CHEMICAL AND MICROBIOLOGICAL ASSAY OF PENICILLIN V

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Chemical Assay

This account is concerned firstly with the investigation of the iodimetric assay of penicillin V (phenoxymethylpenicillin), especially of samples associated with the preparation of a pure assay standard, and secondly with a method for the assay of penicillin V in the presence of penicillin G (benzylpenicillin).

Experimental and Results

Description of Methods of Assay

Two iodimetric methods were used, one involving alkali and the other penicillinase, as inactivating agents. The reader is referred to descriptions by Alicino[1] and Ortenblad[2] for further information about iodimetric assay.

Alkali inactivation method. Approximately 100 mg. of sample were accurately weighed, dissolved in 100 ml. of 0·067M *p*H 7·0 phosphate buffer solution and made up to 500 ml. with distilled water. *Blank.* To a 20 ml. aliquot of the sample solution was added 20 ml. of 0·01N iodine in 20 per cent. w/v potassium iodide solution, and the whole was titrated immediately with 0·01N sodium thiosulphate previously standardised against potassium iodate. A 1 per cent. starch solution in 20 per cent. w/v aqueous potassium chloride solution was used as an indicator. *Test.* To a 20 ml. aliquot of the sample solution was added 5 ml. of N sodium hydroxide and the resulting mixture allowed to stand for 15 minutes at room temperature. After this period 5 ml. of 1·1N hydrochloric acid were added followed by 20 ml. of the 0·01N iodine solution. After a further period of 15 minutes the whole was titrated with the 0·01N sodium thiosulphate using the starch solution as indicator.

Penicillinase Inactivation method. A freeze dried penicillinase product was found to be non-iodine absorbing and particularly suitable for iodimetric assays. The penicillin sample solutions were prepared as described in the alkali inactivation method. *Blank.* To a 20 ml. aliquot of sample solution was added 30 ml. of 0·01N iodine solution in McIlvaine *p*H 4·4 buffer of 1·5 times the usual concentration. This was then allowed to stand for 30 minutes in the dark and titrated with the standardised 0·01N sodium thiosulphate solution using the starch solution as indicator. *Test.* To a 20 ml. aliquot of sample solution was added 1 ml. of a penicillinase solution, prepared by adding 20 ml. water to a 30 mega-unit vial

of penicillinase. This solution was allowed to stand for 15 minutes after which time 30 ml. of the 0·01N iodine solution was added. After a further 30 minutes standing in the dark, the titration was carried out with 0·01N sodium thiosulphate using starch solution as indicator.

Calculation of assay results. The difference between "blank" and "test" sodium thiosulphate titrations was taken as a measure of the penicillin content and used in terms of "ml. 0·01N iodine absorbed per mg. of sample after inactivation" in order to compare the various samples.

The Preparation of Pure Penicillin V for use as a Standard. Assay of Samples

First series. The preparation of samples A, B and C is described elsewhere[9]. From these samples duplicate assays of single solutions were obtained on five separate days. Assays were made by the two methods and are shown in Table I with a summary of the analysis of variance in Table II.

TABLE I

IODIMETRIC ASSAY OF PENICILLIN V, SAMPLES A, B, C, D, AND E

		ml. 0·01N iodine absorbed per mg. of sample after inactivation											
		Penicillinase inactivation						Alkali inactivation					
		Day						Day					
Sample	Time of assay	1	2	3	4	5	Mean	1	2	3	4	5	Mean
A	—	2·36 2·36	2·37 2·37	2·37 2·36	2·37 2·37	2·36 2·39	2·37	2·49 2·48	2·53 2·50	2·51 2·51	2·50 2·51	2·45 2·45	2·49
B	—	2·42 2·44	2·43 2·43	2·41 2·41	2·40 2·40	2·40 2·40	2·41	2·54 2·54	2·58 2·57	2·54 2·56	2·56 2·55	2·54 2·59	2·56
C	—	2·41 2·44	2·46 2·45	2·43 2·44	2·47 2·44	2·39 2·41	2·43	2·58 2·56	2·58 2·59	2·59 2·59	2·57 2·61	2·55 2·57	2·58
D	Morning	2·40 2·43	2·39 2·37	2·39 2·39	2·39 2·39	2·42 2·40	2·39	— —	— —	— —	— —	— —	—
	Afternoon	2·41 2·39	2·39 2·39	2·37 2·36	2·36 2·36	2·39 2·39		— —	— —	— —	— —	— —	—
E	Morning	2·49 2·48	2·44 2·44	2·47 2·46	2·47 2·47	2·48 2·46	2·46	— —	— —	— —	— —	— —	—
	Afternoon	2·46 2·46	2·46 2·46	2·44 2·46	2·44 2·44	2·45 2·44		— —	— —	— —	— —	— —	—

It was found that the Sample x Day interaction was significant ($p = 0·05$) using the penicillinase inactivation method, almost significant using the alkali inactivation method, and was probably due to variation in the time interval between dissolving samples and making assays. Accordingly this interaction was used in the "t" test to compare the means of sample A with sample B and sample B with sample C. By both methods of assay, sample B was shown to have a higher iodine absorption than sample A, this difference being highly significant ($p = 0·005$). Sample C was not shown to have a significantly ($p = 0·05$) higher iodine absorption than sample B, but further experimentation would possibly show a significant difference.

Second series. In a second series were sample D, a sample of Distillers Company (Biochemicals) Limited production material and E, the same

material after the purification stages described elsewhere[9]. A single solution of each was prepared on each of five days and duplicate assays were made in the morning and afternoon. The significant Sample × Day interaction shown in the first series was thought to be due to variation in the time interval between dissolving samples and making assays, therefore a time effect was included to test this possibility. Assays were carried out by the penicillinase inactivation method only (for reasons given below), and are also shown in Table I with a summary of the analysis of variance in Table II. The difference between samples was highly

TABLE II

IODIMETRIC ASSAY OF PENICILLIN V, SAMPLES A, B, C, D, AND E. SUMMARY OF ANALYSIS OF VARIANCE OF RESULTS GIVEN IN TABLE I

Samples	Method	Source of variance	Degrees of freedom	Mean square
A, B, and C	Penicillinase inactivation	Between samples (S)	2	0·010675
		Between days (D)	4	0·000604
		S × D interaction	8	0·000436
		Residual	15	0·000139
	Alkali inactivation	Between samples (S)	2	0·021841
		Between days (D)	4	0·000925
		S × D interaction	8	0·000568
		Residual	15	0·000230
D and E	Penicillinase inactivation	Between samples (S)	1	0·049070
		Between days (D)	4	0·000923
		Between times (T)	1	0·002608
		D × S interaction	4	0·000156
		T × S interaction	1	0·000002
		D × T interaction	4	0·000450
		D × T × S interaction	4	0·000030
		Residual	20	0·000066

significant ($p = 0{\cdot}001$), in addition to which the Day × Time interaction was very significant ($p = 0{\cdot}01$), when both were compared with the residual, but the Between Times effect was not significant when compared with this interaction. Inspection of the results showed that assays made in the afternoon were lower than the morning assays on four days, but that on the second day this was not the case, and probably accounted for the significant Day × Time interaction term. If the assays made on the second day were omitted, the Between Times effect would almost certainly have been significant. The appropriate interaction terms were used in the "t" test to compare the means of samples C and E and sample E was shown to have a significantly greater iodine absorption ($p = 0{\cdot}05$).

Of all the samples assayed, sample E had the highest iodine absorption after inactivation, and in conjunction with evidence given in Parts 1 and 3 of this paper was probably the purest sample. This sample E with an iodine absorption after inactivation of 2·46 ml. per mg. was therefore considered suitable as an assay standard.

Determination of Penicillin V in the Presence of Penicillin G

The investigation of the iodimetric determination of penicillin V in the presence of penicillin G was based upon the difference in the rates of inactivation of the two penicillins at *p*H 2·0.

The initial experiments involved the use of three different solutions, the

first containing penicillin G (A.S.C. III standard) only, the second containing penicillin V, and the third a mixture of the two. Each solution was adjusted to *p*H 2·0 for different periods of time, re-adjusted to *p*H 7·0 and then assayed according to the alkali inactivation method. After 1 hour at *p*H 2·0 the solution of penicillin G gave a negative assay; such a result could be explained by the difference in treatment of "blank" and "test" in this method, and was later shown to be due in particular to the differences in *p*H level of "blank" and "test" during iodine absorption. Subsequent experiments and in fact those concerning the assay of samples D and E were conducted using the penicillinase inactivation method only, where "blank" and "test" were treated identically as far as possible.

In the next series of experiments concerned with inactivation at *p*H 2·0 it was found that there was no period at which all the penicillin G was inactivated leaving the penicillin V unaffected. Therefore there appeared to be two possible ways in which the determination could be carried out. The first would consist essentially of inactivation at *p*H 2·0 for a period long enough to decompose all the penicillin G followed by a determination of the residual penicillin V, and the use of a factor to find the original content. The second would consist of a shorter period of inactivation at *p*H 2·0 to cause partial decomposition of the two penicillins, and a comparison with values obtained with mixtures of known proportions of penicillin V and penicillin G. The former method was thought to be subject to less variation and was therefore investigated further.

At *p*H 2·0 and temperatures of approximately 20° C. with solutions of about 350 units/ml. inactivation of penicillin G took 3 hours. The solutions were adjusted to *p*H 7·0 for assay. The residual penicillin V varied between 80 and 85 per cent. of the original content. Table III shows results of determinations made on solutions of penicillin G, penicillin V and mixtures of equal volumes of the two on six different days using the A.S.C. III sodium penicillin G standard and sample E. The standard deviation of the results obtained from the solutions of penicillin V only was $\pm$ 1·9 per cent. whereas the corresponding standard deviation for the mixture was $\pm$ 3·2 per cent. This method is under investigation.

The Purity and Potency of the A.S.C. III Sodium Penicillin G Standard and its Relation to Penicillin V

The A.S.C. III sodium penicillin G standard was assigned a potency of 1682 units/mg. and has been in use since October, 1953. For the purpose of control of biological assay a sample of potassium penicillin has been assayed against this standard periodically since January, 1954. A total of 35 sets of six assays of this sample was carried out using the A.S.C. III standard between the period January—June, 1954. From the mean range for this period 95 per cent. confidence limits for day ranges and means were calculated[3]. The mean assay of those falling within these limits was 1625·6 units/mg. after eliminating eight day means which were above the upper p= 0·975 limit. The 95 per cent. confidence limits of this mean were $\pm$ 6·0 units/mg. and thus its potency was significantly higher than the expected 1609 units/mg. Over the next 6 months period 36 sets of

TABLE III

IODIMETRIC ASSAY OF SOLUTIONS OF PENICILLIN G ONLY, PENICILLIN V ONLY AND OTHER MIXTURES OF EQUAL VOLUMES OF THE TWO

Solution	Penicillin estimated	Residual penicillin after 3 hours at *p*H 2·0 (per cent. of the original)					
		Day					
		1	2	3	4	5	6
Penicillin G	Penicillin G	0·2	0·1	0·3	0·0	0·0	0·0
Penicillin V	Penicillin V	82·5	85·0	80·0	84·0	82·0	84·5
Penicillin G and Penicillin V	Penicillin V	81·0	88·0	79·0	83·0	82·5	85·5

assays were made and the mean was 1624·9 $\pm$ 5·6 units/mg. ($p = 0{\cdot}05$). Over this second period therefore this sample was again shown to have a potency significantly higher than that expected. This evidence may therefore indicate that the A.S.C. III standard contains 1 per cent. of impurities.

Over a period of approximately four months the A.S.C. III sodium penicillin G standard was assayed almost daily by the penicillinase inactivation method, and the mean of all these results, 734 units/ml. 0·01N iodine solution or 2·29 ml. 0·01N iodine solution per mg. were therefore suitable factors to use where comparisons were required with this standard. Using this factor, the best estimates of the assays of samples A, B, C, D, and E in terms of units/ mg. relative to the A.S.C. III standard were A 1740, B 1772, C 1785, D 1753 and E 1804.

On the basis that pure sodium penicillin G contains 1682 units/mg. these estimates may be 1 per cent. high.

Accuracy of Iodimetric Assay using the Penicillinase Inactivation Method

The mean square terms for the Sample $\times$ Day interaction and Day $\times$ Time interaction in Table II were the best estimates of assay error in the two series of assays made. Both were similar and indicate a standard deviation of 0·87 per cent. Over the same period in which these assays were made the A.S.C. III standard was also assayed daily giving a corresponding standard deviation of 0·84 per cent. over independent assays. The accuracy of the method was therefore similar for both penicillins and from the two analyses of variance it is probable that the accuracy could be improved if the interval between dissolving the sample and its assay were reduced.

MICROBIOLOGICAL ASSAY

With the renewed interest in penicillin V (phenoxymethylpenicillin) it is necessary to reconsider the terms by which potencies of penicillin preparations are expressed. The use of the term "unit" was appropriate before the chemical structure of penicillin was known, but to-day the penicillin unit refers to a given weight of a purified chemical compound. Expression of the activity of penicillin G in terms of weight has been advocated[6] and with the introduction of penicillin V this procedure is necessary to avoid confusion since penicillin V has a different activity

from penicillin G against a number of test organisms. The confusion which is likely to arise from the use of the microbiological unit is not new since the earlier known penicillins X, F, A and K have different activities from that of G. However the problem has not previously been of practical importance because these other penicillins have not been developed commercially. Already penicillin V has been given several different microbiological potencies[4,7] from which it appears that the value of penicillin V in terms of penicillin G is dependent on both the strain of assay organism and the conditions of the test.

This section deals with an examination of the activity of penicillin V in terms of penicillin G against several recognised assay organisms, and discusses the confusion which is inherent in the use of the penicillin unit. The use of a penicillin V reference standard of the highest purity is advocated in order that potencies may be expressed on a weight basis.

Experimental and Results

Penicillins. Penicillin G (benzylpenicillin) A.S.C. III sodium salt which has been assigned a potency of 1682 units per mg. Penicillin V (phenoxymethylpenicillin free acid), sample E described elsewhere[9]. Solutions of each penicillin were prepared daily by dissolving 47·6 mg. in a final volume of 100 ml. of *p*H 6·0 M/20 phosphate buffer. Further dilutions were made in *p*H 7·0 M/20 phosphate buffer.

Assay method. Assays were carried out by the cavity-plate method developed in this department[8]. The volume of medium was increased to 180 ml. per plate. The following assay organisms and inocula were used. *Staphylococcus aureus.* Inocula were broth cultures grown for 24 hours at 37° C. having opacities* between 1 and 2; the amounts were as follows. Strain 209P 0·3 ml. per plate, Oxford H 1·0 ml. per plate, NCIB 8244 0·3 ml. per plate. *Bacillus subtilis.* Inocula were spore suspensions in distilled water having opacities* between 7 and 10, prepared from nutrient agar slopes; the amounts were as follows. Strain 288 0·1 ml. per plate, ATCC 6633 0·1 ml. per plate, I.C.I. Pen D/C8 0·1 ml. per plate. The inoculated medium was held at 60° C. for 10 minutes before pouring the plates. *Sarcina lutea.* The inoculum medium was the same as for *Staph. aureus.* The inoculum was a broth culture grown for four days at 29° C. A 1 to 10 dilution had an opacity* between 2 and 3. Strain NCIB 8553 2·0 ml. per plate.

Dose-response curves. Before attempting the quantitative assessment of penicillin V in terms of penicillin G, it was necessary to examine the form of the response curves for the two substances. This was done for one strain of each of the three species of organism. The plate design was modified to accommodate the same four levels of concentration by weight of each penicillin and each level was filled into eight cavities to a randomised design. The response curves for the averages of six plates of *Staph. aureus* 209P and five each of *B. subtilis* 288 and of *Sarcina lutea*, obtained by plotting inhibition zone diameter against logarithm of concentration, are given in Figure 1. The results were analysed statistically and the main conclusions are

* Brown's scale, Burroughs Wellcome and Company, Limited opacity tubes.

summarised as follows. The curves for *B. subtilis* 288 were substantially linear and parallel. The curves for *Staph. aureus* 209P were linear and parallel over the range of the three upper levels. There was a slight deviation at the lowest level of penicillin V with the average of the six plates used, but this was unimportant since it was below the range used for the assay of penicillin V against penicillin G. For *Sarcina lutea* the response curves to penicillins G and V differed in slope and curvature and therefore in these tests one penicillin could not be expressed in terms of the other. That the response curves for *B. subtilis* and for *Staph. aureus* were parallel was confirmed by a detailed analysis of variance for one plate of each of the six organisms in the course of the following experiment on the assessment of the activity of penicillin V. A similar analysis for plates of *Sarcina lutea* confirmed the non-parallelism of the penicillin V and G response curves for this organism.

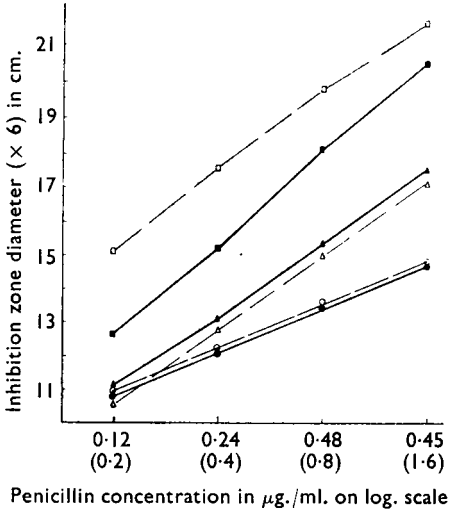

FIG. 1. Response curves for *B. subtilis* 288 ○ ●, *Staph. aureus* 209P △ ▲, and *Sarcina lutea* □ ■ against Penicillin G – – and Penicillin V ——.

Assessment of the activity of penicillin V. The activity of penicillin V was determined in terms of penicillin G for the strains of *Staph. aureus* and *B. subtilis* listed above. Solutions were prepared from two weighings of each penicillin. The four solutions were diluted to "high" and "low" concentrations and were filled in duplicate on each of two plates for each organism. Each "filling" (4 cavities "high" + 4 cavities "low") of penicillin V was calculated against the average of all the "fillings" of penicillin G on the same plate. The averages of the eight results for each organism on each of three days are given in Table IV.

A detailed statistical analysis of the results showed that the potency of penicillin V was (*a*) significantly greater than the potency of G for *Staph. aureus* strains 209P and NCIB 8244 (*b*) not significantly greater for the Oxford H strain and (*c*) significantly less for all three strains of *B. subtilis*. The relative potencies of the two penicillins for the three strains of *Staph. aureus* were different one from another and the result for *B. subtilis* ATCC 6633 was significantly lower than those for the other two strains. The activity of penicillin V against the Oxford H strain used was lower than expected from published results and this difference may have been associated with the method of maintenance. Cultures of this strain obtained from other sources are being examined. There was no difference, apart

from size, in the appearance of the zones for penicillins V and G on the same plate.

Inactivation of penicillin V by penicillinase. The inactivation of penicillin V by the penicillinase treatment described under "Chemical Assay" was examined microbiologically using *Staph. aureus* 209P and *B. subtilis* 288, and was found to be complete.

TABLE IV

ACTIVITY OF PENICILLIN V FREE ACID IN UNITS/MG. AGAINST PENICILLIN G SODIUM SALT AT 1682 UNITS/MG.

	Staph. aureus			*B. subtliis*		
Day	209P	Oxford H	NCIB 8244	288	ATCC 6633	ICI Pen.D/C8
1	1830	1704	1960	1514	1317	1556
2	1897	1788	2044	1578	1376	1628
3	1822	1573	1970	1642	1330	1601
Mean (±17)	1850	1688	1991	1578	1341	1595

Stability of penicillin V to acid. The stability of penicillin V to the acid treatment described under "Chemical Assay" was assessed microbiologically. On each day the following duplicate solutions were prepared: Solutions 1 and 2 of penicillin G 350 units per ml. (208 μg. per ml.); 3 and 4 of penicillin V 208 μg. per ml.; 5 and 6 of a mixture of penicillin G 175 units per ml. (104 μg. per ml.) and penicillin V 104 μg. per ml. (total 208 μg. per ml.). After treatment, the 6 solutions were assayed against *Staph, aureus* 209P and *B. subtilis* 288. The figures given in Table V show that penicillin V is more stable to acid than penicillin G confirming the results obtained under "Chemical Assay." Each figure is an average of 5 or 6 assays on different plates. The percentages given for the activity remaining for mixtures of G and V have been corrected for the residual G and therefore indicate only the residual V. Confirmation that penicillin G was not completely inactivated in solutions 1 and 5 for day 2 is presented in Figure 3 of the paper by Stephens and Grainger[10].

TABLE V

MICROBIOLOGICAL ACTIVITY AFTER 3 HOURS AT *pH* 2·0

Activity remaining as per cent. of original activity. Results for the mixtures of G and V are based on V

Day	Temperature of inactivation °C.	Assay organism	Penicillin G		Penicillin V		Penicillin G + V	
			Soln. 1	Soln. 2	Soln. 3	Soln. 4	Soln. 5	Soln. 6
1	Room temp. 20–21	*B. subtilis*	1·9	3·2	88·0	86·2	83·9	85·1
		Staph. aureus	1·8	3·3	92·8	90·0	89·6	88·5
2	Room temp. 20–21	*B. subtilis*	3·9	4·4	92·8	83·0	89·7	93·7
		Staph. aureus	4·1	4·3	90·6	82·3	81·0	85·7
3	25	*B. subtilis*	0·2	0·2	76·5	75·0	78·8	77·0
		Staph. aureus	0·1	0·2	80·5	77·8	82·8	83·8
4	25	*B. subtilis*	0·0	0·5	78·8	76·5	78·2	82·8
		Staph. aureus	0·0	0·4	82·8	77·2	80·0	81·0

Discussion

Earlier evidence[4,5] indicated that iodimetric assay of the various penicillins varied only according to the ratio of their respective molecular weights. The results of assays described in this paper do not support this theory. Sample E, for example, the purest of those prepared, assayed about 5·6 per cent. higher than anticipated from the assays of the A.S.C. III standard or 4·6 per cent. higher if the probable impurity of this standard is allowed for. However amongst other factors, iodimetric assays are known to vary with *p*H, with the time for iodine absorption, and, in the presence of much impurity, with the excess of iodine added. Thus it is equally likely that a change in the molecule could cause a change in the rate of iodination, particularly when the reaction between the penicilloic acid produced by inactivation and iodine is not stoichiometric.

When the microbiological activity of penicillin V is assessed in terms of penicillin G the result obtained depends not only on the species but also on the strain of the assay organism. A similar conclusion may be drawn from previously published work[4,7] in which activities ranging from 1670 (for the sodium salt) to 2695 units per mg. are given. It also appears that different activities in terms of penicillin G may be obtained in different laboratories using the same strain of test organism. For example, the figure of 2250 units per mg. for the Oxford H strain of *Staph. aureus* reported by Brunner[4] is appreciably higher than the activity found in the present tests. In the case of *Sarcina lutea* the potency of penicillin V in relation to G could not be expressed accurately because the response curves differed in slope.

It is evident that disagreement and confusion will occur if potencies of penicillin V preparations are expressed in terms of penicillin G by different laboratories using different methods and test organisms. It is therefore recommended that the potency of a penicillin preparation should be expressed on a weight basis in relation to a purified specimen of the same penicillin which has been established as a reference standard.

Summary

1. Iodimetric assays were made on various samples of penicillin V obtained during the purification of this material. A significant increase in purity, as judged by iodimetric assays, was obtained. The purest sample, E, was considered suitable as a reference standard and absorbed 2·46 ml. 0·01N iodine per mg. after inactivation using the penicillinase inactivation method. The difference in assay between this and the sample with the next highest purity was just significant ($p = 0·05$).

2. A method was developed for the assay of penicillin V in the presence of penicillin G based upon the difference in the rates of inactivation at *p*H 2. The greater stability of penicillin V was confirmed microbiologically.

3. A cavity-plate assay method was used to compare the activity of penicillin V in terms of penicillin G against several recognised test organisms. The potency varied according to the strain of *Staph. aureus* and *B. subtilis* used. It is concluded that penicillin V preparations should be

assayed against a pure standard of the same penicillin and the potency expressed on a weight basis.

4. Assays using *Sarcina lutea* were invalid.
5. Penicillin V was shown to be completely inactivated by penicillinase.

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REFERENCES

1. Alicino, *Industr. Engng Chem.*, (*Anal.*), 1946, **18**, 619.
2. Ortenblad, *Acta. chem. scand.*, 1950, **4**, 518.
3. Dudding and Jennett, B.S. 600R.
4. *Sci. pharm.*, 1954, **22**, 151.
5. Brandl and Margreiter, *Öst. ChemikerZtg.*, 1954, **55**, 11.
6. Noel, *Antibiotics and Chemotherapy*, 1954, **4**, 923.
7. Behrens *et al.*, *J. biol. Chem.*, 1948, **175**, 793.
8. Brownlee, Loraine and Stephens, *J. gen. Microbiol.*, 1949, **3**, 347.
9. Parker, Cox and Richards, *J. Pharm. Pharmacol*, 1955, **7**, 683.
10. Stephens and Grainger, *ibid.*, 1955, **7**, 702.