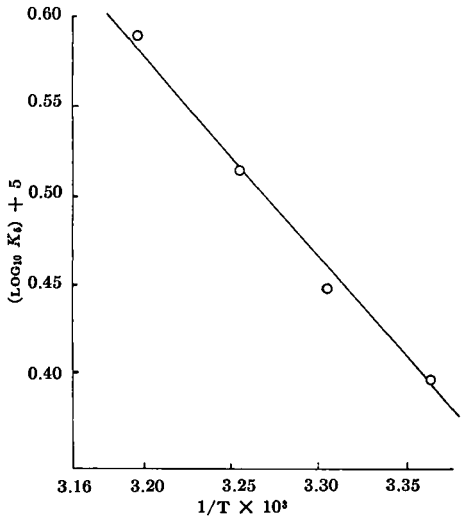

Fig. 4.—Arrhenius plot of the rate constants obtained for the dissolution of benzoic acid in water using the automatic recording apparatus.

values for the energy of dissolution as reported by Edwards' and those obtained in this study. The data from Edwards' paper give a standard deviation of 750 cal./mole, while the results obtained using the recording apparatus gave a standard deviation of 200 cal./mole. Thus, the apparatus appears to be particularly useful for working at elevated temperatures since it eliminates the problem of temperature change during the collection and subsequent handling of samples.

TABLE VI.—RATE CONSTANTS AS A FUNCTION OF TEMPERATURE FOR THE DISSOLUTION OF BENZOIC ACID IN WATER

Temp., °K.	K_4	Weight Needed for Saturation[a]	$K_5 \times 10^5$
298	0.07227	2565	2.818
303	0.09890	3075	3.216
308	0.13410	3623	3.701
313	0.18271	4163	4.389

[a] Taken from Edwards' data (4), and given as mg. needed to saturate 750 ml. of water.

One further advantage for the apparatus is that it can be cleaned and assembled for further use within 15 minutes after the completion of a run. This is a vast saving in time over that generally needed to clean volumetric glassware used in a kinetic study. This apparatus is therefore suggested for use in the study of rapid reactions in which one chemical species can be assayed spectrophotometrically with no interference from other species in solution.

REFERENCES

(1) Noyes, A. A., and Whitney, W. R., *J. Am. Chem. Soc.*, **19**, 930(1897).
(2) Hixson, A. W., and Crowell, J. H., *Ind. Eng. Chem.*, **23**, 923(1931).
(3) Davies, O. L., "Statistical Methods in Research and Production," 2nd ed., Oliver & Boyd, London, 1949, p. 153.
(4) Edwards, L. J., *Trans. Far. Soc.*, **47**, 1191(1951).

Investigation of the Mechanism of Urea-Induced Hemolysis

By DALE E. WURSTER and PAUL H. SHAPIRO†

The hemolysis of mammalian erythrocytes in concentrated urea solutions and in isotonic sodium chloride and dextrose solutions containing thirty per cent urea was investigated. Increased concentrations of sodium chloride, dextrose, and other osmotic agents inhibited the urea-induced hemolysis. The existence of urea–dextrose complexes in water, methanol, and methanol–water systems containing high dextrose concentrations were demonstrated.

DURING the last decade, Javid and Settlage, *et al.*, developed a remarkably effective method for reducing elevated intracranial and intraocular pressures. The method involves the intravenous administration of 30 per cent urea solutions (1, 2). In preliminary work with animals they observed that the administration of concentrated solutions caused a hemolysis which was detectable by the resulting hemoglobinuria. This would be expected to happen since the erythrocyte is permeable to urea, and thus the urea solution can exert no osmotic pressure. However, it was found that hemolysis also occurred when the urea was dissolved in an isotonic solution of sodium chloride or dextrose. In experimenting with the addition of various con-

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centrations and combinations of salts and sugars it was found that 10 per cent invert sugar was a suitable agent for preventing this hemolysis.

The purpose of this investigation was to obtain additional information on the mechanism of the urea-induced hemolysis. Equally important was a desire to study the effects produced by sugars in these systems and to determine in what manner they inhibited hemolysis. Since beef erythrocytes were used in this study, a quantitative transfer of the experimental data to a system containing human erythrocytes is not possible; however, it is reasonable to expect that the data would apply, qualitatively.

Hemolysis can be caused by two general methods, the lowering of the osmotic pressure of the medium in which the cells are suspended and by the direct action of a substance on the cell membrane. There are other means of causing hemolysis, but these methods are the most important.

In the early stages of the study it was suggested that urea might cause hemolysis by the latter method. Since the erythrocyte is permeable to urea, hemolysis occurs when erythrocytes are suspended in a solution containing any concentration of urea in water. Since hemolysis also occurred in a system containing 0.9 per cent sodium chloride plus 30 per cent urea, it seemed possible that the urea was attacking the cell membrane. Urea has previously been reported to complex with proteins and long chain fatty acids (3) and these substances are, of course, present in the cell membrane (4). Therefore, the possibility that such a complex was causing the disruption of the cell membrane was strongly considered. It was also possible that the sugars were adsorbed on the cell membrane and thereby protecting it from the urea or else forming a complex with the urea and thereby blocking its hemolytic action on the cell membrane. Several investigators have noted the inhibition of hemolysis by nonelectrolytes (5–9).

As the study progressed, however, there was reason to believe that urea caused hemolysis not by exerting any specific action on the cell membrane, but by the general action of a penetrating molecule. The study was then approached from this viewpoint.

EXPERIMENTAL

Source of the Blood.—The blood used in this study was obtained from beef cattle immediately after killing. The blood was collected in 125-ml. glass-stoppered Erlenmeyer flasks into which had previously been placed 0.25 ml. of heparin sodium injection U.S.P. After mixing, the flasks were immediately refrigerated.

Hemolysis Conditions.—The 100-ml. volumetric flasks containing 49 ml. of the particular solution were placed in a shaking apparatus in a 30° constant temperature bath. After allowing sufficient time for the solutions to reach 30°, 1 ml. of the whole blood was pipeted into each flask. The shaker was started and the flasks were maintained in the bath for 1 hour. Then, about 15 ml. of each suspension was poured into 15-ml. centrifuge tubes which were immediately centrifuged at 2000–2500 r.p.m. for about 15 minutes in a Servall type SP/X angle centrifuge.

Determination of Hemoglobin.—The analytical method for the determination of hemoglobin was a modification of that described by Finholt (10). Ten milliliters of the supernatant liquid from the centrifuge tubes was removed by pipet and placed in 25-ml. volumetric flasks. To these solutions was added 1 ml. of a 0.2% potassium cyanide–1% potassium ferricyanide solution. After waiting 15 minutes for the cyanomethemoglobin to form, the absorbance of the solutions was read at 545 mμ on a Cary model 11 recording spectrophotometer using 1-cm. silica cells.

Calculation of Per Cent Hemolysis.—The hemoglobin was assayed for the relative, not the absolute concentration. In each experiment one sample was included which contained 2% blood in water or in 30% urea, depending upon whether or not the other samples contained urea. The absorbance of this sample was taken as the 100% hemolysis reading. The per cent hemolysis of the samples was determined by dividing the absorbance of the sample by the 100% hemolysis reading.

Urea-Dextrose Complex in Methanol.—In the case of 100% methanol as the solvent, 2 Gm. of dextrose was placed in a series of 30-ml. vials along with varying amounts of urea and 20 ml. of methanol. The vials were placed in a shaker in a 30° constant temperature bath. After three days, 10 ml. was withdrawn from each vial and placed in tared containers. After the solvent was evaporated, the weight of dextrose was determined by subtracting the appropriate weight of urea. When 80 and 90% methanol were used as the solvent, 10 Gm. of dextrose was added and a 2-ml. aliquot was withdrawn.

Urea-Dextrose Complex in Water.—Varying amounts of urea were weighed into 4-dram vials. Into each vial was placed 3 Gm. of dextrose and 5 ml. of a saturated solution of dextrose. The vials were maintained with agitation in a 25° constant temperature bath for at least one week.

Upon removal from the bath, the entire contents of each vial was filtered through a 15-M sintered glass filter into a 100-ml. volumetric flask. Then the interior of the vial and the contents of the filter were washed repeatedly with 95% ethanol until the 100-ml. volumetric flask was filled. Three milliliters of this solution was diluted to 1,000 ml. with water. Two milliliters of this dilution was assayed for dextrose. In this case, the dextrose was determined according to the method of Nelson (11) and Somogyi's modification (12).

RESULTS AND DISCUSSION

Effect of 30 Per Cent Urea Plus Varying Sodium Chloride Concentration.—Since the erythrocyte is

TABLE I.—PER CENT HEMOLYSIS WITH 30% UREA AND VARYING SODIUM CHLORIDE CONCENTRATION

Age of Blood, Days	Per Cent Hemolysis — Sodium Chloride Concentration					
	0.7%	0.9%	1.1%	1.3%	1.5%	1.8%
0	100	100	74	24	24	14
1	100	100	74	15	15	19
2	100	97	61	12	12	10
3	100	92	43	4	4	4
4	100	100	97	16	16	19

TABLE II.—PER CENT HEMOLYSIS WITH 0.9% SODIUM CHLORIDE AND VARYING UREA CONCENTRATION

Age of Blood, Days	Per Cent Hemolysis — Urea Concentration					
	20%	22%	24%	26%	28%	30%
0	11	16	51	67	93	98
1	3	20	42	68	94	100
2	5	17	37	69	95	100
3	3	14	36	67	93	97
4	8	14	44	67	94	97

permeable to urea, if urea is the only dissolved substance in the suspension then hemolysis will occur regardless of the urea concentration. It was previously thought, however, that this permeability would not affect the cell adversely as long as the urea was dissolved in an isotonic solution of sodium chloride. Since hemolysis did occur under these conditions, it appeared possible that urea caused the hemolysis by attacking the cell membrane. However, it was found that when beef erythrocytes were suspended in medium containing 30% urea plus higher concentrations of sodium chloride, the per cent of hemolysis decreased with an increase in the sodium chloride concentration. The results of one such experiment are shown in Table I. Several of the earlier experiments were repeated on five consecutive days to determine what effects storing the blood in a refrigerator would have on the results.

On each day, the degree of hemolysis decreased as the sodium chloride concentration increased. As the sodium chloride concentration increased, increasing volumes of water necessarily left the erythrocyte. When the suspension medium contained 30% urea, a large volume of urea molecules diffused into the erythrocyte. When the sodium chloride concentration was low, the cell membrane was not able to accommodate the large increase in cellular content and it finally broke. Presumably, if the sodium chloride concentration was elevated sufficiently, a large enough quantity of water would leave the cell to enable the cell membrane to accommodate a large volume of urea without breaking.

For some of the intermediate concentrations of sodium chloride, the per cent hemolysis decreased daily for four days. This may be due to the constant leakage of potassium from the erythrocytes, as reported by Davson and Danielli (14). It is possible that the increased degree of hemolysis on the fifth day was due to some deterioration in the cell membrane.

Effect of Varying the Urea Concentration at a Constant Sodium Chloride Concentration.—The critical volume of the erythrocyte is about 1.6 times greater than the normal volume (15). If urea caused hemolysis as was described, there should be a minimum concentration below which no hemolysis should occur at any one concentration of sodium chloride. Table II shows the results of an experiment in which the urea concentration was varied, but the sodium chloride concentration was maintained constant at 0.9%.

The results indicated that when beef erythrocytes were suspended in 0.9% sodium chloride, the cells could accommodate approximately 20% urea before the critical volume was reached. As the concentration of urea was increased above 20 per cent, the degree of hemolysis increased rapidly.

Effect of Methylurea.—If urea caused hemolysis by the bulk of its molecules increasing the volume of the cell to the breaking point, then methylurea, having a greater molecular volume, should show a somewhat more pronounced effect. An experiment in which equal molar concentrations of the two compounds were compared showed that this was the case. The compilation of results from several experiments are given in Table III.

TABLE III.—A COMPARISON OF THE PER CENT HEMOLYSIS WITH UREA AND METHYLUREA

Molar Concentration	Per Cent Hemolysis	
	Urea	Methylurea
0.25	9	50
0.292	5	11
0.33	9	17
0.375	23	34
0.392	33	89
0.409	54	100
0.417	75	100

Effects of Dextrose.—It has been shown that urea-induced hemolysis can be inhibited by increasing the sodium chloride concentration in the medium. It was assumed that this effect could be produced by adding an osmotic equivalent of any nonpenetrating compound, but this assumption was not completely correct. Dextrose proved to be a far more effective agent in preventing hemolysis. Table IV shows a comparison of equal osmotic concentration of sodium chloride and dextrose.

It is interesting to note that the presence of urea affected the results only quantitatively. It was mentioned previously that erythrocytes are reported to have increased osmotic resistance when

TABLE IV.—COMPARISON OF HEMOLYSIS OF SODIUM CHLORIDE AND DEXTROSE WITH AND WITHOUT UREA

Concentration of Osmotic Agent		Per Cent Hemolysis	
		30% Urea	No Urea
Isotonic concn. 0.6	0.54% Sodium Chloride	100	71
	3.03% Dextrose	34	5
	0.585% Sodium Chloride	100	41
Isotonic concn. 0.65			
	3.28% Dextrose	13	2
	0.63% Sodium Chloride	100	21
Isotonic concn. 0.7			
	3.54% Dextrose	18	0

placed in a solution of a nonelectrolyte. These results with and without urea further indicate the similarity between osmotic hemolysis and urea-induced hemolysis.

The observation that erythrocytes behave abnormally when placed in a solution of a nonelectrolyte implies that it is the absence of electrolyte that causes the difference. To investigate this further, solutions were prepared which contained combinations of sodium chloride and dextrose such that the combination of the two compounds yielded a solution that was 0.6 isotonic. No urea was included. These results are given in Table V.

TABLE V.—PER CENT HEMOLYSIS WITH VARYING COMBINATIONS OF SODIUM CHLORIDE AND DEXTROSE

Concn. Sodium Chloride, %	Concn. Dextrose, %	Hemolysis, %
0.27	2.02	30
0.18	2.52	18
0.09	3.03	11
0	3.54	2

The increase in sodium chloride concentration from zero to 0.18 per cent caused a marked response even though this was only a 0.03 molar solution. This suggested that the dextrose did not affect the cell membrane directly, but acted only by allowing electrolyte to be excluded from the medium.

Effects of Other Sugars.—Dextrose was the primary sugar studied in this investigation. The results in Table VI show that hypotonic lactose and sucrose solutions cause an increased osmotic resistance similar to the effect produced by dextrose.

TABLE VI.—PER CENT HEMOLYSIS IN HYPOTONIC LACTOSE AND SUCROSE SOLUTIONS

Concentration of Osmotic Agent		Per Cent Hemolysis
Isotonic concn. 0.6	5.85% Lactose	7
	5.55% Sucrose	6
	6.34% Lactose	3
Isotonic concn. 0.65	6.01% Sucrose	3
	6.82% Lactose	2
Isotonic concn. 0.7	6.48% Sucrose	2

Blood in isotonic fructose (5.05%) plus 30% urea showed only 6% hemolysis. Thus, it is evident that all of these sugars gave very similar results. It is probable that all four sugars act simply by allowing electrolyte to be excluded from the medium.

Other Effects of Sugars.—On several occasions when blood was suspended in an isotonic solution of dextrose, lactose, sucrose, or inositol, within 15 minutes the red cells aggregated into large masses which immediately settled to the bottom of the flask. Upon the addition of very small quantities of one of several salts such as sodium chloride, sodium bromide, or potassium bromide, it was immediately possible to resuspend the red cells to form an apparently normal suspension. The addition of sufficient sodium chloride to give a concentration of only 0.18% was sufficient to bring about this reversal.

Effects of Other Electrolytes.—The only electrolyte reported on extensively thus far has been sodium chloride. Therefore, an attempt was made to compare the other electrolytes with sodium chloride to determine whether the erythrocyte reacted similarly to electrolytes in general. Table VII is a compilation of the results of several experiments on several different samples of blood.

The results showed that whenever the anion was divalent or trivalent, there was little or no hemolysis. There was a danger, of course, that in working with such a wide variety of salts in unbuffered solutions, that changes in the pH of the medium would become important. However, the results suggested that perhaps it was not the presence of

TABLE VII.—PER CENT HEMOLYSIS WITH HYPOTONIC CONCENTRATION OF VARIOUS SALTS

Salt	Concn., %[a]	Hemolysis, %
NaCl	0.54	80
KCl	0.741	76
NaI	1.42	94
KI	1.55	46
$NaC_2H_3O_3$	0.708	96
$NaNO_3$	0.816	62
KNO_3	0.972	76
$KClO_4$	1.13	93[b]
$CaCl_2 \cdot 2H_2O$	1.02	74
Calcium Lactate	2.70	79
$NaH_2PO_4 \cdot H_2O$	1.47	72
Na_2HPO_4 (anhyd.)	1.05	3
Na_2SO_4	0.986	4
K_2SO_4	1.27	2
$CuSO_4 \cdot 5H_2O$	4.11	0[c]
$MgSO_4$	3.78	2
Sodium Citrate	1.81	0
$Na_2B_4O_7 \cdot 10H_2O$	1.56	0
$Na_2S_2O_3$	1.79	0

[a] 0.6 of isotonic. [b] Turned brown in color. [c] Cupric ion reacted with the cyanide ferricyanide reagent. However, visual observation indicated little or no hemolysis.

electrolyte *per se* which determined whether or not the erythrocyte behaved normally, but that the anion was the important moiety.

Urea-Dextrose Complex.—It was mentioned previously that a urea-dextrose complex could be a contributing factor to the ability of dextrose to inhibit urea-induced hemolysis. Since the erythrocyte is impermeable to dextrose, such a complex would serve to reduce the effective concentration of urea. The problem was to demonstrate the existence of such a complex. The small size of both molecules combined with their very high water solubility and low solubility in solvents made it very difficult to detect a complex. Because of the small molecular size, it was not possible to use a semipermeable membrane to follow the complex formation. The high water solubility also made it very difficult to measure changes in concentration in two immiscible solvents as the complex formed. However, it was possible to demonstrate the existence of urea-dextrose complex in water, methanol, and water-methanol mixtures by using the solubility method. Unfortunately, it was necessary to use saturated solutions of dextrose to do so. It would have been much more desirable to use the same concentrations of dextrose that were used in the hemolysis studies.

In the case where the complex was studied in water, a further complication was provided by the large variation in the volume of the various samples caused by the amount of urea included. This large fluctuation in volume made it extremely difficult to determine the concentrations of urea and dextrose in the solutions. Since it was necessary to remove the vials from the bath for the dextrose determination, the complex in water was thermostated at 25° instead of 30°. The former temperature being closer to room temperature made it less likely that a temperature change would disturb the complex.

Figure 1 shows that as urea was added to a saturated solution of dextrose in water, the quantity of dextrose in solution increased. Since the precise concentrations of urea and dextrose were difficult to determine, it was not possible to calculate the equilibrium constant of the complex. The plateau of the curve was used to calculate the stoichiometric ratio of the complex (16). The ratio of urea to dextrose was probably in the neighborhood of

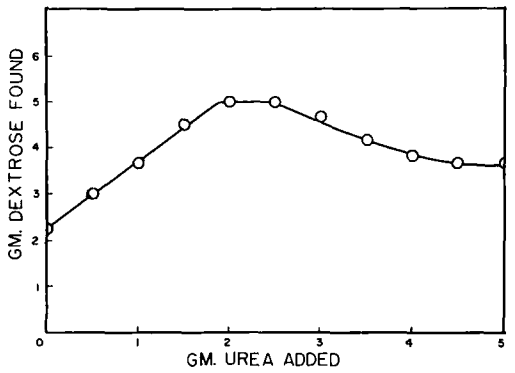

Fig. 1.—Phase diagram of dextrose–urea system in water at 25°.

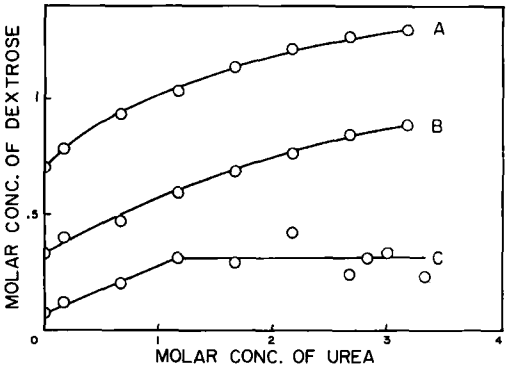

Fig. 2.—Phase diagrams of dextrose–urea systems in methanol–water and methanol at 30°. (Curve *A* is 80% methanol, curve *B* is 90% methanol, and curve *C* is 100% methanol.)

3:1 to 4:1. The uncertainty arose from the difficulty in ascertaining the exact length of the plateau.

The low solubility of dextrose in methanol made it easier to demonstrate the existence of a complex in this solvent. Figure 2 shows the results that were obtained with three concentrations of methanol. In curve A, 80% methanol was used as the solvent; curve B represents 90%; and pure methanol was employed as the solvent in curve C.

Thus, there was little doubt that under certain conditions a urea-dextrose complex did indeed exist. However, because of the aforementioned solubility problems, it was not possible to demonstrate the existence of this complex at low dextrose concentrations in water. If urea did complex with dextrose at low dextrose concentrations this would become a very important factor in the inhibition of urea-induced hemolysis.

SUMMARY

Various aspects of urea-induced hemolysis have been investigated. It was shown that high concentrations of urea cause hemolysis in the presence of isotonic concentrations of other compounds by a sufficient volume of urea molecules diffusing into the cell to cause it to rupture. Therefore, a sufficiently large concentration of any nonpenetrating compound in the medium will inhibit urea-induced hemolysis.

Experimental evidence was obtained which indicates that it is necessary for electrolyte to be present in the medium in order to maintain a normal suspension of the erythrocytes. There is also evidence that the anion may be the important ion of the electrolyte in maintaining the integrity of the cell.

The existence of a urea-dextrose complex has been demonstrated at high dextrose concentrations. If this complex forms at low dextrose concentrations it would be an important factor in the ability of dextrose to inhibit urea-induced hemolysis.

REFERENCES

(1) Javid, M., and Settlage, P., *J. Am. Med. Assoc.*, **160**, 943(1956).
(2) Javid, M., and Anderson, J., *Surg. Forum*, **9**, 686 (1959).
(3) Ponder, E., and Ponder, R., *Acta Haematol.*, **12**, 282(1954).
(4) Williams, H. H., Erickson, B. N., and Macy, I. G., *J. Lab. Clin. Med.*, **26**, 996(1941).
(5) Yeager, J. F., *Quart. J. Exp. Physiol.*, **19**, 219(1929), cited by Ponder, E., "Hemolysis and Related Phenomena," Grune and Stratton, New York, N. Y., 1948, p. 274.
(6) Morita, K., *Shikoku Acta Med.*, **14** (1), 29(1959), cited by *Biol. Abstr.*, **35** (23), 66261(1960).
(7) Jacobs, M. H., and Parport, A. K., *Biol. Bull.*, **65**, 512(1933).
(8) Jacobs, M. H., and Willis, M., *Federation Proc.*, **6**(1), 137(1941).
(9) Hendry, E. B., *Edinburgh Med. J.*, **55** (7), 427 (1948).
(10) Finholt, P., *Norg. Apotekerforen. Tidssk.*, **61**, 384 (1953).
(11) Nelson, N., *J. Biol. Chem.*, **153**, 375(1944).
(12) Somogyi, M., *ibid.*, **195**, 19(1952).
(13) "Merck Index," 7th ed., Merck and Co., Inc., Rahway, N. J., 1960, p. 1545.
(14) Davson, H., and Danielli, J. F., "The Permeability of Natural Membranes," 2nd ed., Syndics of the Cambridge University Press, London, Eng., 1952, p. 58.
(15) Castle, W. B., and Deland, G. A., *Arch. Internal Med.*, **60**, 949(1937).
(16) Higuchi, T., and Lach, J. L., THIS JOURNAL, **43**, 349(1954).

Softening and Liquefaction Temperature of Suppositories

By IVO SETNIKAR and SERGIO FANTELLI

A method is described for testing the softening and liquefaction temperature of suppositories. The data obtained yield the maximum environmental temperature at which suppositories retain sufficient firmness for handling and for ordinary storage. The physical properties of 44 bases were investigated by this new method. The melting point was determined by the open capillary method. Of 27 fatty bases, only 8 present properties which are as satisfactory as or better than those of theobroma oil. Most of the 17 water-soluble bases investigated have very satisfactory firmness, but their melting point is above body temperature so that, for liquefaction, they must dissolve in water drawn from the rectal mucosa.

RECTAL suppositories, if well formulated, should melt, soften, or dissolve when introduced into the rectum and yet retain their firmness and shape in ordinary storage conditions.

Melting, softening, or dissolving may be tested in an apparatus which reproduces the physical conditions of the rectum (1). This apparatus, however, does not give indications as to the firmness of the suppositories. The following characteristics may be examined in order to test firmness.

Melting temperature.—Melting temperature may be determined (*a*) by the open capillary method for fatty substances (*cf.* U.S.P. XVI, p. 926, class II) or by the sealed capillary method (2). Only suppository bases can be tested and the results are influenced by the technique used in the preparation of the capillary (2). (*b*) The suppository may be put in an aqueous environment whose temperature is raised until the suppository melts (3–6). In these conditions only strictly water-insoluble suppositories can be tested. Suppositories containing water-soluble substances soften or dissolve in the aqueous environment irrespective of temperature. (*c*) The temperature of the air surrounding the suppository may be gradually raised until melting. This method was followed by Erbe (7) who used an apparatus described by Bogs (8).

Hardness—Malangeau (3) molded a cylinder with a diameter of 9 mm. of the same composition of the suppository and determined the temperature at which the cylinder collapsed under a weight of 500 Gm. (8 Gm./-mm.^{2}). Münzel (9) and other authors (2, 10, 11) used the penetrometric method described in U.S.P. XIV, p. 693. Büchi and Oesch (12) stated that a suppository, at a temperature of 22°, should not be deformed by a weight of 500 Gm.

The first two methods cannot be used directly on suppositories while the method of Büchi and Oesch can, though it gives only limited information on the maximal temperature at which a suppository still shows sufficient mechanical stability. We describe here a method for determining the softening and melting temperatures directly on suppositories.

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