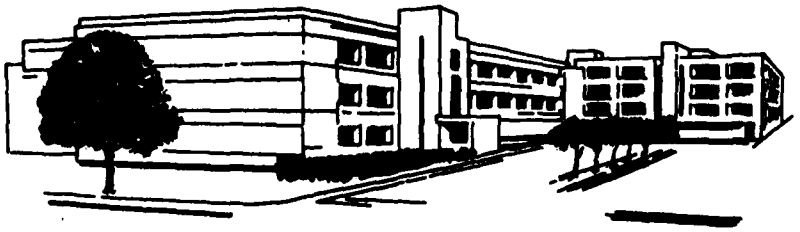

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Quantitative determinations of sucrose in plasma required preliminary protein precipitation with $Ba(OH)_2$ - $ZnSO_4$ or ethyl alcohol followed by centrifugation. Aliquots of the resultant protein-free supernate were then concentrated to dryness at 80 C with a manifold evaporator after which endogenous monosaccharides were destroyed by the addition of 0.1 ml of 30% KOH and heating for 10 min at 100 C. Upon cooling, optimum sucrose-anthrone color development was achieved by the addition of 3.0 ml of anthrone reagent (0.15% anthrone in 75% H_2SO_4 -

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20. Abstract (cont)

25% H_2O mixture), followed by heating at 40 C for 30 minutes, cooling, and the recording of optical density at 625 nm. The reaction followed Beer's Law over the range of 10 to 80 μg of sucrose. The effectiveness of the procedure in estimating anatomic and physiologic parameters of extracellular space was explored.

ABSTRACT

Quantitative determinations of sucrose in plasma required preliminary protein precipitation with $Ba(OH)_2$ - $ZnSO_4$ or ethyl alcohol followed by centrifugation. Aliquots of the resultant protein-free supernate were then concentrated to dryness at 80 C with a manifold evaporator after which endogenous monosaccharides were destroyed by the addition of 0.1 ml of 30% KOH and heating for 10 minutes at 100 C. Upon cooling, optimum sucrose-anthrone color development was achieved by the addition of 3.0 ml of anthrone reagent (0.15% anthrone in a 75% H_2SO_4 - 25% H_2O mixture), followed by heating at 40 C for 30 minutes, cooling, and the recording of optical density at 625 nm. The reaction followed Beer's Law over the range of 10 to 80 µg of sucrose. The effectiveness of the procedure in estimating anatomic and physiologic paramaters of extracellular space was explored.

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INTRODUCTION

Sucrose has been used frequently for indicator dilution measurements of the rapidly equilibrating fraction of extracellular fluid volume (1-4). Its attractiveness for such endeavors, however, has been diminished somewhat by a variety of problems associated with the measurement of sucrose concentration in blood plasma. In recent years the availability of ^{14}C-labeled sucrose has eliminated many of the technical difficulties that were encountered with earlier chemical procedures (5-7). But radiolabeled sucrose may present problems. The available procedures do not address potential, albeit small, errors attributable to sucrose catabolism (7-9), and in certain circumstances the use of radioactive material may be precluded by health-safety considerations.

Most of the original chemical procedures for measuring plasma sucrose concentration involved modifications of existing methods for blood sugar determinations. Keith, Power and Peterson (10), for example, used the Shaffer-Hartmann copper-iodometric procedure (11), and Kruhoffer (12) used a ferricyanide modification described by Hagedorn and Jensen (13). In both instances acid hydrolysis of sucrose was required as a preliminary step to the determination of total reducing substances. The presence of endogenous reducing substances, principally blood glucose, tended to compromise the utility and accuracy of these iodometric determinations of sucrose. To reduce the magnitude of the problem, later investigators, such as Deane et al (2) and Cotlove (14), used modifications of the Roe's (15) resorcinol method for determining fructose. Interference by monosaccharides was reduced by the hot dilute NaOH contained in the resorcinol reagent, but sucrose hydrolysis was still required as a preliminary step to determination of the fructose moiety.

With the advent of Dreywood's anthrone reagent (16) an improved procedure for the chemical determination of sucrose appeared feasible.

This reagent, according to Dreywood (16), provided a 10 to 40-fold increase in sensitivity as compared to the conventional iodometric methods. More importantly, non-carbohydrate reducing substances did not react with anthrone, and the highly acid nature of the reagent eliminated the need for preliminary acid hydrolysis of polysaccharides. The initial attempts to use it for selective determinations of monosaccharides in a mixture, however, were frustrated by a mutual interference; glucose, fructose, galactose, maltose, lactose, sucrose, and glycogen thus reacted with the anthrone reagent to nearly the same extent (17). Furthermore, when the reagent was added to aqueous solutions variable amounts of heat were generated and this, in turn, resulted in variable color development. Bloom and Wilcox (18), and subsequently Young and Raisz (19) solved the problem of monosaccharide interference by the preliminary treatment with hot concentrated alkali. In determinations of plasma dextran (20) and inulin (21) such treatment effectively oxidized glucose and other alkali labile chromogens. Seifter et al (20) tried to eliminate the thermal effects by controlling temperature during reagent addition and color development. Others, such as Mokrasch (21), used controlled heating in attempts to measure individual monosaccharides in a mixture selectively. The latter efforts did not achieve much acceptance.

The anthrone reagent was first used for measuring plasma sucrose concentration in the extracellular volume studies of Raisz, Young and Stinson (3). It was rarely used thereafter by other workers, perhaps because it offered no major advantage over the older resorcinol and iodometric procedures. Such an advantage seemed to appear in 1968 when Van Handel (22) reported a micromethod for the selective determination of sucrose in plant materials and honey. The attractiveness of his method, aside from sensitivity and specificity at the microgram level, rested in its simplicity. In the present communication, a modification of Van Handel's micromethod for the measurement of sucrose in plasma is described. The modification is evaluated in terms of the effectiveness of different procedures for removing plasma protein interference, the time-temperature characteristics of sucrose-anthrone color development, and the applicability of the sucrose-anthrone procedure for estimating the anatomic and physiologic parameters of extracellular space.

MATERIALS AND METHODS

Reagents

Sucrose standard. Dissolve 4.0 g of reagent grade sucrose in distilled water and dilute to 100 ml in a volumetric flask. Until used this stock standard is kept frozen in a tightly capped plastic bottle. A working standard is prepared at weekly intervals by diluting 1.0 ml of stock standard in 100 ml of distilled water; it is stored at 4 C when not in use. The working standard, at room temperature, contains 400 µg of sucrose per ml.

Zinc sulfate, 5%. Dissolve 25 g $ZnSO_4 \cdot 7H_2O$ in 500 ml distilled water.

Barium hydroxide, 0.3N. Dissolve 23.7 g $Ba(OH)_2 \cdot 8H_2O$ in 500 ml distilled water and filter to remove any $BaCO_3$ that may be present. This solution must neutralize the $ZnSO_4$ volume for volume, titrate 5.0 ml of 5% $ZnSO_4$ in 50 ml water with 0.3N $Ba(OH)_2$ to a phenolphthalein end point. On the basis of this titration the solution that is more concentrated is diluted to match the other.

Anthrone reagent, 0.15%. To 25 ml of distilled water, add 75 ml of concentrated H_2SO_4. When cool, add 150 mg of anthrone (MCB Manufacturing Chemists, Cincinnati, Ohio) and mix. At room temperature the reagent deteriorates, hence it should be prepared fresh every few days. It remains stable for two months at 4 C in the dark, according to Young and Raiz (19), but this should be verified.

Potassium hydroxide, 30%. Dissolve 15 g of KOH in 50 ml of distilled water.

Equipment and Supplies

Spectrophotometer. A Beckman Acta III (Beckman Instruments, Fullerton, California) double-beam, recording spectrophotometer was used to obtain the spectral characteristics of the anthrone-sucrose reaction and to measure sucrose concentration. Other simpler instruments would suffice for routine measurements of plasma sucrose concentration.

Temperature control. A thermoregulated cuvet holder (Beckman Instruments, Fullerton, California) was used to evaluate the effects of temperature on anthrone-sucrose color development. Cuvet temperatures were monitored with a thermister thermometer. Temperature control during routine plasma sucrose concentration measurements was achieved with two test tube heating blocks (Brownwill, Van Waters and Rogers, San Francisco) with aluminum modules for 13 x 100 mm test tubes. The temperature of one block was set at 100 C for alkali digestion of plasma reducing substances. The other block was set at 40 C for sucrose-anthrone color development.

Manifold evaporator. A ten place, N-Evap (Organomation Assoc., Northborough, Massachussetts) test tube evaporator was used to concentrate (dry) $Ba(OH)_2$ - $ZnSO_4$ plasma filtrates. The bath temperature was set at 80 C and compressed air or N_2 was used to accelerate drying.

Miscellaneous equipment and supplies. Vortex mixer, automatic pipets; disposable Pasteur pipets; disposable centrifuge tubes; disposable 13 x 100 mm plastic test tubes with snap caps; disposable 13 x 100 mm glass test tubes; heparin (100 units/ml).

PROCEDURES AND RESULTS

Sample Concentration

The procedure described by Van Handel (22) called for concentration of aqueous sucrose-containing samples by heating at 120 C on a hot plate until a final volume of 0.05 to 0.10 ml remained. He cautioned against complete drying. Since the exact volume of the concentrated samples was uncertain, this procedure appeared to introduce a variable error into the determination of sucrose concentration. Consequently, anthrone-sucrose color development following complete sample drying was compared to color development following sample concentration to less than 0.1 ml. In both instances a manifold dryer, set at 80 C, was used to reduce or dry samples of the standard sucrose solution contained in 13 x 100 mm test tubes. Subsequently, 3.0 ml of anthrone reagent was added, color was developed at 40 C for 15 minutes and was read at 620 nm as recommended by Van Handel (22). The following results are shown in Table 1.

Table 1. Comparison of sample concentration to sample drying on the anthrone-sucrose reaction.

Tube	Sucrose Standard (µg)	Sample Condition	Optical Density (620 nm)
1	50	wet	0.555
2	50	wet	0.560
3	50	dry	0.572
4	50	dry	0.578

On the basis of these results it was apparent that complete drying of the sample had little effect on the color development; complete drying gave slightly higher values than incomplete drying. Complete drying, therefore, was used in all subsequent evaluations of the anthrone procedure.

Time-temperature Characteristics

Color development with the anthrone reagent varies with both time and temperature (18), but the interaction of the two has not been precisely described. To obtain this information 40 µg samples of sucrose standard, taken to dryness, were mixed with 3.0 ml of room temperature anthrone reagent and color development was followed as a function of time at 30, 40, 50, and 60 C in the spectrophotometer (set at 620 nm).

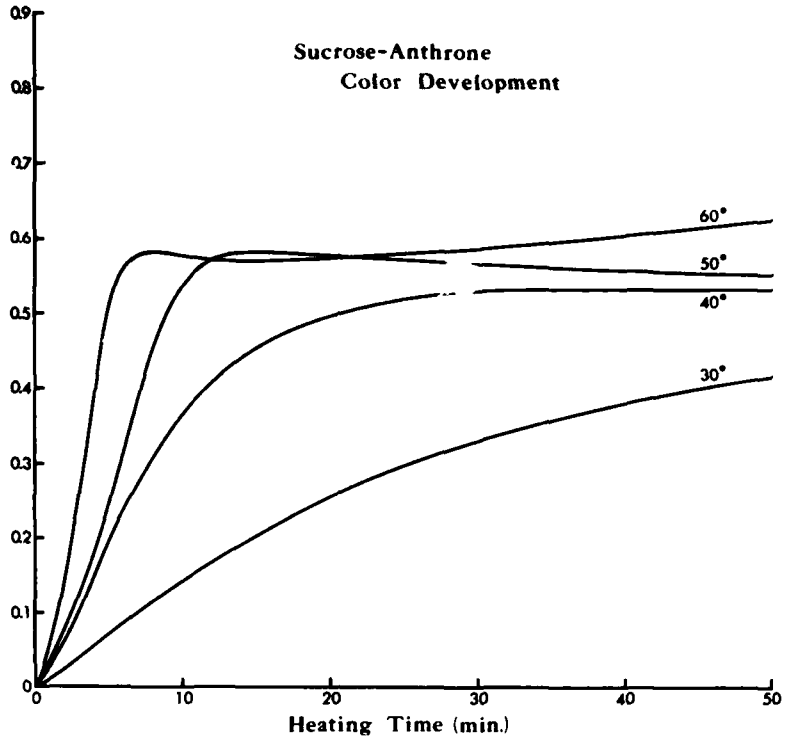

Figure 1. Effects of time and temperature on anthrone-sucrose reaction. Ordinate values are expressed in optical density units.

The results as shown in Figure 1 indicated that optimal color development for analytical purposes was achieved when the samples were heated at 40 C for 30 to 40 minutes. Below this temperature color developed slowly and failed to reach asymptotic values. Above 40 C color development proceeded more rapidly, but curves did not stabilize.

Effects of Plasma Protein

Initially, an attempt was made to measure sucrose concentration in plasma samples without removal of plasma protein. These efforts proved fruitless; the samples containing plasma developed an intense brown rather than a green color when mixed with anthrone. Figure 2 shows the results of such an attempt. In this instance, 0.2 ml of plasma was added to 50 µg of sucrose in one sample tube and 0.2 ml of water was added to 50 µg of sucrose in a second (control) tube. Both were taken to dryness, 0.1 ml of 30% KOH was added to each and they were heated for 10 minutes at 100 C to remove interference by reducing substances

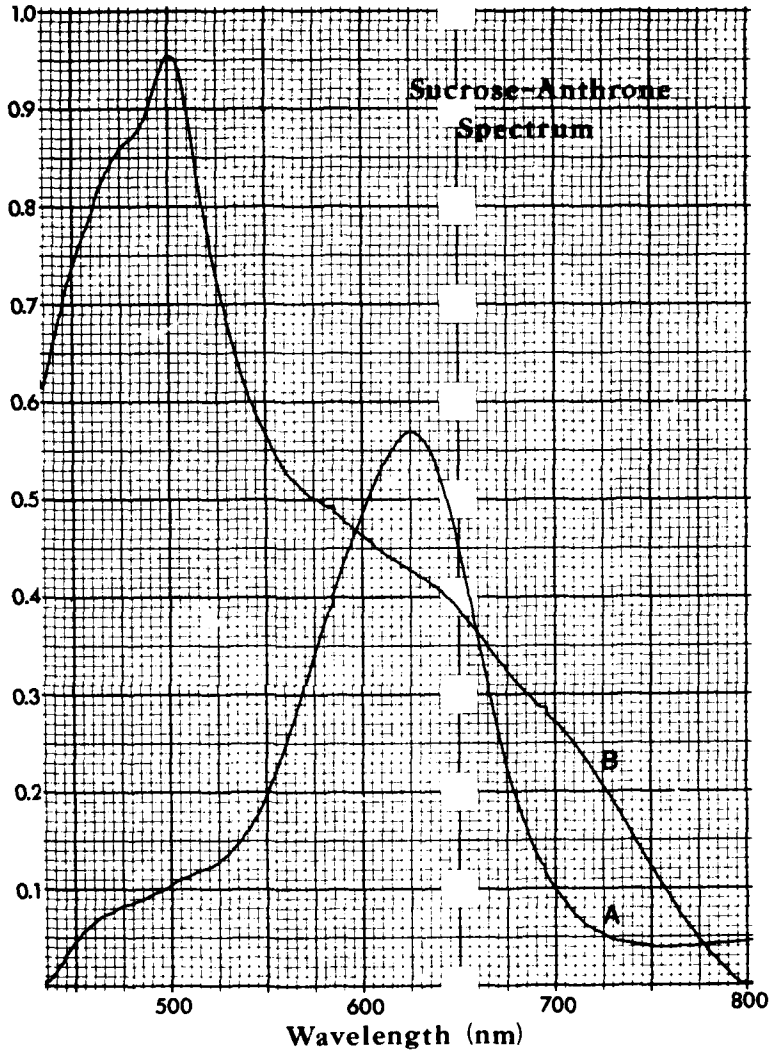

Figure 2. Spectral characteristics of the sucrose-anthrone reaction in the presence (Curve B) and absence of plasma protein. Ordinate values expressed in optical density units.

(presumably in the tube containing plasma). After cooling 3.0 ml of anthrone were added and color was developed at 40 C for 30 minutes. Curve A depicts the spectral characteristics of the anthrone-sucrose reaction in the absence of plasma. Curve B, which had to be displaced downward approximately 0.5 optical density units to be recorded, shows the impact of plasma protein. After cooling, 3.0 ml of anthrone were

added and color was developed at 40 C for 30 minutes. Curve A depicts the spectral characteristics of the anthrone-sucrose reaction in the absence of plasma. Curve B, which had to be displaced downward approximately 0.5 optical density units to be recorded, shows the impact of plasma protein.

Three procedures for removal of plasma protein interference were attempted. The first involved protein precipitation with 25% trichloroacetic acid. This attempt was unsuccessful since the trichloroacetic acid itself reacted with anthrone. It also interfered with the subsequent alkali removal of reducing substances. The second procedure involved plasma protein removal with ethyl alcohol and the third with $Ba(OH)_2$ and $Zn(SO_4)$ as described by Somogyi (23). Accordingly, three test tubes were set up as follows: the first (control) contained 3.0 ml of water; the second, 1.0 ml of plasma to which 2.0 ml of 100% ethyl was added; the third, 1.0 ml of plasma to which 1.0 ml of 0.3N $Ba(OH)_2$ followed by 1.0 ml of 5% $ZnSO_4$ were added. The precipitated protein in tubes two and three were sedimented by centrifugation and supernatant fluid (plasma filtrate) was collected with Pasteur pipets. One milliliter aliquot of the two filtrates and the tube sample were transferred to a second set of test tubes, each containing 50 µg of sucrose standard. The contents of these tubes were taken to dryness as before, 0.1 ml of 30% KOH was added and they were heated for 10 minutes at 100 C to remove interference by reducing substances. Finally, 3.0 ml of anthrone reagent was added to each, color was developed over a 30-minute period at 40 C and the color spectrum between 450 and 800 nm was recorded. Figure 3 shows the results that were obtained. It is evident from these curves both $Ba(OH)_2$ - $ZnSO_4$ (curve B) and ethyl alcohol (curve C) are effective in removing plasma protein interference with the anthrone-sucrose reaction. In both instances maximum optical density was at nearly the same wave length, i.e., 625-630 nm, as that obtained in the control sample (curve A). Furthermore, when allowance was made for intentional vertical displacement of the spectra to depict the results better, all three curves gave essentially the same maximum optical density reading for equivalent amounts of sucrose. At optical densities above and below the maximum, $Ba(OH)_2$ - $ZnSO_4$ seemed to be more effective than alcohol in removing spectral distortion of the sucrose-anthrone reaction. Also, the $Ba(OH)_2$ - $ZnSO_4$ seemed to be more effective than alcohol in removing spectral distortion of the sucrose-anthrone reaction. Also, the $Ba(OH)_2$ - $ZnSO_4$ sample had maximum optical densities at the same wave length (625 nm), whereas, the maximum for the alcohol sample was slightly higher (630 nm). Thus, if one were to choose between the two methods of plasma protein precipitation, the $Ba(OH)_2$ - $ZnSO_4$ method would appear to offer some superiority.

Standard Curve

To establish compliance of the sucrose-anthrone reaction to Beer's Law, 1.01 ml of plasma was added to each of nine plastic test tubes with snap caps. This wa· followed, in order, with 40, 80, 120, 160, 200,

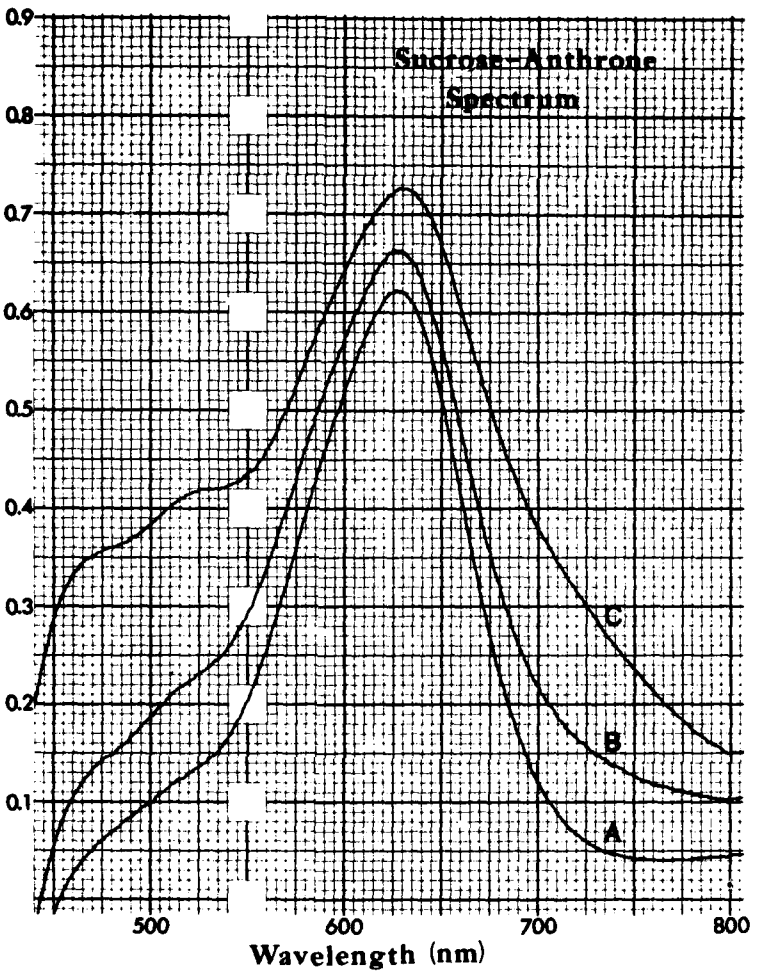

Figure 3. Special characteristics of the sucrose-anthrone reaction in the absence of plasma protein (Curve A) and when plasma protein was removed by $Ba(OH)_2$ - $ZnSO_4$ (Curve B) and ethyl alcohol (Curve C) precipitation. Ordinate values expressed in optical density units.

240, 280, and 300 µg of sucrose with sufficient water to bring the volume in each tube to 2.01 ml. Next 1.0 ml of $Ba(OH)_2$ was added, the tubes were thoroughly stirred with a vortex mixer after which 1.0 ml of $ZnSO_4$ was added, the tubes capped, and the contents thoroughly mixed by shaking. After sitting for 5 to 10 minutes to allow precipitate flocculation, the tubes were centrifuged for 10 minutes at 3000 to 4000 rpm. The protein-free supernatants were collected with Pasteur pipets and transferred to a set of appropriately labeled glass test tubes. One milliliter aliquots were then dried with the manifold evaporater after which reducing substances were removed by the addition of 0.1 ml of 30% KOH and heating at 100 C for 10 minutes. After cooling, 3.0 ml of

anthrone reagent were added to each tube and color was developed for 30 minutes at 40 C. Finally, the optical density of each tube was read at 625 nm. The results of this experiment (Figure 4) showed adherence

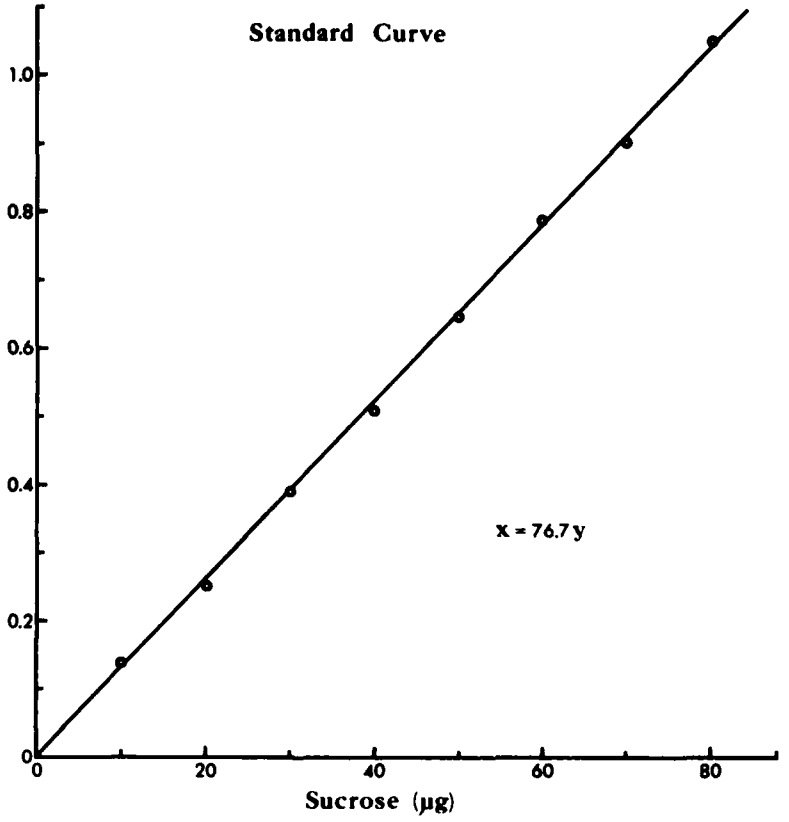

Figure 4. Sucrose-anthrone compliance with Beer's Law at 625 nm. Ordinate values expressed in optical density units.

to Beer's Law over a sucrose concentration range of 10 to 80 µg. The concentration values shown in this figure represent the amount of sucrose contained in 1 ml of protein-free supernatant fluid. If sucrose were to be determined per milliliter of original plasma, these values would have to be multiplied by four.

Subsequent studies showed that addition of equal volumes of plasma, $Ba(OH)_2$ and $ZnSO_4$ for protein removal did not always produce a completely clear supernatant fluid after centrifugation. The reason for this was uncertain. However, the problem can be eliminated by combining two volumes of $Ba(OH)_2$ and $ZnSO_4$ with one volume of plasma.

In Vivo Applications

The potential utility of the foregoing procedures in assessing anatomic and functional characteristics of the extracellular space was tested in a dog, a domestic pig, and a human. In all three instances, a 50 percent (W/V) sterile sucrose solution was rapidly injected intravenously and blood samples were taken serially at predetermined times after injection for measurement of plasma sucrose concentration. The dog was maintained under light sedation with xylazine. The sucrose solution was injected into the cephalic vein, and blood samples were obtained from a catheter placed in an exteriorized carotid artery. The pig was maintained under nitrous oxide anesthesia, the sucrose solution was injected into the femoral vein catheter and blood samples were obtained from a femoral artery catheter. No sedatives or anesthetics were used in the human during the procedure. The sucrose solution was injected into the cubital vein of one arm and blood samples were obtained by means of a catheter inserted into the cubital vein of the other arm. In each species the amount of injected dose of sucrose was determined by accurately weighing the injection syringe. The weight difference before and after injection was divided by the density of the sucrose solution to obtain the volume injected and this, in turn, was divided by two to obtain the grams of sucrose injected.

Since 1.0 ml plasma samples were needed for determinations of plasma sucrose concentration, venous blood samples of 2.5 to 3.0 ml were adequate, except for an initial sample of 10 ml, taken before sucrose injection, which was used to obtain plasma for the spectrophotometer blank and for optical density measurements of the sucrose standard. Heparin was added (0.025 ml of 1000 units/ml solution) to prevent coagulation of the serial samples until they had been centrifuged for plasma collection. The $Ba(OH)_2$ - $ZnSO_4$ protein-free supernatants were prepared as indicated in Table 2.

Table 2. Preparation procedure for obtaining protein-free samples to be used in extracellular volume measurements.

	Reagent Blank		Standards		Dilution Samples
Addition	1	2	3	4	5 thru N
Plasma (ml)	1.0	1.0	1.0	1.0	1.0 ea
Standard (ml)	0	0	0.5	0.5	0
H_2O (ml)	1.0	1.0	0.5	0.5	1.0 ea
$Ba(OH)_2$ (ml)	2.0	2.0	2.0	2.0	2.0 ea
$ZnSO_4$ (ml)	2.0	2.0	2.0	2.0	2.0 ea

Aliquots (1.0 ml) of protein-free supernatant fluid were dried, freed of reducing substances with KOH, and treated with anthrone reagent as described above. Figure 5 shows results obtained in these experiments.

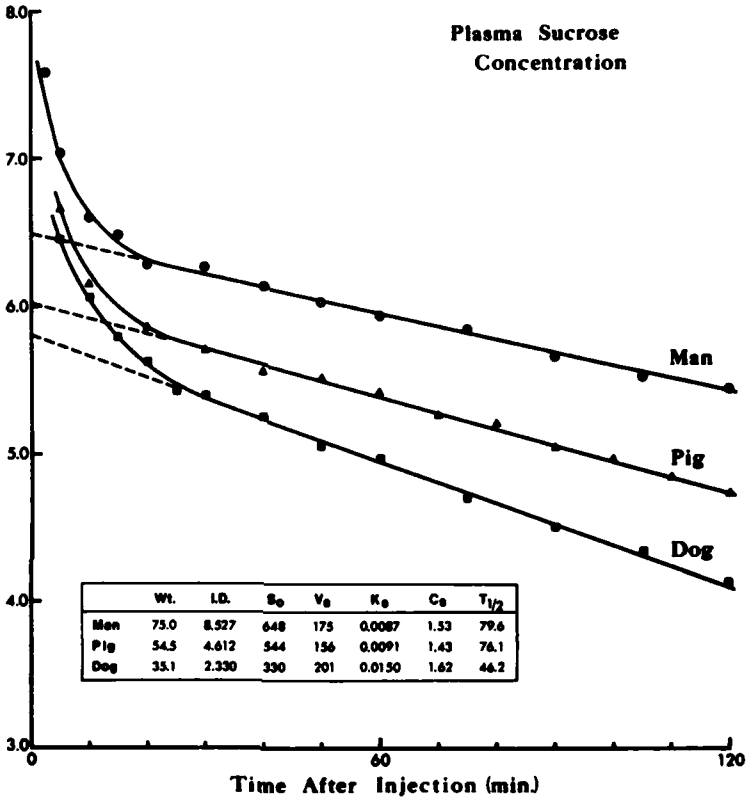

Figure 5. Use sucrose to determine anatomic and physiologic parameters of extracellular space. Ordinate values express plasma sucrose concentration as ln mg/l.

In all three species a progressive decrease in plasma sucrose concentration was observed as a function of time after injection. Initially, the decline proceeded quite rapidly and presumably reflected the movement of sucrose from the blood to the interstitial space. After 20 to 30 minutes, the slope of plasma sucrose concentration curve became linear when plotted semi-logarithmically. The concentration decrements observed during this period presumably reflected renal clearance of sucrose. On the basis of the linear portions of the plasma concentrations

curves theoretical zero-time plasma sucrose concentrations were determined by extrapolation. When the injected dose (I.D.) was divided by the zero-time concentration (S_o), the distribution volume of sucrose, i.e., extracellular volume, was obtained. In Figure 5 the distirbution volumes (V_s) were expressed per kilogram of body weight. The linear portions of these curves were also used to calculate the rate constant (K_s) for plasma sucrose disappearance, plasma sucrose clearance (C_s) expressed as milligram per kilogram of body weight and half-time ($T_{1/2}$) expressed in minutes for the decay curve. These calculations were all based on the equation:

$$S_t = S_o e^{-Kt}$$

where, S_t = the plasma sucrose concentration at any time t, S_o = the zero-time concentration and K = the rate constant.

DISCUSSION

The foregoing experiments show that plasma sucrose concentration can be accurately determined with the anthrone reagent and that the procedure can be used for evaluating parameters of extracellular anatomy and function, provided: (a) plasma protein is first removed from the samples by precipitation; (b) glucose and other interfering chromogens are removed by hot alkali; and (c) sucrose-anthrone color development is carefully controlled with respect to time and temperature.

Protein interference with the anthrone reaction was first noted by Dreywood (16). Morris (17) reported that the protein-anthrone reaction produced a red color. Graff et al (24) conducted a detailed study of the reaction with plasma proteins and showed that the albumin-anthrone reaction had an absorption peak at 520 nm, while globulin-anthrone reaction had peaks at both 520 and 620 nm. The absorption at 620 nm was attributable to the carbohydrate moiety of mucroprotein, i.e., α-1-globulin (24). In the present study both $Ba(OH)_2$-$ZnSO_4$ and ethyl alcohol were effective in removing plasma protein. Trichloroacetic acid was unsuitable because it reacted with anthrone and, consequently, interefered with color development. The $Ba(OH)_2$ - $ZnSO_4$ procedure gave somewhat better results than alcohol, at least in terms of the spectral characteristics of the reaction. In terms of absorption maximum for a given sucrose concentration, however, there was little to choose between the two. Thus, if only plasma sucrose is to be determined, ethyl alcohol may offer an attractive and, perhaps, simple alternative procedure for plasma protein precipitation. It was so used in the studies of Graff et al (24) but no technical details were provided. More recently, Van Handel (22,25) employed 66 percent alcohol in water as a solvent for the extraction of certain carbohydrates from biological samples. He did not investigate the effectiveness of alcohol as an agent for protein precipitation nor the solubility characteristics of sucrose in the solvent. In 100 percent alcohol, sucrose has a maximum solubility of 90 µg/ml and, in 20 C water, it has a

solubility of 204 mg/ml (25); hence, one would expect fairly high solubility in a water-alcohol mix. In a limited test, two parts alcohol to one part plasma effectively precipitated plasma protein and allow accurate determination of plasma sucrose concentrations up to 90 µg/ml. Presumably, much higher plasma sucrose concentrations could have been determined with the alcohol precipitation procedures, but this needs verification.

Several other protein precipitation agents have been used in the development of anthrone procedures for carbohydrate analysis, but the results have been contradictory. In an attempt to remove protein interference with anthrone determinations of tissue glycogen, Seifer et al (20) utilized $Cd(OH)_2$ and $Zn(OH)_2$ to prepare protein-free filtrates. Their efforts were unsuccessful. Subsequently, White and Samson (27) used 25 percent trichloroacetic acid to remove plasma protein prior to the determination of plasma insulin concentration. They mention no analytical difficulties with the procedure, yet Young and Raisz (19) encountered analytical errors, not only when trichloroacetic acid was employed as a protein precipitating agent, but also when $CdSO_4$ or tungstic acid was used. They achieved analytical success only with Somogyi's (28) $ZnSO_4$-NaOH procedure. Elsewhere Zipf and Waldo (29) and Handelsman and Sass (30) developed anthrone procedures for blood sugar determination based on tungstic acid filtrates but provided no information on possible interference by chromogens carried over in the protein-free filtrate. Clearly, these alternative techniques for protein precipitation should be thoroughly reinvestigated before they are used as a preliminary step to anthrone determinations of plasma sucrose concentration.

In the present experiments, successful use of hot alkali to remove interference by glucose and other reducing substances was consistent with the reports of Bloom and Wilcox (19), Young and Raisz (20), Van Handel (22), Kruhoffer (31), and Haddlesman and Drabkin (32). Van Handel (22) showed that 0.05 ml (1 drop) of 30 percent KOH and a heating time of 10 minutes were sufficient to remove all monosaccharide interference; such conditions completely eliminated the potential effects of 5000 µg of glucose on the anthrone determination of 50 µg of sucrose. Two alternative methods have been used to eliminate or correct for errors attributable to the presence of endogenous reducing substances in the plasma sample. One, employed for instance by White and Samson (27), involves preincubation of the sample with yeast (so-called "yeasting") to catabolize any glucose or other hexose that may be present. This is not only time-consuming, but also tends to introduce analytical errors because of the sample transfers that are required. The second alternative involves measurement of endogenous materials and subsequent correction for the contribution of endogenous material to the sucrose measurement. This technique was commonly incorporated in the older iodometric (11,13) and resorcinol (12,15) procedures for determining sucrose or other polysaccharide concentration. Its major disadvantage is the additional plasma requirement for the measurement of endogenous

material interference, an issue of prime importance in extracellular volume determinations which usually require multiple plasma samples.

The rate and extent of anthrone reaction with various carbohydrates are highly dependent on the temperature of the reacting mixture (17,21). In the presence of heat and a strong mineral acid, H_2SO_4 in the case of the anthrone reagent, disaccharides and polysaccharides are hydrolyzed to form monosaccharides. In addition, water is split off from the latter to form hydroxaldehyde. With further water loss and ring formation the hydroxyaldehyde is converted to a hydroxymethylfurfural which in the presence of anthrone yields the characteristic green chromophore associated with completion of the reaction sequence (33). In early studies, anthrone was mixed with concentrated sulfuric acid, and when this reagent was added to aqueous containing samples, exergonic heat production served to facilitate the reaction sequence (16,17,24). This procedure, as might be anticipated, led to erratic and inconsistent results because heat production and loss in the reaction vessel was influenced by a variety of extraneous variables. To achieve better control, most subsequent workers added the anthrone reagent slowly while the reaction vessel was cooled in tap water or ice water and thereafter initiated the reaction sequenced by placing the vessel in hot or boiling water (18-21,24,27,29,30,32). Some (17,21,33, 34) used the rate and extent of color development in attempts to qualitatively identify individual sugars in a mixture, but these attempts achieved only limited success. Virtually no effort was made to assess the possibility of using controlled time and temperature to quantitively distinguish between sugars until Von Handel (35) showed in 1967 that fructose moiety could be determined accurately in the presence of glucose if the anthrone-hexose reaction was carried out at room temperature. A key feature of his modified procedure was the evaporation of the aqueous sugar-containing sample to near dryness. For all practical purposes, this eliminated the problem of exergonic heat production during addition of the anthrone reagent to the sample. In 1968, Von Handel (22) reported a further modification in which he was able, by controlling reaction temperature at 40 C for a period of 10 to 15 minutes, to measure the fructose moiety of sucrose quantitatively in the presence of a large excess of glucose. The present investigation confirms Von Handel's (22) conclusion that 40 C is an optimum reaction temperature for the quantitative determination of sucrose with the anthrone reagent. His conclusion that maximum color development is achieved in 10 to 15 minutes was not confirmed; at least 30 minutes at 40 C was required. The reason for this discrepancy is uncertain. Von Handel (22) did not present any data on the rate of color development, hence it was not possible to make a direct comparison of his results with those presented here. The apparently more rapid color development in Von Handel's experiments may have been due to the small amount of water contained in his concentrated samples and consequent, albeit limited, heat production at the stage of reagent addition.

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