THE ASSAY OF POLYMYXIN AND ITS PREPARATIONS

By R. E. A. Drey, G. E. Foster and G. A. Stewart

From The Wellcome Chemical Works, Dartford, Kent

Received June 17, 1955

In 1947 Benedict and Langlykke[1] reported the antibacterial properties of cultures of *Bacillus polymyxa* and Stansly, Shepherd and White[2] announced the isolation from such cultures of a new antibiotic, which was named polymyxin. Almost simultaneously Ainsworth, Brown and Brownlee[3], working independently, obtained aerosporin from media in which *Bacillus aerosporus* had grown. These two antibiotics proved to be polypeptides of similar composition. It was subsequently found that different strains of *B. polymyxa* gave rise to a series of closely related antibiotics and this led to a re-investigation[4] of the taxonomic derivation of *B. aerosporus* and *B. polymyxa*, the two being found identical and the latter the specific name.

Five antibiotics known as polymyxins A, B, C, D and E have now been isolated and characterised by the amino-acids formed from them on hydrolysis. Table I summarises data by Jones[5] and Catch, Jones and Wilkinson[6,7].

TABLE I

The amino-acid components of the polymyxins

Polymyxin	D-Leucine	D-Phenyl-alanine	L-Threonine	D-Serine	L-α:γDiamino-butyric acid
A	+	−	+	−	+
B	+	+	+	−	+
C	−	+	+	−	+
D	+	−	+	+	+
E	+	−	+	−	+

Chemical analysis of the hydrolysates established that in addition to the amino-acids a fatty acid, identified by Wilkinson[8] as D-6-methyl-octan-1-oic acid, is present in all polymyxins. Polymyxin A, originally known as aerosporin, has the same qualitative amino-acid composition as polymyxin E but Jones[9] was readily able to differentiate the intact antibiotics from each other by paper chromatography.

Brownlee, Bushby and Short[10,11] studied the chemotherapeutic and pharmacological properties of the polymyxins and showed that polymyxins A, C and D all cause severe proteinuria in animals when administered by injection and were too toxic for clinical use. Polymyxins B and E, however, are less nephrotoxic and their sulphates have been used in medicine. Polymyxin B sulphate is now established as the most widely used polymyxin preparation in medical practice and, on this account, its identification and assay have become of importance. In the present communication methods, which have been successfully used in our laboratories for some years, are described in the hope that the information may be of assistance to other workers in this field.

Tests for Identification and Purity

Identification of the polymyxins is best achieved by the methods of Jones[5,6,7] and his colleagues using paper partition chromatography for examination of the intact and hydrolysed antibiotics. Hydrolysis is conveniently carried out by a process subsequently employed by Foster, Macdonald and Jones[12] in their work on the ergot alkaloids. For polymyxin B sulphate the following tests are appropriate.

(*a*) Shake together four volumes of *n*-butanol, one volume of glacial acetic acid and five volumes of water, allow to separate and transfer the lower layer to a dish placed on the floor of an air tight chamber. When the atmosphere of the chamber is saturated, suspend in it a strip of Whatman No. 1 filter paper on which has been placed 0·005 ml. of solution, prepared by dissolving 5 mg. of the polymyxin sulphate in 0·5 ml. of water. Develop the chromatogram with the upper layer of the mixed solvents for about 16 hours. Dry the chromatogram in air, spray with a 0·1 per cent. solution of ninhydrin in water saturated *n*-butanol and heat in an air oven at 90° C. for about 5 minutes. The developed chromatogram shows only one spot characteristic of polymyxin B.

(*b*) Dissolve 5 mg. in 1 ml. of 5N hydrochloric acid. Transfer the solution to a small ampoule, seal and heat at 120° C. for 6 hours. Transfer the resulting solution to a small evaporating dish and evaporate to dryness on a steam bath. Continue to heat until the residue no longer gives off hydrogen chloride. Dissolve the residue in 0·5 ml. of water and place 0·005 ml. of the solution on a strip of Whatman No. 1 filter paper. As a control, prepare a solution containing 10 μg. each of leucine, phenylalanine, threonine and serine per 0·005 ml. Similarly place 0·005 ml. of this control solution on the same strip. Develop the chromatogram, as described under identification test (*a*) and locate the spots by means of the ninhydrin reagent. Definite spots for leucine, phenylalanine, threonine and $\alpha:\gamma$-diaminobutyric acid should be identified. The latter is indicated by a slow moving spot ($R_F = 0{\cdot}1$) near the starting line. No spot for serine should be present.

These tests are also applicable to polymyxins A, C, D and E, the amino-acids formed on hydrolysis being indicated in Table I. Salts of the polymyxins afford reactions characteristic of the acids present.

When the antibiotics are tested for purity by the chromatographic technique it has been found advantageous to make tests with increasing amounts (5, 10, 15 μl.) of their 1 per cent. aqueous solutions on the paper. For the examination of bacitracin in the intact state a mixture of equal volumes of acetone and water is a suitable developing solvent. The sensitivity of these tests may be increased by using the modified ninhydrin reagent proposed by Lewis[13]. Separation of phenylalanine and leucine on the chromatograms of the hydrolysates is often incomplete but they may be distinguished by the different coloured spots which they give with the ninhydrin reagent.

Biological Assay of Polymyxin B Sulphate

A plate diffusion method for the assay of polymyxin using a strain of *Escherichia coli* as test organism was first described by Stansly and

Schlosser in 1947[14]. Since *Escherichia coli* grows very rapidly and polymyxin diffuses slowly through agar it was found necessary to assay by incubating the plates for 16 to 18 hours at 25° C., followed by a further 6 hours at 37° C., in order that clearly defined zones of inhibition could be measured. Benedict and Stodola[15] found that *Brucella bronchiseptica*, NRRL Strain B-140, was a more suitable test organism since it showed the same order of sensitivity to polymyxin as *Escherichia coli* and grew more slowly. Hence it was possible to assay by incubation at one temperature only, namely, 37° C., for 14 to 16 hours. Existing methods of assay were reviewed in 1949 by Reese and Eisenberg[16]. The present United States Food and Drug Administration's (F.D.A.) schedule for the assay of polymyxin[17] is largely based on the work of Stansly and Schlosser, and of Benedict and Stodola. The assay plates contain a base layer of pancreatic digest of casein, papain digest of soybean, sodium chloride, dipotassium phosphate and dextrose in a 2 per cent. agar gel. The seed layer medium is similar to that of the base layer with a reduction in the agar content to 1·2 per cent. and with the addition of Tween 80. The *p*H of both layers is 7·3. The organism is a 24-hour culture of *Brucella bronchiseptica*, American Type Culture Collection 4617, incubated at 32 to 35° C. Whilst the original workers used a glycine-hydrochloric acid buffer at *p*H 2·0 for preparing the solutions of polymyxin to be assayed, the present official schedule requires the use of a phosphate buffer at *p*H 6·0.

The method employed in our laboratories for the assay of polymyxin is in principle similar to that described by Benedict and Stodola[15] but differs in experimental detail.

Assay Procedure

The assay plate consists of a 7 in. × 7 in. sheet of good quality window glass framed in 1 in. × ¼ in. duralumin. A loose cover of 22 S.W.G. duralumin is provided into which is inserted a sheet of blotting paper held in position by brass wires running along its length. The blotting paper absorbs any moisture from the medium during the period of incubation.

The organism used is *Brucella bronchiseptica*, Wellcome Culture No. 385, maintained on 7-ml. slopes of nutrient agar* in 20-ml. screw-capped bottles. The culture is transferred to a fresh slope once each week. For the assay a "loopful" is placed in 7 ml. of a nutrient broth† 48 hours previously and incubated at 37° C. Two ml. of this culture is added to 140 ml. of the nutrient agar at 47° C. and the bulk inoculum is then poured into a levelled assay plate. Cavities may be cut in the agar using an 8-mm. cork-borer, after which they are filled with 0·15 ml. of polymyxin solution, or "fishspine" refractory insulating beads No. 2, size 4 mm. × 3·7 mm. diameter[18], may be used to pick up by capillarity 0·017 ml. of the solution of polymyxin, and the beads containing the solution then applied to the surface of the uncut nutrient agar.

* A protein hydrolysate to which horse muscle extract, sodium chloride and 1·2 g. of New Zealand agar per litre are added.

† A papain digest of horse meat.

The polymyxin standard employed is a sub-standard assayed in terms of the proposed British standard for polymyxin B, obtainable from the Department of Biological Standards, National Institute for Medical Research, London, N.W.7. The latter has a potency of 7871 units per mg. when assayed in terms of the United States Food and Drug Administration's Standard of polymyxin B (7700 F.D.A. u/mg.).

All samples are diluted before assay with a glycine-hydrochloric acid buffer at *p*H 2·0. If the cavity-plate technique is used the standard is prepared in solution at two concentrations, namely 400 units per ml. and 100 units per ml. respectively, and the test sample is prepared at similar concentrations on the basis of the potency assumed for it. If the "fish-spine" bead method is employed then the respective concentrations of the standard and test preparations should be 1000 units per ml. and 250 units per ml.

The four solutions are applied to the assay plate in the order determined by any 2 + 2 latin square design. Diffusion of the polymyxin from the centres of application is allowed to proceed for 5 hours at room temperature, during which time the growth of the *Brucella bronchiseptica* is considerably retarded. The plates are then incubated for 10 hours at 37° C., and the zones of inhibition read after magnifying them 15·5-fold. Even with this high magnification the edges of the zones are clearly defined.

The potency of a sample is usually derived as the statistical mean calculated from the data obtained from two assay plates, giving 8 responses at each dose.

Assay of Certain Pharmaceutical Preparations

This method may be employed for the assay of pharmaceutical preparations containing polymyxin.

Polymyxin B Sulphate Otic Solution

The otic solution is a preparation containing 10,000 units of polymyxin B sulphate per ml. in propylene glycol acidified with Acetic Acid B.P. It is highly effective against *Pseudomonas aeruginosa* commonly found in ear infections. The preparation may be assayed simply by dilution with glycine-hydrochloric acid buffer (*p*H 2·0) to the concentrations of polymyxin required for the assay. At these concentrations the other substances present in the solution do not interfere. The results of the assay of 5 recent samples are presented in Table II.

Ointment Containing Polymyxin B Sulphate and Bacitracin

The ointment contains 10,000 units of polymyxin B sulphate and 500 units of bacitracin per g. of a soft paraffin base. Since bacitracin is active against gram-positive micro-organisms the ointment has a very wide bacterial spectrum and may be used topically for many infections with an extremely low risk of obtaining bacterial resistance.

(*a*) *Assay for polymyxin B sulphate.* About 1 g. of the ointment is accurately weighed in a centrifuge tube. 10 ml. of chloroform and 17 ml.

TABLE II

ASSAY OF OTIC SOLUTION OF POLYMYXIN B SULPHATE
(Labelled potency, 10,000 units per ml.)

Sample from batch	Found Potency ÷ Expected Potency (per cent.)	Limits of error ($p = 0{\cdot}95$) of Found Potency ÷ Expected Potency (per cent.)
1	95·1	82·9–109·0
2	104·8	97·0–113·4
3	94·2	85·6–103·6
4	98·7	88·7–109·9
5	102·5	91·3–115·2

of glycine-hydrochloric acid buffer (*p*H 2·0) for each g. of ointment are added. The tube is stoppered and shaken vigorously for one hour in an automatic shaker and the contents of the tube are then centrifuged. The chloroform layer is discarded and a further 10 ml. of chloroform is added and the extraction is repeated.

The final solution of the polymyxin in glycine-hydrochloric acid buffer is assayed by the method previously described using cavity plates.

(*b*) *Assay for bacitracin.* The extraction procedure given for the extraction of polymyxin B sulphate is employed except that 50 ml. of phosphate buffer (*p*H 6·8) is used in place of the 17 ml. of glycine-hydrochloric acid buffer for each g. of ointment. The solution of bacitracin in phosphate buffer is assayed in cavity plates by a method similar to that given for the assay of polymyxin, using the American National Type Culture No. 7743 of *Micrococcus flavus* as test organism. The organism is maintained on nutrient agar slopes (similar to that described for the assay of polymyxin) which are sub-cultured weekly. 48 hours before the assay one "loopful" is inoculated into 7 ml. of nutrient broth and 3 ml. of this 48-hour culture is added to 140 ml. of nutrient agar and poured into a levelled assay plate.

The standard of bacitracin at present employed by us is a batch from Société Industrielle pour la Fabrication des Antibiotiques, containing 54·5 units per mg.

The results obtained on both polymyxin and bacitracin contents of five samples from recently manufactured batches of the ointment are given in Table III.

TABLE III

ASSAY OF AN OINTMENT CONTAINING POLYMYXIN B SULPHATE AND BACITRACIN
(Labelled potency, 10,000 units of polymyxin B sulphate and 500 units of bacitracin per g. of ointment)

Sample from batch	Polymyxin content		Bacitracin content	
	Found potency÷ Expected potency (per cent.)	Limits of error ($p = 0{\cdot}95$) of Found potency÷ Expected potency (per cent.)	Found potency÷ Expected potency (per cent.)	Limits of error ($p = 0{\cdot}95$) of Found potency÷ Expected potency (per cent.)
1	100·3	95·7–105·2	102·2	97·0–107·8
2	97·5	93·0–102·3	96·0	92·5– 99·6
3	97·6	91·2–104·6	102·8	93·6–113·2
4	101·4	97·6–105·4	103·3	96·0–110·9
5	108·1	102·1–114·5	98·9	95·3–102·7

Chemical Estimation of Polymyxin

While numerous methods have been described for the chemical determination of proteins[19], little work of a comparable nature has been carried out on the assay of naturally occurring polypeptides, particularly those of a bacterial origin. Polymyxin B has been estimated by the ninhydrin reaction[20] and fractions of this antibiotic obtained in counter-current distribution experiments[21] have been estimated by measurement of their ultra-violet absorption at $\lambda = 259$ mμ. Other methods which are available for estimation of polypeptides include elementary analysis, particularly determination of nitrogen by the method of Kjeldahl, and colorimetric assay by the biuret reaction. These methods have the disadvantage either of being insufficiently specific or of lacking reproducibility or both.

A search was therefore made for a method in which these disadvantages would be reduced to a minimum and experiments indicated that gravimetric estimation with phosphotungstic acid, taken in conjunction with the results of chromatographic examination of the material would achieve this object. The proposed method is of limited application should the results of chromatographic examination reveal the presence of more than traces of impurities which give insoluble phosphotungstates. In this case the phosphotungstate factor must be determined for the actual batch of polymyxin present in the preparation under examination. None the less, in our experience with polymyxins and bacitracin, the method is capable of giving reproducible results and is applicable not only in the presence of non-nitrogenous substances but also when the polypeptide is accompanied by ammonium salts, nitrates and organic compounds containing one primary amino-group only, such as monoamino-acids. It is also applicable to pharmaceutical preparations of polypeptides and may be regarded as a useful adjunct to the biological method.

As we could find no publication describing the use of phosphotungstic acid for the assay of polypeptides of bacterial origin, an investigation was made to establish the optimum conditions of precipitation, washing and drying of the phosphotungstates. The effects of systematic changes in the following variables were studied in turn, (1) acidity of precipitation medium, (2) quantity of precipitant, (3) volume of liquid to be filtered, (4) volume of wash liquid, (5) acidity of wash liquid and (6) conditions for drying of the phosphotungstates. It was found that the phosphotungstates are virtually insoluble in water (e.g., the solubility of polymyxin B phosphotungstate is 0·0011 per cent. at 20° C.) and are even less soluble in acid medium. They are, however, soluble in organic solvents such as methanol, ethanol and acetone.

A 10 per cent. w/v solution of choline chloride in 2 per cent. w/v sulphuric acid proved a useful reagent for testing washings for the presence of phosphotungstic acid, one part of acid in 50,000 parts of solution being detected. Experiments using several batches of AnalaR phosphotungstic acid proved that the results were consistent when this quality of acid was employed. The precipitates are unaffected by heating for prolonged periods at 100 to 110° C. and are readily obtained in the anhydrous state.

The following general method was adopted for polymyxin B sulphate, polymyxin E sulphate and bacitracin.

Procedure

Transfer about 65 mg. accurately weighed to a 50-ml. beaker and dissolve in 12 ml. of water. Add 5 ml. of dilute sulphuric acid and 6 ml. of a 5 per cent. w/v solution of AnalaR phosphotungstic acid in water, previously filtered through a Whatman No. 5 filter paper. Allow to stand for 10 minutes, stirring intermittently, and filter with the aid of suction through a tared No. 4 sintered glass crucible. Completely transfer the precipitate to the crucible with three portions (20 ml. each) of 2 per cent. w/v sulphuric acid and wash the residue with two portions (20 ml. each) and one portion (10 ml.) of water. Maintain the precipitate in a wet condition during the entire filtering and washing operations and allow it to be dried by suction only after the final washing. Dry the precipitate either at 50° C. for 2 hours or over phosphorus pentoxide *in vacuo* at room temperature for 4 hours; finally heat at 110° C. for 1½ hours, cool and weigh.

Phosphotungstate Factor

For the purpose of assay work a factor equal to the weight of anhydrous polypeptide equivalent to 1 g. of anhydrous phosphotungstate complex, was carefully determined with samples of the purest antibiotics which we had available. Our results are summarised in Table IV.

TABLE IV

Polypeptide	Temperature at which "loss on drying" was determined	Potency of anhydrous material (u./mg.)	Phosphotungstate factor
Polymyxin B sulphate ..	37° C.	8150	0·265
Polymyxin E sulphate ..	37° C.	8430	0·259
Bacitracin	60° C.	76·1	0·322

The Assay of Otic Solution of Polymyxin B Sulphate (10,000 *u./ml.*)

The chemical assay has been applied to the assay of production batches of otic solution, of the composition described under the section on biological assay. For this purpose the following procedure was found to be satisfactory.

Transfer an accurately weighed quantity of otic solution, equivalent to about 360,000 units (approximately 37·5 g.) to a 250-ml. beaker. Add 100 ml. of water and 20 ml. of dilute sulphuric acid with stirring, followed by 5 ml. of a previously filtered 10 per cent. w/v solution of AnalaR phosphotungstic acid in water. Allow to stand for 30 minutes and filter with the aid of suction through a tared No. 4 sintered glass crucible. Complete the assay as described under the assay of polymyxin B sulphate. Calculate the potency of the otic solution using the following formula:—

Potency in units per ml. $= \frac{1000\ \mathrm{D.F.P.W.}}{\mathrm{M}}$, where D = specific gravity

of solution; F = phosphotungstate factor; P = potency of polymyxin, used in preparation of solution, expressed as units per mg. of anhydrous material; W = weight in g. of anhydrous phosphotungstate obtained in assay; M = weight in g. of sample taken for assay.

When the above procedure is carefully followed assay results usually fall within 99 to 101 per cent. of the expected value. For routine checking of strengths of solutions during manufacturing operations it may be more convenient to use the standard phosphotungstate factor of 0·265 without determination of the factor for each batch of polymyxin B sulphate used. In our experience the results obtained by this modification are often close to the theoretical figures but an error of not more than 5 per cent. may occur.

Assay of Tablets of Polymyxin B Sulphate (500,000 *units*)

The procedure may be applied to tablets providing the tablet contains no base which will interfere with the assay. For tablets containing 500,000 units of polymyxin B sulphate per product, we have proceeded as follows.

Take a sample of 10 tablets and determine the average weight. Powder the sample and transfer an accurately weighed quantity, equivalent to about 400,000 units of polymyxin B sulphate, to a small beaker. Add 10 ml. of water and 3 ml. of dilute sulphuric acid and triturate the powder with the liquid. Filter through a Whatman No. 2 paper, washing the residue and filter with two 10-ml. portions of 2 per cent. w/v sulphuric acid and two 5-ml. portions of water. Add to the filtrate 5 ml. of dilute sulphuric acid and 8 ml. of a previously filtered 5 per cent. w/v solution of AnalaR phosphotungstic acid in water. Complete the assay as described under the assay of polymyxin B sulphate.

Discussion

Development of assay procedures for the polymyxins has followed a course similar to that experienced with many other antibiotics. The antibacterial properties of polymyxin and its preparations are of major importance and it will therefore be necessary to depend upon the microbiological assay until such time as physical or chemical techniques of equal specificity are available. A serial dilution method of assay, originally used, was abandoned for the plate diffusion method, now widely accepted, with the advantages of accuracy and simplicity. The results given in Tables II and III indicate that polymyxin B may now be assayed in conformity with the general requirements of the B.P. 1953 for the biological assay of antibiotics; namely that the estimated potency shall not be less than 90 per cent. and not more than 111 per cent. of the stated potency and the limits of error of the estimated potency ($p = 0{\cdot}95$) shall lie within 80 and 125 per cent. of the stated potency.

The chemical assay, employing phosphotungstic acid, was adopted by us after the use of other precipitating agents, such as ammonium reineckate and picric acid, had been explored. Conditions for the precipitation of polymyxin B as the phosphotungstate were carefully investigated

and the method, as described, may have wider applications for water-soluble polypeptides, particularly those of bacterial origin. It is intended that the chemical assay should supplement and not replace the microbiological assay which, at this stage of our knowledge, is fundamental. In order to obtain the most accurate results it is recommended that the phosphotungstate factor for each batch of polymyxin B sulphate should be determined, but the standard factor given may be employed usefully for much work involving the study of manufacturing operations. Polymyxin B phosphotungstate dissolves in some organic solvents and, for this reason, the application of the chemical assay to liquid preparations needs careful study. For example, it is necessary to dilute the otic solution to the extent described in order to prevent low assay figures being obtained owing to the solubility of the precipitate in the presence of propylene glycol, a constituent of the preparation.

In recent years studies on the uptake of polymyxin by bacterial cells have been reported and some of the data provided may form the basis of further assay procedures. Newton[22] and Few and Schulman[23] have shown that bacteria sensitive to polymyxin liberate substances which absorb ultra-violet light at $\lambda = 260$ mμ, when polymyxin is taken up by them. They found that within a suitable range of polymyxin concentrations the amount of light absorbing material released into the surrounding medium was a function of the amount of antibiotic present. Newton[24] has also shown that when washed cells of *Pseudomonas æruginosa* are suspended in a weak solution of *N*-tolyl-α-naphthylamine-8-sulphonic acid the addition of polymyxin produces an immediate fluorescence, which as a percentage of the maximum is linearly related to the dose of polymyxin added to the suspension of cells. These methods would appear to repay further investigation.

The methods of assay described in this paper deal especially with polymyxin B but there is little doubt that they may be extended for use with the other polymyxins. In particular, we have applied both the microbiological and the phosphotungstate assay to samples of polymyxin E sulphate with satisfactory results. No official standard preparation of polymyxin E is yet available and we have found it convenient, under our experimental conditions, to assay this antibiotic against the provisional British Standard polymyxin B sulphate and to express the potency in terms of units of polymyxin B sulphate.

It has been emphasised that these polypeptides should be substantially free from interfering impurities if the phosphotungstate assay is to yield reliable results. We have employed paper chromatography as a test of purity but it must be remembered that "chromatographically pure" is a relative term and workers must be for ever vigilant for the presence of unsuspected impurities. For example, we have been unable to separate polymyxins B and E in the intact state on paper chromatograms in spite of trials with a large number of developing solvents, while the composite nature of polymyxin B has been indicated by the recent work of Hausmann and Craig[21] using their counter-current distribution technique.

SUMMARY

1. The development of the series of antibiotics known as polymyxins is reviewed.

2. Details are given for the identification of the polymyxins by paper partition chromatography.

3. A plate diffusion microbiological assay using *Brucella bronchiseptica* as test organism has been developed and its application to the assay of pharmaceutical preparations is described.

4. Results are recorded to show that the proposed assay method conforms to the general limits of error of the B.P. 1953 for the biological assay of antibiotics.

5. A chemical method is described for the determination of polymyxin B by precipitation as phosphotungstate.

6. The application of the chemical assay to otic solution and tablets of polymyxin B sulphate is described.

7. Reference is made to the extension of the microbiological and chemical assay to other polymyxins, particularly polymyxin E. Some preliminary work with bacitracin is also described.

REFERENCES

1. Benedict and Langlykke, *J. Bact.*, 1947, **54**, 24.
2. Stansly, Shepherd and White, *Johns Hopk. Hosp. Bull.*, 1947, **81**, 43.
3. Ainsworth, Brown and Brownlee, *Nature, Lond.*, 1947, **160**, 263.
4. Brownlee and Jones, *Biochem. J.*, 1948, **43**, xxv.
5. Jones, *ibid.*, 1948, **43**, xxvi.
6. Catch, Jones and Wilkinson, *ibid.*, 1948, **43**, xxvii.
7. Catch, Jones and Wilkinson, *Ann. N.Y. Acad. Sci.*, 1949, **51**, 917.
8. Wilkinson, *Nature, Lond.*, 1949, **164**, 622.
9. Jones, *Ann. N.Y. Acad. Sci.*, 1949, **51**, 909.
10. Brownlee, Bushby and Short, *ibid.*, 1949, **51**, 952.
11. Brownlee, Bushby and Short, *Brit. J. Pharmacol.*, 1952, **7**, 170.
12. Foster, Macdonald and Jones, *J. Pharm. Pharmacol.*, 1949, **1**, 808.
13. Lewis, *Biochem. J.*, 1952, **52**, 330.
14. Stansly and Schlosser, *J. Bact.*, 1947, **54**, 585.
15. Benedict and Stodola, *ibid.*, 1948, **55**, 286.
16. Reese and Eisenberg, *Ann. N.Y. Acad. Sci.*, 1949, **51**, 968.
17. Compilation of Regulations for tests and methods of assay and certification of antibiotic and antibiotic containing drugs. United States Department of Health, Education and Welfare, Food and Drug Administration, Vol. I, Sec. 141.112, p.B.19.
18. Stewart and Thorp, unpublished data.
19. Kirk, *Advances in Protein Chemistry*, 1947, **3**, 139; Taylor, *The Proteins*, Neurath and Bailey, Academic Press, New York, 1953, I, 14.
20. Newton, *Biochem. J.*, 1953, **55**, x.
21. Hausmann and Craig, *J. Amer. chem. Soc.*, 1954, **76**, 4892.
22. Newton, *J. gen. Microbiol.*, 1953, **9**, 54.
23. Few and Schulman, *ibid.*, 1953, **9**, 454.
24. Newton, *ibid.*, 1954, **10**, 491.

DISCUSSION

The paper was presented by MR. G. A. STEWART.

DR. G. BROWNLEE (London) said that as polypeptides of identical chemical composition could have different activities there would be grave danger if the chemical method were ever practised in the absence of the microbiological method.

MR. T. D. WHITTET (London) suggested that it would be helpful if the authors could extend their work to studying the stability of various preparations as there were conflicting reports in the literature.

MR. D. JACK (Harrow) emphasised the importance of the strain of *Brucella bronchiseptica* used in the assay. Using the British strain he had found that he could only estimate concentrations of between 100 and 400 u./ml. With the American strain it was possible to determine concentrations of the order of 25 to 50 u./ml. That was important in the work being undertaken because it was a low potency preparation.

DR. G. E. FOSTER (Dartford) said that there was information available concerning the stability of polymyxin and its preparations.

It was rather unfortunate that the first official standard for polymyxin had to appear in the United States Pharmacopeia XV. Unfortunately, there was no official British standard preparation of polymyxin B sulphate available at the time of the preparation of a monograph for the B.P.C. 1954.

MR. D. STEPHENSON (Dartford) said that polymyxin B sulphate in freeze dried powder retains its potency for many years. Aqueous solutions may be autoclaved with losses of activity not greater than 10 per cent. Compressed tablets of polymyxin E sulphate and polymyxin B sulphate have retained full potency for about three years. The otic solution and the anhydrous ointment may be kept at room temperature for two years without significant loss of activity. It was desirable to prepare ointments at as low a temperature as possible because if soft paraffin were heated during the incorporation of the antibiotics, some loss of activity occurred.

MR. G. A. STEWART, in reply, said that he had not found it necessary to use a more sensitive strain than the Wellcome culture for assaying preparations of polymyxin. However when very low concentrations of antibiotic have to be estimated one would seek for the most sensitive strain of test organism with which to conduct the assay. Results with the American strain of *Brucella bronchiseptica* were in agreement with those obtained by him when assaying similar polymyxin preparations.