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A P P E N D I X

APPENDIX A

A Photometric Adaptation of the Somogyi Method for the Determination of Glucose [1](17)

Reagents

1. Copper Reagent

1.1 Copper Reagent A

Dissolve 25 g of Na_2CO_3 (anhydrous), 25 g of Rochelle salt (sod. potassium tartrate), 20 g of $NaHCO_3$, and 200 g of Na_2SO_4 (anhydrous) in about 800 ml of water and dilute to 1 liter. Filter if necessary.

1.2 Copper Reagent B

15 per cent $CuSO_4 \cdot 5H_2O$ containing one or two drops of concentrated sulfuric acid per 100 ml.

1.3 Copper Reagent

25 parts of Reagent A + 1 part of Reagent B (prepared in the day of use).

2. Arsenomolybdate color reagent

Dissolve 25 g of ammonium molybdate in 450 ml of dis-

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[1](17) - Number of Reference.

tilled water, add 21 ml of concentrated H_2SO_4, mix, add 3 g of $Na_2HASO_4 \cdot 7H_2O$ dissolved in 25 ml of H_2O, mix and place in an incubator at 37°C for 24-48 hours. This reagent should be stored in a glass-stoppered brown bottle.

Procedure

1 ml of Copper reagent is added to 1 ml of sample in a test tube. The solution is mixed and heated for 20 minutes in a boiling water bath. At the end of 20 minutes the tubes are cooled in a pan of cold water. 1 ml of the arsenomolybdate reagent is then added. The color develops very rapidly. The mixture is then diluted with 2 ml of distilled water, mixed, and read in a photoelectric colorimeter at 520 mμ. The color is very stable and may therefore be read at convenience.

APPENDIX B

Buffer solutions

1. Ammediol (2-Amino-2-methyl-1, 3-propanediol) Buffer (9).

Stock Solutions

A : 0.2 M solution of 2-amino-methyl-1, 3-propanediol (21.03 g in 1000 ml).

B : 0.2 M HCL.

50 ml of A + x ml of B, diluted to a total of 200 ml.

x	pH	x	pH
2.0	10.0	22.0	8.8
3.7	9.8	29.5	8.6
5.7	9.6	34.0	8.4
8.5	9.4	37.7	8.2
12.5	9.2	41.1	8.0
16.7	9.0	43.5	7.8

2. Acetate Buffer (30)

Stock Solutions

A : 0.2 M solution of acetic acid (11.55 ml in 1000 ml)

B : 0.2 M solution of sodium acetate (16.4 g of $C_2H_3O_2Na$ or 27.2 g of $C_2H_3O_2Na.3H_2O$ in 1000 ml).

x ml of A + y ml of B, diluted to a total of 100 ml.

x	y	pH
46.3	3.7	3.6
44.0	6.0	3.8
41.0	9.0	4.0
36.8	13.2	4.2
30.5	19.5	4.4
25.5	24.5	4.6
20.0	30.0	4.8
14.8	35.2	5.0
10.5	39.5	5.2
8.8	41.2	5.4
4.8	45.2	5.6

3. Phosphate Buffer (25)

Stock Solutions

A : 0.2 M solution of monobasic sodium phosphate (27.8 g in 1000 ml).

B : 0.2 M solution of dibasic sodium phosphate (53.65 g of $Na_2HPO_4.7H_2O$ or 71.7 g of $Na_2HPO_4.12H_2O$ in 1000 ml).

x	y	pH	x	y	pH
93.5	6.5	5.7	45.0	55.0	6.9
92.0	8.0	5.8	39.0	61.0	7.0
90.0	10.0	5.9	33.0	67.0	7.1
87.7	12.3	6.0	28.0	72.0	7.2
85.0	15.0	6.1	23.0	77.0	7.3

x	y	pH	x	y	pH
81.5	18.5	6.2	19.0	81.0	7.4
77.5	22.5	6.3	16.0	84.0	7.5
73.5	26.5	6.4	13.0	87.0	7.6
68.5	31.5	6.5	10.5	90.5	7.7
62.5	37.5	6.6	8.5	91.5	7.8
56.5	43.5	6.7	7.0	93.0	7.9
51.0	49.0	6.8	5.3	94.7	8.0

APPENDIX C

Disc electrophoresis by the method of Davis (5)

Reagents

(1) Acrylamide [1](Eastman 5521). Acrylamide is a white crystalline solid that is best stored in a cool, dark, dry place to reduce slow spontaneous polymerization and hydrolysis. The average shelf life of commercial samples is several years.

(2) N, N'-Methylenebisacrylamide [1](Eastman 8383) (BIS). BIS is a white crystalline solid best stored in a cool, dark, dry place. Slow spontaneous polymerization may occur during long storage.

(3) 2-Amino-2-(hydroxymethyl)-1, 3-propanediol : also known as tris (hydroxymethyl) aminomethane [2](TRIZMA BASE) (TRIS).

(4) N, N, N', N'-Tetramethylethylenediamine [1](Eastman 8178) (TEMED).

(5) Riboflavin (Eastman 5181).

(6) Hydrochloric acid (HCl), reagent grade, one Normal (N).

(7) Ammonium persulfate, reagent grade.

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[1]Eastman No. - the product of Distillation Products Industries, Division of Eastman Kodak Company, Rochester, N.Y.

[2]TRIZMA BASE - the product of Sigma Chemical Company, St. Louis, Mo.

(8) Glycine (Ammonia-free).

(9) Glacial acetic acid.

(10) Amido Schwarz : also known as Naphthol Blue Black, Color Index Number 20470.

(11) Bromphenol Blue.

(12) Sucrose.

Stock Solutions

These solutions are prepared using distilled water and are filtered and stored in brown glass bottles in a refrigerator. The shelf life of these solutions is up to several months.

Table 1

Stock Solutions

(A)			(B)		
1 N HCl	48	ml	1 N HCl	approximately 48 ml[3]	
TRIS	36.6	gm	TRIS	5.98	gm
TEMED	0.23	ml	TEMED	0.46	ml
water	to 100	ml	water	to 100	ml
	(pH 8.9)			(pH 6.7)	
(C)			(D)		
Acrylamide	28.0	gm	Acrylamide	10.0	gm
BIS	0.735	gm	BIS	2.5	gm
water	to 100	ml	water	to 100	ml
(E)			(F)		
Riboflavin	4	mg	Sucrose	40	gm
water	to 100	ml	water	to 100	ml

[3] pH adjusted by titrating with 1 N HCl.

Working Solutions

These solutions should be used the day they are prepared, with the exception of the persulfate solution which should be used within 7 days of preparation. The persulfate solution should be stored in a refrigerator.

Table 2

Working Solutions

Small-pore solution # 1	Small-pore solution # 2	Large-pore solution	Stock buffer solution for reservoirs
1 part A	Ammonium persulfate	1 part B	TRIS 6.0 gm
2 parts C	0.14 gm	2 parts D	Glycine 28.8 gm
1 part water	water to 100 ml	1 part E	water to 1 liter
pH 8.9(8.8-9.0)		4 parts F	pH 8.3
		pH 6.7(6.6-6.8)	

Wash-solution for destaining and storing gels		Fixative-stain solution	
Glacial acetic acid	70 ml	Amido Schwartz	1 gm
water	to 1 liter	7% Acetic acid	100 ml

The dye is added to the acetic acid, the mixture stirred and filtered. This solution can be reused a number of times. As the solution is reused, a black insoluble precipitate forms

that may coat the gels. This precipitate should be removed by filtering the solution prior to reuse. It should be noted that the fixative-stain solution, after it has been reused a number of times, will tend to stain the protein black rather than the expected dark blue.

Polyacrylamide solution for destaining

Acrylamide	6.0	gm
Riboflavin	0.5	mg
TEMED	0.05	ml
water to	100	ml

This solution is exposed to a daylight fluorescent light in a 100 ml beaker at a distance of about 3 inches. Photopolymerization is allowed to proceed for about 1 1/2 hours. The polymer solution is then diluted with an equal volume of distilled water, thoroughly mixed, and stored in a brown glass bottle.

Procedure

(1) The stock solutions and small-pore solution # 2 are removed from the refrigerator and permitted to warm to room temperature before use.

(2) A sample gel solution composed of about 0.15 to 0.20 ml of large-pore solution and about 3 to 4 µl of sample (about

200 gamma of protein) are gently but thoroughly mixed. Each sample is mixed in an individual container and protected from strong light. After all the sample mixtures are prepared, each is then added to the well of a rubber stopper attached to the tube stand previously described.

The total amount of protein in the sample volume should not usually exceed 200 gamma. Tuberculin syringes and needles, the latter fitted with lengths of small-pore polyethylene tubing, serve as useful devices for measuring, mixing, storing, and delivering the sample gel solution into the stopper wells.

(3) After the stopper wells have been filled with the sample solutions, the gel tubes are inserted into the wells, care being taken that the tubes are vertically positioned.

Prior to use, the gel tubes are cleaned in a detergent solution, and the inner walls vigorously cleaned with a cotton-tipped applicator stick. The tubes are then rinsed, first in distilled water and then in a solution of distilled water containing 1 part in 200 of [4]Kodak Photo-Flo solution. The gel tubes are drained and allowed to dry. Rapid drying can be accomplished by attaching the gel tubes to a vacuum line. This cleaning procedure must always be repeated prior to reuse of the gel tubes.

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[4]Kodak Photo-Flo solution - the product of Eastman Kodak Company, Rochester, N.Y.

(4) A water layer is now placed on top of the gel solution. This is accomplished by means of a syringe barrel or pipette previously described. The needle with the attached syringe containing 1 to 2 ml of distilled water is introduced into the top of the gel tube so that the needle rests against the wall and the hub against the top of the tube. The water will flow slowly and evenly down the inner wall of the gel tube and will layer smoothly on top of the denser sample gel solution. A water layer 3 to 4 mm in height is adequate.

If the tubes are not clean or adequately coated with Photo-Flo solution, the water-layering step cannot be performed readily. The water, instead of flowing smoothly and evenly down the glass walls, will tend to collect into a bolus that will then drop rapidly and thus penetrate into, and dilute, the sample gel solution.

(5) Following water layering, the tube stand is placed directly under a daylight fluorescent bulb; the bulb is so positioned that it is about 1/2 to 1 inch above the tips of the gel tubes. The sample gel solutions are exposed to this light for about 30 to 45 minutes. After about the first 5 minutes light scattering can be seen in the gel solutions, indicating photopolymerization.

(6) Following photopolymerization, the tube stands are removed from the light. They are inverted so that the water layer and the adjacent small fraction of inhibited gel solution

flow down the walls of the tubes to the open ends. A piece of absorbent cloth or paper, preferably as lint free as possible, is touched to the open ends of the tubes to absorb the liquid. The inner walls of the tubes are now rinsed with a large-pore solution in which the monomer (D) and the sucrose (F) stock solutions have been replaced by distilled water; that is the tubes are one-half filled with these solutions by means of a pipette and the tube stands rocked gently several times so that all areas of the walls are washed. The tube stands are then inverted and drained as described. The wash procedure is performed twice.

(7) A spacer gel is prepared by adding about 0.15 ml of large-pore solution to the gel tubes. This large-pore solution is water layered as described in step (4). The tube stands are again placed under a fluorescent light, as described in step (5), and exposed to light for about 20 to 30 minutes.

In instances when a very dilute protein sample is used and the sample gel volume, and therefore height, are increased, the height of the spacer gel column must be increased proportionately.

(8) Following photopolymerization of the spacer gel the tube stands are removed from under the lamp. The tube stands are then inverted for about one minute with the gel tubes resting on absorbent cloth or paper so that the water layer and adjacent inhibited large-pore solution flow down the tube walls and onto

the absorbent material. While the tubes are draining, small-pore gel solution is prepared by mixing equal volume of small-pore solutions # 1 and # 2. A total final volume of about 20 ml is sufficient for washing and filling 12 gel tubes. Promptly after this solution is prepared all of the gel tubes are half filled by means of a pipette, and the tube stands are rocked and tilted gently several times so that the surface of the spacer gel and all areas of the tube walls are washed. The small-pore gel solution is removed by inverting the tube stands, permitting the solution to drain down and out onto absorbent meterial. The wash procedure is performed twice. The gel tubes are now completely filled and an excess of small-pore gel solution is added so that a "bead" of solution rests on top of the upper ends of the gel tubes. The tubes are capped by placing on top of each, by means of forceps, a previously cut 10 mm square of [5]Saran Wrap film. Sufficient excess of solution is added so that on capping a tube, a small amount of solution runs down the outside of the tube wall and no air bubble is trapped under the film. The gel tubes are protected from strong light and permitted to stand undisturbed for about 30 minutes, at the end of which time electrophoresis can be performed.

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[5]Saran Wrap - the product of the Dow Chemical Company, Midland, Mich.

The gel time of this small-pore gel mixture is about 15 to 20 minutes. The time between the preparation of the small-pore gel solution and the capping of the tubes should not exceed 10 minutes, and the gel solution should reside undisturbed in the gel tubes for at least 5 to 10 minutes prior to the onset of gelation.

If the gel time exceeds the stated limits when prepared from fresh reagents, it can be corrected by making minor adjustments in the concentration of TEMED.

Alternative Procedure

In certain instances the use of a sample gel may not be possible, that is, the protein sample may contain inhibitory substances that prevent the formation of a sample gel. In other instances, the incorporation of the sample into a solution undergoing vinyl polymerization may not be desirable.

In both instances an alternative procedure can be employed in which the formation of a sample gel is bypassed and the sample is, instead, layered on top of a spacer gel just prior to electrophoresis.

The procedure is identical to that just described except for the following differences. (A) In the method outlined, the sample gel solution is replaced by a 40 per cent sucrose solution on top of which the spacer gel solution is layered and then polymerized. (B) Since the surface of the spacer gel formed against

the sucrose solution is rarely flat and smooth, it is necessary, following the polymerization of the small-pore separation gel, to insert the gel tubes, separation gel down, into a tube stand and to drain off the sucrose solution. After several brief washes with the large-pore solution to remove excess sucrose, additional large-pore solution is added on top of the spacer gel. A column about 4 mm in height of large-pore solution is pipetted on top of the spacer gel. This gel solution, when water layered and photopolymerized, forms a smooth, flat surface upon which the sample is placed just prior to electrophoresis.

(9) When polymerization is complete, following step (8), the gel tubes are removed from the tube stand, caution being exercised to avoid stressing and distorting the gel column to prevent inadvertent separation from the gel tube wall. Removal is accomplished by pressing and tilting the tube against one side of the flexible cap wall so as to provide a space through which air can enter the hollow of the cap as the gel tube is removed. Polymerization of a zone, 0.5 to 1.0 mm in height, of the sample gel adjacent to the base of the stopper well is generally inhibited. This watery solution is removed by inverting the gel tubes, sample gel down, and touching the open end to absorbent material.

(10) Electrophoresis should preferably be started within 1 hour after the separation gel has been prepared. The tubes, sample gel uppermost, are inserted into the grommets of the upper

buffer reservoir, and this reservoir is filled with about 200 ml of the stock buffer solution diluted to 1/10 strength with distilled water. One ml of 0.001 per cent Bromphenol Blue in water is stirred into the upper buffer. Any air spaces in the gel tubes above the sample gel are displaced with buffer by means of a pipette. Next a hanging drop of buffer is placed on the bottom of each gel tube to prevent trapping of bubbles, and the upper reservoir is then lowered so that the bottoms of the gel tubes are immersed about 1/4 inch in the buffer (also 1/10 strength) of the lower reservoir. The lower reservoir should be filled to within 1/2 inch of the top.

In the instance when a sample gel is not used, the volume of the tube above the spacer gel is first filled with buffer, the tubes are attached to the upper reservoir, and the upper reservoir is filled with buffer. The sample solution is picked up in a pipette and the pipette introduced through the buffer solution and into the gel tubes to a point about 4 mm above the surface of the spacer gel. The sample is gently expelled and permitted to layer between the top of the surface of the spacer gel and a less dense buffer solution above. The gel tubes must be carefully positioned so that the protein solution is evenly distributed across the face of the spacer gel.

(11) The power supply is connected, cathode to the upper reservoir. The current is adjusted to about 2 to 5 m.Amp. per tube. Currents higher than 5 m.Amp. per tube should be avoided

since excessive ohmic heating may result in the formation of pattern artifacts. Electrophoresis is carried out until the front of the light blue albumin disc has migrated about 25 mm into the separation gel and the free Bromphenol Blue dye has therefore migrated about 30 mm ; with a current of about 5 m. Amp. per tube, the time for electropheresis is about one-half hour. In the instance when the sample solution is layered above the spacer gel, the current should not initially exceed 2 m.Amp. per tube so as to prevent convective losses of the sample into the upper reservoir. After the sample has entered the spacer gel, the current is then increased to 5 m.Amp. per tube.

When electrophoresis is carried out at room temperature with the apparatus, reagents, and conditions described here, the temperature within the separation gel containing the sample fractions is in the range of about 35°to 40° Centigrade. If the sample to be separated contains heat labile substances, e.g. enzymes, which may be inactivated at the temperature of the operating conditions described, it may be necessary to reduce both the ambient temperature and the current. At a current of 1 m.Amp. per gel tube, heating within the sample volume of the separation gel is negligible; but, for the same distance of migration, the time for separation must be increased to about 2½ hours.

(12) At the completion of electrophoresis, after the power supply is turned off, the buffer solutions are decanted.

If fewer than 12 sampels are run, the buffer solutions may be reused (see Reagent section), but the upper and lower buffer solutions must not be pooled or positions reversed in the apparatus in future runs since this would result in contamination of the cathodic buffer reservoir by gel catalyst substances and chloride ions which migrate into the anodic reservoir during electrophoresis.

(13) The gel tubes are removed from the upper reservoir and the gels removed from the tubes by rimming under water. The water lubricates the gel surface and prevents mechanical damage to the gel by the rimming wire or needle. The wire is slowly introduced into the bottom of the gel tube between the small-pore gel and the tube wall for a distance of about 5 to 10 mm while continuously rotating the gel tube. The wire is then withdrawn with a slight pressure against the gel, stretching the gel so that it protrudes about 2 mm beyond the end of the tube. The needle is then withdrawn completely. It is then introduced into the other end and a continuous rimming action is again applied as the needle advances until the gel slips out of the tube.

(14) Each gel is immersed in at least 2 ml of fixative-stain solution for a minimum of 1 hour. At the end of 1 hour, the fixative-stain solution is decanted and the gels are rinsed for a few minutes in tap water.

(15) Electrophoretic destaining is performed in the same apparatus. The gels, sample gel uppermost, are placed in

the destaining tubes, which have been attached by their wide ends to the upper buffer reservoir. The gels slide down in the tubes and should wedge firmly against the constricted ends of the tubes. In order to reduce convective disturbances and backflow of free dye up the tube, polyacrylamide solution for destaining may be added to each tube by means of a pipette up to the top of the sample gel. No air bubbles should remain in the tubes after adding polyacrylamide solution. The space above the gel in the destaining tube is carefully filled with wash solution by means of a pipette. If the difference between the i.d. of the destaining tube and that of the gel is 1 mm or less, no polyacrylamide solution is necessary; a small cotton plug should be inserted into the upper end of the destaining tube after filling with wash solution. About 200 to 300 ml of wash solution are then added to each reservoir, and 20 to 30 microliters of fixative-stain solution are mixed into the upper reservoir. The electrodes are connected, cathode to the upper reservoir. On applying a voltage, the unbound dye migrates down the gels and into the lower reservoir. Destaining is completed within about 1 hour, using the power supply. This time can be reduced to about 20 minutes if the current is adjusted to about 15 m.Amp. per tube by using a higher voltage power supply.

(16) At the completion of destaining, the power supply is shut off, the wash solutions are decanted, and the gels are transferred to small test tubes containing wash solution for storage.

APPENDIX D

Hugh and Liefson medium (For demonstrating oxidation or fermentation of a sugar) (3)

Base

Tryptone (Difco)		2.0	g
NaCl		5.0	g
K_2HPO_4		0.3	g
Powdered agar		3.0	g
Brom-cresol purple 1.2% aqueous		1.0	ml
Distilled water	to	1000	ml

Dissolve ingredients. Adjust pH to 7.1, tube in 5 ml amounts in 4 x ½ in. tubes, and sterilize at 10 lb for 10 minutes. Add 0.5 ml of a 10 percent solution of the required sugar.

APPENDIX E

Protein Measurement with the Folin Phenol Reagent by the method of Lowry et al (15)

Reagents

Reagent A, 2 per cent Na_2CO_3 in 0.10 N NaOH.

Reagent B, 0.5 per cent $CuSO_4 \cdot 5H_2O$ in 1 per cent sodium or potassium tartrate.

Reagent C, alkaline copper solution. Mix 50 ml of Reagent A with 1 ml of Reagent B. Discard after 1 day.

Reagent D, carbonate-copper solution, is the same as Reagent C except for omission of NaOH.

Reagent E, diluted Folin reagent.

Titrate [1]Folin-Ciocalteu phenol reagent with NaOH to a phenolphthalein end-point. On the basis of this titration dilute the Folin reagent (about 2-fold) to make it 1 N in acid. Working standards may be prepared from human serum diluted 100 to 1000-fold (approximately 700 to 70γ per ml). These in turn may be checked against a standard solution of [2]crystalline bovine albu-

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[1] Folin-Ciocalteu phenol reagent - the product of Eimer and Amend, Fisher Scientific Company, New York.

[2] Crystalline bovine albumin - the product of Armour and Company, Chicago.

min; 1γ is the equivalent of 0.97γ of serum protein. Dilute solutions of bovine albumin have not proved satisfactory for working standards because of a marked tendency to undergo surface denaturation.

Procedure

To a sample of 5 to 100γ of protein in 0.2 ml or less in a 3 to 10 ml test tube , 1 ml of Reagent C is added. Mix well and allow to stand for 10 minutes or longer at room temperature. 0.10 ml of Reagent E is added very rapidly and mixed within a second or two. After 30 minutes or longer, the sample is read in a colorimeter or spectrophotometer. For the range 5 to 25γ of protein per ml of final volume, it is desirable to make readings at or near λ = 750 mμ , the absorption peak. For stronger solutions, the readings may be kept in a workable range by reading near λ = 500 mμ .

APPENDIX F

The Photometric Microdetermination of Glucose with Glucose Oxidase by Saifer *et al* (24)

Reagents and Apparatus

1. Glucose sample.

2. Zinc sulfate, 5 per cent solution of reagent grade $ZnSO_4 \cdot 7H_2O$.

3. Barium hydroxide 0.3 N solution; 10.0 ml of this solution should exactly neutralize a 10.0 ml aliquot of the 5 per cent zinc sulfate solution using phenolphthalein as an indicator.

4. [1]Glucostat reagent. The reagent is freshly prepared in sufficient quantity for each day's run exactly as described in the accompanying circular. It contains glucose oxidase, horseradish peroxidases, and phosphate buffer in one vial and O-dianisidine in a second vial. Each set of two vials is sufficient for 100 ml of reagent. For routine determinations, it is desirable to prepare a more concentrated reagent by diluting the contents of the vials to 47.5 ml plus 2.5 ml of 0.4 M phos-

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[1]Glucostat reagent - obtainable from the Worthington Biochemical Corporation, Freehold, N.J.

phate buffer, pH 7.0 (139.2 gm Na_2HPO_4 and 20.5 gm KH_2PO_4 per liter of solution).

5. Glucose standards : 100 mg per cent and 200 mg per cent glucose solutions. These are prepared by dilution with 0.1 per cent benzoic acid from a stock standard of 1.000 gm per cent glucose (reagent grade) made up in 0.10 per cent benzoic acid solution and are stable at room temperature.

6. Spectrophotometers : Beckman D.U. or equivalent instrument for use in research studies.

Procedure

Prepare the filtrate to remove the reducing substances by adding to each 0.50 ml of sample, 7.50 ml of distilled water and 1.00 ml each of the 5 per cent zinc sulfate and barium hydroxide. Mix and centrifuge, or filter, the precipitate formed.

To 1.00 ml of the 1:20 zinc filtrate, add 6.0 ml of dilute glucostat reagent, mix, and let tube stand in a water bath at 37° C for 30 minutes. Then add 1.0 ml of 0.5 N sulfuric acid solution and mix by inversion. Let tube stand at room temperature for 5 minutes and read in the Beckman D.U. at 395 mμ against a distilled water blank treated in the same manner as the sample. Duplicate 100 and 200 mg per cent glucose standards are also run with the procedure simultaneously with each set of unknowns. Optical density readings are directly proportional

to glucose concentrations in the 25 to 400 mg per cent range.

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