THE APPLICATION OF ENZYME INHIBITION TO THE ESTIMATION OF SMALL QUANTITIES OF DRUGS POSSESSING ANTICHOLINESTERASE ACTIVITY

The Assay of Injection of Neostigmine Methylsulphate

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When highly active substances are administered, it is often necessary to use tablets for oral administration or solutions for injection. These preparations contain accurately known small quantities or low concentrations of the drug and may present a considerable problem to the analyst. Neostigmine methylsulphate is officially assayed by determination of the quantity of elementary nitrogen in 0·5 g. of the sample. Since 0·5 g. of the substance corresponds to 1000 1-ml. ampoules of the official strength, determination of nitrogen as a method of assay for the injection is unsatisfactory. Further, while determination of nitrogen is a fairly satisfactory method of assay for a substance the purity of which can be established by a melting point determination, it constitutes a much less satisfactory method for assaying a preparation in which the drug may have undergone decomposition to a greater or lesser amount without loss of nitrogen.

Such difficulties may sometimes be overcome by the use of sensitive colorimetric or spectrophotometric methods. The drug may not, however, be coloured and may not contain a chemical grouping enabling conversion to a coloured derivative to be effected satisfactorily. Further, injections may contain, in a concentration large in comparison with the concentration of the drug, bactericides such as chlorocresol or phenylmercuric nitrate which interfere with the spectrophotometric assay. In some instances biological assay may be necessary. Biological assays are often time-consusming, costly and yield results with wider limits of error than are suitable for control or public analytical work. During the last few decades it has become established that a number of drugs exert their actions by inhibition of enzyme systems, in some, in very low concentrations. In 1942 Vincent and Morgin[1] used cholinesterase inhibition as the basis of a method for estimating physostigmine in calabar bean and extended the process to the estimation of other drugs possessing anticholinesterase activity such as morphine and codeine. The method was used in toxicological work by Vincent and Beaugar[2]. A similar process was used by Ellis Plachte and Strauss[3] for the estimation of physostigmine in 10^{-6} to 10^{-8} M concentration. Enzyme estimations are inexpensive and can be carried out in a chemical laboratory with as little trouble as, for example, the estimation of nitrogen. The limits of error usually lie between those of chemical and classical biological assays.

It seemed advisable, therefore, to investigate the extent to which

enzyme inhibition might be used for the assay of drugs in tablets and injections. As a result of the work of Stedman[4] relating chemical structure to anticholinesterase activity, Aeschlimann and Reinert[5] investigated a series of substituted esters of alkyl carbamic acids. Neostigmine proved to be the compound showing the most promising pharmacological properties. The pharmacological activity of neostigmine was shown to be closely related to its anticholinesterase activity; the kinetics of the enzyme inhibition have been extensively studied and, as mentioned above, it is inhibitory in such low concentrations as 10^{-7} M (see also below). The British Pharmacopœia lacks an assay of Injection of Neostigmine Methylsulphate. It seemed logical, therefore, in the first place to investigate the possibility of devising a satisfactory process based upon the inhibition of cholinesterase for the assay of this official preparation.

Experimental Methods

Materials. All the water used had been distilled in an all-glass still.

Cresol Red Solution. A stock solution, containing 0·8 g. of cresol red dissolved in 2·7 ml. 0·1 N NaOH and diluted with water to 100 ml., was diluted with three times its volume of water before use. Since 1 ml. of this product was used in each 50 ml. digest, this corresponded to a concentration of 0·004 per cent. cresol red.

Substrate. A salt of acetylcholine (ACh) was used as substrate for the pseudocholinesterase. In the earlier experiments the chloride was employed but later the non-hygroscopic perchlorate[6] was found to be more convenient. Glick[7] has shown that the rate of enzymic hydrolysis of ACh is independent of the nature of the associated anion. Both salts were of commercial quality.

Pseudocholinesterase (ψChE). Sterile horse serum[8,9] obtained commercially in 25 ml. vaccine bottles was used as the source of ψChE. It may be preserved with 0·2 per cent. chlorocresol (see below) or by saturation with chloroform[10]. When kept at 4° C. it retains sufficient activity for at least 6 months.

Determination of ψChE Activity. Cholinesterase activity is determined by the rate at which ACh (or some analogue) is hydrolysed.

Hestrin[11] used a colorimetric method to estimate the residual ACh after the period of hydrolysis. This method not only possesses the errors and difficulties of a colorimetric assay, but it is more suitable for experiments in which the bulk of the ACh is used up than for assays based on first order kinetics achieved by limiting the extent of hydrolysis of the substrate to one-third.

More usually, the acid liberated by the hydrolysis of the ACh is determined in one of several ways. (1) The acid may be caused to liberate CO_2 from $NaHCO_3$ solution and the volume of CO_2 determined either manometrically in the Warburg apparatus[12] or, in the case of microdetermination, by use of the Cartesian Diver technique[13]. Such methods, because of the specialised apparatus required, were considered to be unsuitable for an official assay method, especially since there is

no great increase in accuracy. (2) the acid liberated may be titrated with standard alkali either: (*a*) at the end of the hydrolysis, or (*b*) continuously during hydrolysis.

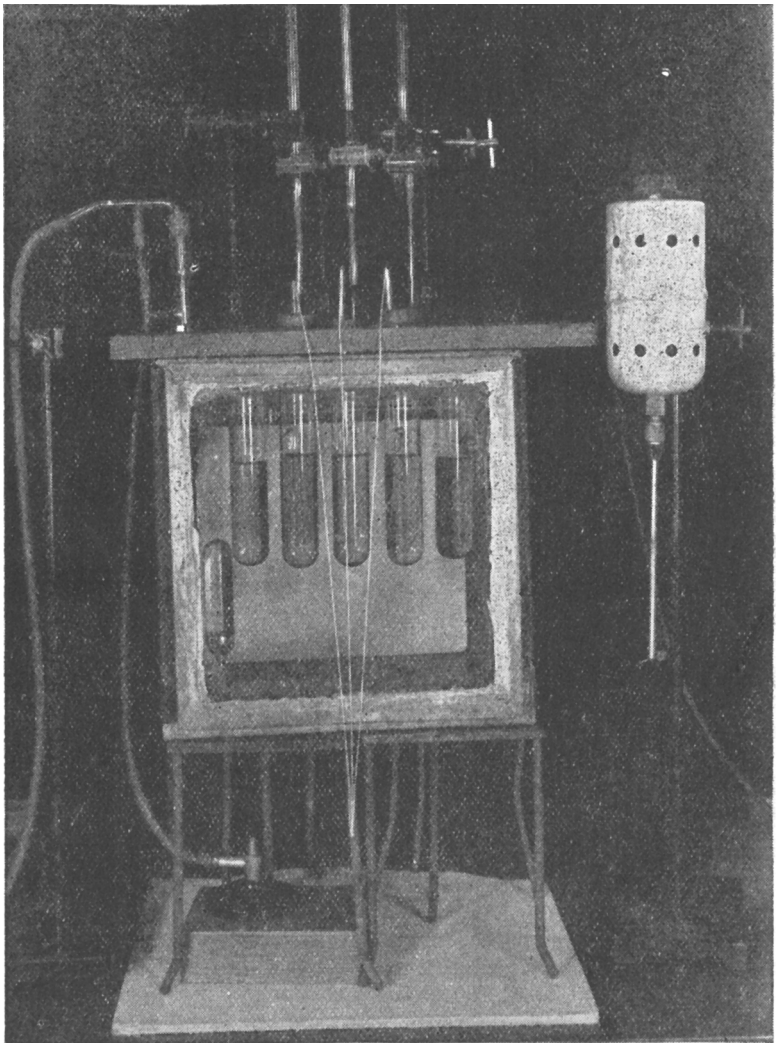

FIG. 1. Photograph of the apparatus used in the determination of ψChE activity.

Method (*a*) has the drawback that the pH and consequently the activity of the enzyme varies during the period of digestion unless a buffer is added, in which case determination of the end-point of the titration is difficult. In method (*b*) the pH is maintained constant during the digestion period. The maintenance of pH may be controlled by use of an indicator or electrometrically. Using an indicator 2 or 3

assays may be carried out simultaneously (Fig. 1) (e.g. with and without addition of inhibitor), whereas electrometrically this is difficult. It was found that variations were smaller between simultaneous replicates than between successive replicates. For these reasons a method based on (2) (*b*) using an indicator was adopted. The method, apparatus (Fig. 1) and technique are similar to those described previously for lipase[14].

One ml., accurately measured, of horse serum was mixed with 43 ml. of water containing 1 ml. of cresol red solution contained in a 3 × 20 cm. Pyrex boiling tube fitted with a stopper with two holes, one for the drawn out tip of a microburette containing 0·025 N NaOH and the other for a short glass tube through which passed a thread operating a glass-coil stirrer. The tubes were placed in a water bath at 40° C. and by addition of alkali the pH was rapidly adjusted to 7·9 (by comparison with the colour of two Pyrex tubes in the water bath containing respectively 1 ml. of cresol red solution and 49 ml. of B.P. buffer pH 7·8, and 1 ml. of cresol red solution and 49 ml. of B.P. buffer pH 8·0). After 15 minutes for temperature equilibration, 5 ml. of a 3 per cent. solution of ACh perchlorate was added (concentration in digest $1{\cdot}22 \times 10^{-2}$ M) the pH readjusted to pH 7·9 and the burette reading noted. The pH was then maintained between 7·8 and 8·0 by dropwise addition of alkali, the burette reading being noted after 5, 10 and 15 minutes. Maintenance between the two pH units is possible because the 1 ml. of serum has a buffering action such that addition of 1 drop of the alkali does not change the pH by more than 0·1 unit. All readings were corrected for non-enzymic hydrolysis ascertained by conducting an experiment with addition of 1 ml. of a dilute buffer in place of 1 ml. of serum. The correction was small, amounting to only 0·32 ml. for a 15 minute period. The corrected quantity of alkali added in each of the three 5-minute periods of the assay should be practically the same. If this were so, the alkali consumed in 15 minutes was recorded as a measure of the ψChE activity ("A_{15}" value).

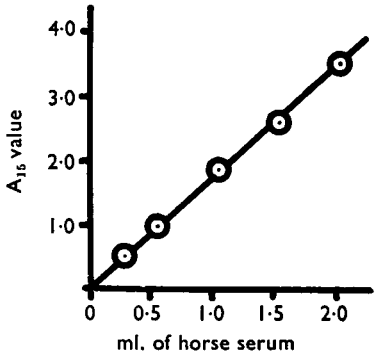

FIG. 2. Relation between the rate of hydrolysis and the quantity of horse serum used. Temperature 40° C. pH 7·9. ACh concentration $1{\cdot}22 \times 10^{-2}$ M.

The suitability of this process for the evaluation of inhibition is established by the straight line graph (Fig. 2) relating enzyme activity to the corresponding A_{15} values. A digest in which the enzyme is inhibited 50 per cent. will obviously require only half the alkali consumed in the absence of inhibition.

The sensitivity of the assay to various factors was determined as follows.

Substrate Concentration. Figure 3 shows that the A_{15} value of a given quantity of serum is practically independent of the substrate concentration.

Temperature. The results presented in Figure 4 show that 40° C. is in the neighbourhood of the optimum temperature under the conditions of the assay.

pH. Using the same sample of serum under identical conditions, A_{15} values of 2·46, 2·53 and 2·55 were obtained by maintaining the digests at pH values of 7·8, 7·9 and 8·0 respectively. The difference between A_{15} values determined at pH 7·8 and pH 8·0 thus amounts to about 1 per cent. This is the maximum percentage error which is likely to arise due to pH variation during the assay.

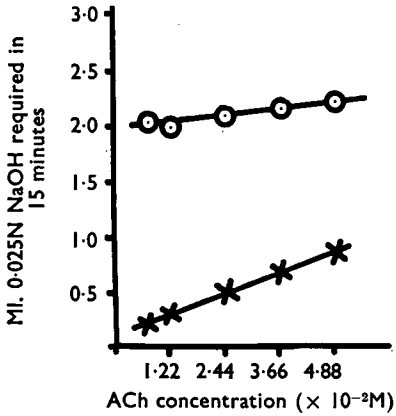

FIG. 3. Relation between the rate of hydrolysis and ACh concentration O—O corrected enzymic hydrolysis. X—X non-enzymic hydrolysis. 1 ml. of horse serum as source of enzyme. Temperature 40° C. pH 7·9.

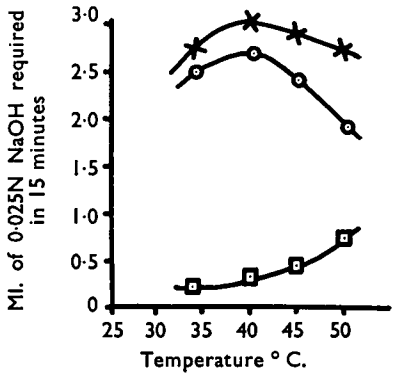

FIG. 4. Relation between the rate of hydrolysis of ACh and temperature ×—× Total hydrolysis. ○—○ Corrected enzymic hydrolysis. □—□ Non-enzymic hydrolysis. ACh concentration 1·22 × 10^{-2}M. pH 7·9. 1 ml. of horse serum as the source of enzyme.

Antiseptics. Chlorocresol 0·2 per cent. or phenylmercuric nitrate 0·002 per cent. may be present as preservatives in Injection of Neostigmine Methylsulphate. Table I shows that such quantities of these preservatives as might thus find their way into the digests of the assay process would be without measurable effect on the ψChE activity.

TABLE I

NEGLIGIBLE ANTICHOLINESTERASE ACTIVITY OF BACTERICIDES 15 MINUTES PRIOR CONTACT OF ENZYME AND INHIBITOR AT 40° C.

Bactericide	Concentration in 50 ml. digest per cent.	A_{15} value	
		Bactericide present	Bactericide absent
Chlorocresol	0·002	2·75	2·76
	0·005	2·72	2·72
	0·01	2·73	2·76
	0·05	2·74	2·72
Phenylmercuric nitrate	0·0002	2·69	2·69
	0·0004	2·71	2·74
	0·001	2·65	2·62
	0·002	2·70	2·73

Accuracy of the Assay Process. Fourteen successive determinations using a single sample of horse serum gave an average A_{15} value of 3·24 ml., with a standard deviation of 0·06.

The ψChE Activity of Different Samples of Horse Serum. Six different samples of horse serum gave average A_{15} values of 3·24, 2·72, 3·04, 2·39, 2·58 and 2·78.

This variation from sample to sample of serum is important because it has been shown that the percentage inhibition caused by a given concentration of an inhibitor varies with the activity of the uninhibited enzyme preparation[15].

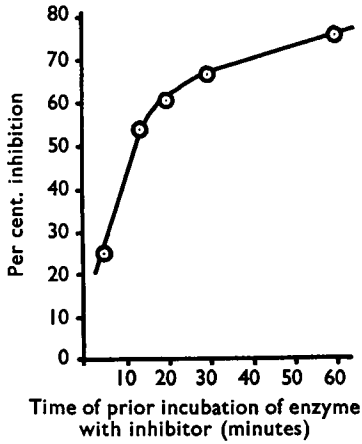

FIG. 5. Relation between the time of prior incubation of neostigmine with enzyme, and the degree of inhibition produced. Temperature 40° C. pH 7·9. ACh concentration $1·22 \times 10^{-2}$M. Neostigmine concentration $6·0 \times 10^{-8}$M. 1 ml. of horse serum as the source of enzyme.

Determination of the Percentage of ψChE Caused by Neostigmine

The percentage inhibition of ψChE caused by neostigmine varies with the time of "prior contact" between the enzyme and inhibitor before the addition of the substrate. Figure 5 shows that under the conditions used, the inhibition is not maximal even after 60 minutes prior contact. However, the curve begins to flatten after 15 minutes and this period of time was chosen for prior contact in the assay. To determine percentage inhibition, two ψChE determinations on the same sample of serum were carried out simultaneously, but whereas the control (A_{15} value = A) contained the usual 43 ml. of water, the test (A_{15} value = B) contained 38 ml. of water plus 5 ml. of a suitable concentration of neostigmine. The per cent. inhibition was $\frac{A-B}{A} \times 100$.

Figure 6 shows that the graph relating the negative logarithm of the molar concentration of neostigmine and the per cent. inhibition is a straight line in the region of 50 per cent. inhibition.

The Assay of Injection of Neostigmine Methylsulphate. According to Figure 6, 50 per cent. inhibition of ψChE is brought about by a $3·6 \times 10^{-8}$ M concentration of neostigmine. Therefore, to assay the Injection or indeed any solution of a salt of neostigmine, it is first quantitatively diluted until the concentration is approximately $3·6 + 10^{-8}$ M. From the experimentally determined per cent. inhibition caused by this dilution, the exact concentration of neostigmine in the digest can be read off from Figure 6 and the concentration in the injection calculated.

An injection was made up containing exactly 0·5 mg. neostigmine methylsulphate in 1 ml. A "first dilution" was prepared by diluting 1 ml.

accurately measured (for greater accuracy the injection may be weighed) to 250 ml. with water. Ten ml. of the first dilution diluted to 100 gave the "second dilution". Five ml. of the second dilution in a 50 ml. digest gave a concentration of $5{\cdot}98 \times 10^{-8}$ M neostigmine, hence from Figure 6 approximately 60 per cent. inhibition should result. Six separate assays on an injection made up to contain exactly 0·5 mg. of neostigmine methylsulphate per ml., gave per cent. inhibitions of 62·6, 62·9, 62·1, 62·3, 60·0 and 61·5: (average value = 61·9). A 2 per cent. variation in inhibition corresponds approximately to a 10 per cent. variation in concentration of inhibitor. Thus, by this method the injection can be assayed to within approximately 10 per cent. This conclusion is confirmed by the results recorded in Table II.

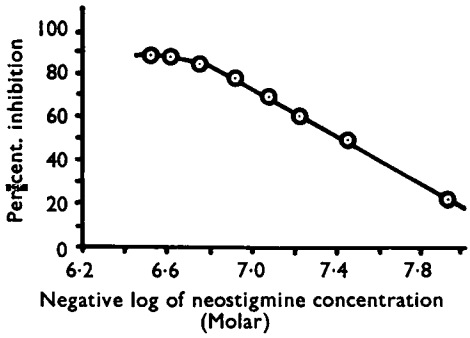

FIG. 6. Relation between concentration of neostigmine and the degree of inhibition produced. Temperature 40° C. pH 7·9. ACh concentration $1{\cdot}22 \times 10^{-2}$ M 1 ml. of horse serum as source of enzyme. Prior incubation of enzyme with neostigmine 15 minutes.

There are several objections to such an assay process. (1) The preparation of the calibration graph (Fig. 6) is time-consuming and since, as pointed out earlier, the per cent. inhibition caused by a given concentration of inhibitor varies with the ψChE activity of the serum used, a new calibration curve would be necessary at least for each sample of

TABLE II

VARIATION IN THE DEGREE OF INHIBITION OF ψChE WITH SMALL CHANGES IN INHIBITOR CONCENTRATION

15 MINUTES PRIOR CONTACT OF ENZYME WITH INHIBITOR AT 40° C.

Concentration of neostigmine in 50 ml. test digest $\times 10^{-8}$ M	A_{15} value		Inhibition per cent.
	Inhibitor absent	Inhibitor present	
5·38	2·29	1·16	49·3
5·68	2·36	1·16	50·1
5·98	2·20	1·06	51·8
6·28	2·28	1·02	55·2
6·58	2·30	1·01	56·1

serum. (2) The process is not very accurate (rather more than 10 per cent. error). It had, however, been noticed that simultaneous replicates gave closer results than successive replicates. Further, by comparing simultaneously a standard injection (of accurately known strength) with the injection to be assayed, it is possible to dispense with the preparation of a calibration curve. Based upon these considerations a process, perhaps best called a standardisation process rather than an assay, was

devised by means of which, in a single experiment, it can be proved that the strength of a sample of Injection of Neostigmine Methylsulphate does not deviate by more than 10 per cent. from the strength stated on the label.

The Standardisation of Injection of Neostigmine Methylsulphate

The Recommended Process

Prepare standard 1st and 2nd dilutions, as described above, from an accurately prepared aqueous 0·05 per cent. w/v solution (0·5 mg. per ml.) of neostigmine methylsulphate.

Prepare a test 2nd dilution from the injection to be examined, but in this case make the dilutions so that, assuming the strength of the neostigmine methylsulphate stated on the label to be correct, the test 2nd dilution will contain exactly the same concentration of neostigmine as the standard 2nd dilution.

In three tubes prepare three digests each containing 1 ml. cresol red solution, and 1 ml. horse serum, but in A 38·5 ml. of water + 4·5 ml. of standard 2nd dilution, and in B 38·0 ml. of water and 5·0 ml. of test 2nd dilution and C 37·5 ml. of water + 5·5 ml. of standard 2nd dilution. After mixing the contents of each tube, place the tubes in a water bath at 40° C. for 15 minutes before determining the remaining ψChE activity as described above. The injection under test is satisfactory if residual enzyme activity of digest B lies between those of digests A and C, i.e., if the A_{15} value of B lies between the A_{15} value of A and C.

Results

Table III shows the results of applying the proposed Method of Standardisation to a sample of Injection of Neostigmine Methylsulphate containing exactly 0·5 mg. per ml.

Two samples of serum were used, differing considerably in activity, but in each one of the 16 experiments the activity of the injection lay between the results corresponding to 10 per cent. excess and 10 per cent. deficiency.

Discussion

From the results reported inhibition of ψChE is a method which can be applied to the determination of anticholinesterase drugs like neostigmine in the dilute solutions used in pharmacy. 0·1 ml. would be more than sufficient for use in the assay of Injection Neostigmine Methylsulphate. Using a calibration curve relating enzymic inhibition of a particular sample of horse serum to the concentration of neostigmine present, this injection may be assayed with an error of not more than 10 to 15 per cent., depending upon the number of assays carried out. Using the comparison method described, the results recorded in Table III establish that a single experiment is capable of deciding whether a solution containing neostigmine (e.g., the injection) is of the strength stated on the label with less than a 10 per cent. error. It seems reasonable to

TABLE III

RESULTS OBTAINED BY TESTING A SAMPLE OF INJECTION OF NEOSTIGMINE METHYLSULPHATE BY THE PROPOSED METHOD. CONCENTRATION OF ACh = 0·0122 M. TEMPERATURE = 40° C. TIME OF PRIOR INCUBATION = 15 MINUTES

Serum	A_{15} digest containing 4·5 ml. of standard second dilution A	Difference A–B	A_{15} digest containing 5 ml. of test second dilution B	Difference B–C	A_{15} digest containing 5·5 ml. of standard second dilution C
A46750	1·48	0·08	1·40		
			1·35	0·13	1·22
	1·47	0·10	1·37		
			1·31	0·06	1·25
	1·54	0·09	1·45		
			1·40	0·03	1·37
E57060	2·56	0·02	2·54	0·04	2·50
	2·32	0·15	2·17	0·04	2·13
	2·50	0·12	2·38	0·06	2·32
	2·35	0·12	2·23	0·11	2·12
	2·27	0·02	2·25	0·14	2·11
	2·35	0·27	2·08	0·14	1·94
	2·35	0·36	1·99	0·06	1·93
	2·40	0·14	2·26	0·13	2·13
	2·32	0·19	2·13	0·13	2·00
	2·32	0·00	2·32	0·22	2·10

suggest that in the future, enzyme inhibition will become a recognised method for the determination of small quantities of certain drugs in such pharmaceutical preparations as tablets and injections.

SUMMARY

1. The suitability of cholinesterase inhibition as a method of assaying pharmaceutical preparations containing small quantities or low concentrations of certain drugs has been discussed and investigated with solutions containing neostigmine.

2. A method of assay for neostigmine accurate to within 10 to 15 per cent. has been elaborated.

3. A standardisation process has been described capable of ensuring, without the necessity of averaging replicate results, that a sample of Injection of Neostigmine Methylsulphate has a strength not more than 10 per cent. in excess or deficiency of the strength stated on the label.

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DISCUSSION

The paper was presented by the CHAIRMAN.

DR. D. C. GARRATT (Nottingham) said that the assay was really a challenge to the analyst in that the drug was simple in structure and should be quite capable of assay by conventional chemical means or, in small quantities, by physical methods. It would seem that the bacteriostatic prevented normal methods being used.

DR. G. E. FOSTER (Dartford) said he had repeated the authors' work, but he had been concerned about the very small titrations obtained. In his view it would be better to dilute the caustic soda to half strength. He considered the results presented in the paper should have been accorded statistical treatment.

MR. K. L. SMITH (Nottingham) said he was concerned that the authors had omitted to use the tools provided by the statistician. It was usual to apply statistical methods to those assays in which the result could not be established by theory and was subject to some variance. The assay under discussion fell into that category. Having had an opportunity of using the method with mustine hydrochloride, his impression was that the slope of the dosage response curve was much flatter than for neostigmine.

MR. A. R. ROGERS (Brighton) expressed the view that 15 minutes of prior incubation represented a short time in regard to the changing slope of the graph (Fig. 5) and that 25 minutes might not perhaps make the assay unduly long. It would give a better chance of obtaining good reproducibility.

The CHAIRMAN, in reply, said the Statistics Department of the University had been consulted, and the error had been assessed as being less than 10 per cent. If it were desired to carry out an estimation of how much nearer than 10 per cent. one could get, statistics would be required. It would be necessary to do a whole series of triplicates or 20 series of separate determinations. Dr. Foster's suggestion about using a more dilute caustic soda solution was a good one. There was no reason why 25 minutes for the prior incubation period should not be used. Accuracy to within 10 per cent. was obtained by using 15 minutes prior incubation.