TABLE I.
Prothrombin Time of Fresh and Stored Human Plasma, Decalcified with Amberlite. Results of Oxalate and Citrate Treatment on This Plasma Passed Through Amberlite to Eliminate Calcium.

Prothrombin Time (sec.)					
Oxalated fresh plasma	1 day stored	Amberlite plasma	1 day stored Amberlite plasma	1 day stored Amberlite plasma + sodium oxalate	1 day stored Amberlite plasma + sodium citrate
12″	15″	13″	13″	18″	18″

substance in blood which protects the P.C.T. against oxidation. It must be a strong reductor. Its destruction by sodium oxalate would explain the slow but effective alteration of stored plasma.

To test this hypothesis the following experiments were carried out. Human blood withdrawn in silicon syringes (Jaques[5]) was passed through Amberlite IR-100-H (Quick[6]) to eliminate all the calcium without using oxalate. Then 0.1 cc of 0.1 M sodium oxalate or 0.125 M sodium citrate to 0.9 cc of plasma were added. A third sample passed through Amberlite (without addition of any other substance) was stored at 5°C. For prothrombin time determination a modified one-stage method of Quick[4] was used.

The results are shown in Table I.

After 24 hours, no change in the prothrombin time of the sample passed through Amberlite was observed. Thus calcium is not a protective element. On the other hand a very important change in the prothrombin time of plasmas passed through Amberlite, and to which oxalate or citrate were subsequently added, was observed. If no calcium was present, both salts must have altered the plasma by another mechanism than by the elimination of calcium. No difference was observed between oxalate and citrate activity, even when 0.125 M solution for the latter was used (0.125 M citrate preserves plasma better than oxalate 0.1 M, when both are used to withdraw blood samples). We postulate the existence of a protective substance, strong reductor, to preserve P.C.T. from oxidation, which is seriously and specifically altered by sodium oxalate and only subsequently by sodium citrate, 0.125 M, when calcium is present. Without calcium, differences of an affinity of this protective factor disappear.

[4] Honorato, C. R., PROC. SOC. EXP. BIOL. AND MED., 1947, **65**, 41.

[5] Jaques, L., *Can. J. Med. Assn.*, 1946, **55**, 26.

[6] Quick, A. J., *Am. J. Physiol.*, 1947, **148**, 212.

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Studies on Polymyxin: An Assay Method for Blood and Urine.*

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Extension of the agar diffusion method[1] for the assay of polymyxin in fermentation liquors and concentrates has resulted in useful methods for the assay of polymyxin in blood and urine. The method to be described is rapid, simple and relatively accurate and has been useful in studies on the absorption and ex-

* Grateful acknowledgment is made to Miss M. E. Schlosser and Mrs. Beverly Gordon for valuable assistance.

[1] Stansly, P. G., and Schlosser, M. E., *J. Bact.*, 1947, **54**, 585.

cretion of polymyxin in experimental animals. It should prove to be equally useful for clinical purposes.

Preparation of seeded plates. Twenty ml of 2% Trypticase-Soy† agar are poured into standard petri plates and allowed to solidify. Four ml of 1.2% agar containing 1% "Tween-80"‡ and 0.5% of a 24-hour, 37°C, Trypticase-Soy broth culture of *Brucella bronchiseptica,* N.R.R.L. B-140, are then spread over the base layer. Prior to use, the plates are placed with lids slightly raised for 45 minutes in a dry 37°C incubator.

Preparation of standard curve for blood. To fresh or citrated normal blood is added an equal volume of 0.2 N HCl. Dry polymyxin hydrochloride is added to an aliquot to give 64 μg per ml.§ Serial twofold dilutions are made using the remainder of the acid-blood as diluent to give a series of standard solutions ranging from 64 to 0.125 μg per ml. To each of 3 sterile filter paper discs (¼ inch diameter, Schleicher & Schuell No. 740E) in a sterile petri dish is added 0.03 ml of one of the 10 standard solutions by means of a 0.1 ml Kahn pipette. One of these replicate discs is placed in each of 3 seeded plates. The remaining standard solutions are treated similarly. Five discs may conveniently be placed on a single seeded plate. Thus, a total of 6 seeded plates are required for a total of 30 discs, representing the 10 standard solutions in triplicate.

The plates containing the discs are then incubated, in a well-humidified incubator, preferably with lids slightly raised, at 37°C for 16-18 hours and the zones measured in mm. The average zone diameter for each group of 3 replicates is determined and a curve relating zone diameter to log of (concentration × 2) constructed on semi-log paper. A typical blood standard curve is illustrated in Fig. 1.

Assay of blood. The manner of collecting blood for assay of polymyxin may be left to the discretion of the individual insofar as the amount of blood removed is concerned and whether the blood is citrated or not. The least amount of blood required is theoretically about 0.02 ml for when diluted with 0.02 ml of 0.2 N HCl, 0.03 ml of solution is available to saturate one disc. If 3 replicates are desired, 0.05 ml of blood is required. Fresh blood may be added directly to the acid or the blood may be citrated and later diluted with the acid. In any case, the saturated discs are treated as described in the preparation of the standard curve. The diameter of the zone is measured in mm and the concentration of polymyxin in whole blood in μg per ml read off the standard curve.

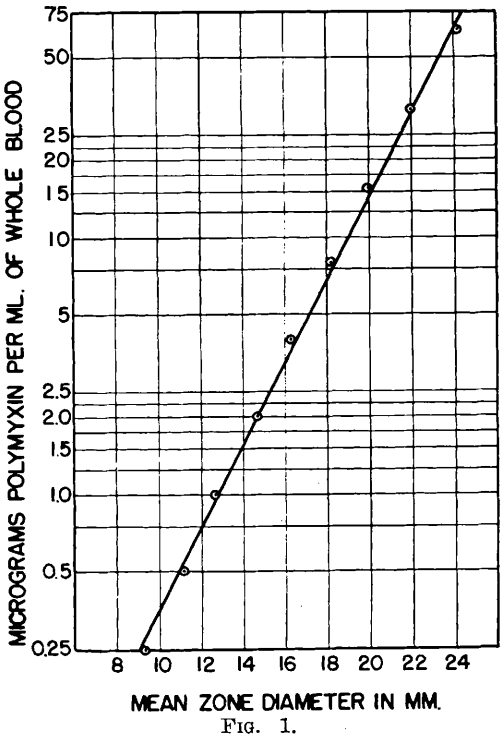

FIG. 1.
Standard curve for assay of polymyxin in blood.

Preparation of standard curve for urine. Serial twofold dilutions of polymyxin from 256 to 0.125 μg per ml are prepared in glycine-hydrochloric acid buffer of pH 2.‖ The subsequent steps are as described for the prepara-

† Baltimore Biological Laboratories.

‡ Polyoxyalkalene derivative of sorbitan monooleate, Atlas Powder Co.

§ Calculated on the basis of 2000 units per mg for "pure" polymyxin. See *Bull. Johns Hopkins Hosp.,* 1947, **81**, 43.

‖ One volume of a solution consisting of 7.505 g of glycine and 5.85 g of NaCl per liter of water is added to an equal volume of 0.1 N HCl.

tion of the blood standard curve.

Assay of urine. The sample of urine is diluted with an equal volume of glycine-hydrochloric acid buffer of pH 1.4¶ and subsequently treated as indicated for the assay of blood with the exception that the standard curve for urine is, of course, used to determine the potency in unknown urines.

Discussion. In the assay of fermentation liquors and concentrates for polymyxin,[1] *E. coli* (MacLeod strain) was recommended as the test organism and the two-dose method of determining potency was suggested as more desirable than the standard curve method. *Brucella bronchisepticus,* N.R.R.L. B-140, was recently introduced by Benedict and Stodola[2] as a more desirable organism for the assay of polymyxin. This organism has the advantage of requiring only one temperature for the incubation of the seeded plates and of having a slower rate of growth than *E. coli.* The latter property of *Br. bronchiseptica* permits the standard curve method to be used with less risk of incurring serious error.[1]

Several points are worth considering in connection with the blood assays. In the first place, blood should either be untreated as far as anticoagulants are concerned or citrated. It should not be oxalated. In our experience, oxalated blood frequently results in a viscous solution upon the addition of an equal volume of 0.2 N HCl and is, therefore, difficult to pipette. This does not occur, or only rarely, with either untreated or citrated blood. Under these conditions, solutions of acid blood remain fluid indefinitely at room temperature or in the refrigerator.

In the second place, solutions of polymyxin in 50% acid blood are stable indefinitely in the refrigerator. Therefore, when mixed with acid, the blood samples may be stored until convenient to assay. The series of polymyxin-blood standards may also be stored in the refrigerator indefinitely. It is our practice to prepare a standard curve daily, although this may not be essential if experience indicates that standard curves do not deviate appreciably from day to day.

A third point of interest is that a 50% acid solution of normal blood, prepared as described, does not produce any inhibition zone when applied to a seeded plate and incubated as described. This is true both of animal bloods (mouse and dog) and of human blood (24 miscellaneous samples tested.**) The pH of such solutions is in the neighborhood of 3.75. If stronger acid is used so as to result in a pH of 2, slight but definite inhibition zones are produced which, of course, would complicate the determination of potency in unknown bloods. It is noteworthy that buffer or urine solutions of polymyxin at pH 2 do not exhibit this phenomenon.

Finally, the nature of the assay method precludes the necessity for strict aseptic precautions in the manipulation of the blood for assay and in the various steps of the procedure.

The only point of special interest in connection with the assay of urine is that urine samples are diluted with an equal volume of pH 1.4 buffer, whereas the standard curve is prepared with buffer of pH 2. The reason for this is that the addition of pH 1.4 buffer to urine of our experimental dogs generally results in a pH of about 2, thus bringing it in line with the pH of the standard solutions. The effect of pH of polymyxin solutions on zone diameter has been discussed.[1]

The following examples illustrate the type of data obtained by the methods described.†† A single subcutaneous dose of 1 mg/K of polymyxin hydrochloride in each of 5 mice resulted in a level of 2γ/ml in pooled blood at the end of 15-30 minutes, declining to about 1 γ/ml at the end of one hour; a single intravenous dose of 5 mg/kg in a dog resulted in a blood level of 5 γ/ml of blood at the end of 1½ hours. A total of 90 ml of urine was col-

¶ 2.5 volumes of a solution consisting of 9.38 g of glycine and 7.32 g of NaCl per liter of water are added to 7.5 volumes of 0.125 N HCl.

[2] Benedict, R. G., and Stodola, F. H., *J. Bact.,* 1948, **55**, 286.

** Thanks are due Dr. N. G. Chaucer and Miss L. P. Morrison of the Medical Department for their cooperation in supplying samples of human blood for testing.

†† We wish to thank Dr. J. T. Litchfield, Jr., of these Laboratories for permission to quote these results.

lected during this time which assayed 220 γ/ml, thus accounting for about 30% of the polymyxin administered. Details of these and other studies will be presented at a later time.

Summary. A filter paper disc method for the determination of polymyxin in blood and urine is described. Using ¼-inch diameter discs, 0.25 μg of polymyxin per ml of blood may be determined in triplicate with samples of blood as small as 0.05 ml.

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Effect of Digitoxin on Contractility of Ventricular Muscle Strips.

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In a summary of his extensive studies on digitalis action, Gold[1] has concluded from the reactions of the papillary muscle of the cat heart that increased systolic force caused by direct action on heart muscle may be taken as the primary mechanism by which digitalis relieves heart failure. Variability of digitalis effect on muscle strips is indicated in numerous reports. Cattell and Gold[2] in carrying out earlier experiments selected as a "therapeutic" dose concentrations which produced augmentation of beat in approximately 50% of trials. Salter and White[3] who used the same procedure found that fresh muscles failed to respond to relatively high concentrations of ouabain, and in a later paper[4] they described alteration of the Ringer solution in addition to prolonged stimulation, so that the muscle might be more readily "fatigued." In their study of digoxin on strips of turtle ventricle, Wedd, Blair, and Dwyer[5] rarely observed pronounced or sustained increase in amplitude of contraction and argued that the constant shortening of systole, electrical and mechanical, might be a factor in reducing contraction height. It is apparent from such studies that augmentation of contractility by digitalis is not due to primary stimulation of that function, such as occurs with epinephrine or veratrine. Further observations indicate that to be enhanced contractility must have first been impaired and experiments to be cited suggest that such effect will occur only after some alteration of muscle metabolism. The present report deals with the effect of digitoxin on strips of turtle ventricle when contractility has been diminished by various physical and chemical means.

Method. Details of the method used in this laboratory to study drug effects on heart muscle have been described.[5] Ventricular strips suspended in a bath are rhythmically stimulated by condenser discharges and contraction recorded on a drum. The Ringer solution was phosphate buffered and had a pH of 7.2; no carbonate or sugar was present. The usual driving rate was 13.5 per minute. Tension was adjusted to record maximum contraction; it was altered if necessary during the control period, but not after the drug was added. It made no difference whether digitoxin was added to the original bath or a fresh one containing the drug was substituted. A concentration of 1:2 million was found generally satisfactory and was used in all experiments.* For each group in which con-

1 Gold, H., *J. Am. Med. Assn.*, 1946, **132**, 547.

2 Cattell, McK., and Gold, H., *J. Pharm. Exp. Therap.*, 1941, **71**, 114.

3 Salter, W. T., and White, W. F., *Fed. Proc.*, 1946, **5**, 200.

4 White, W. F., and Salter, W. T., *J. Pharm. Exp. Therap.*, 1946, **88**, 1.

5 Wedd, A. M., Blair, H. A., and Dwyer, G. K., *J. Pharm. Exp. Therap.*, 1941, **72**, 394.

* The digitoxin used in these experiments was Digitalline Nativelle, generously supplied by the Varick Pharmacal Co.