Brit. J. Pharmacol. (1954), 9, 370.

SOME OBSERVATIONS ON THE METHOD OF SUPERFUSION

BY

G. W. CAMBRIDGE AND J. A. HOLGATE

From the Department of Physiology, University of Leeds

(RECEIVED MAY 18, 1954)

In 1941 Kwiatkowski reported a new technique for the assay of histamine, the tissue being suspended in air and the appropriate fluid, with or without dissolved drug, being run over the surface of the preparation. This was a development of the method described by Gaddum, Jang and Kwiatkowski (1939) for the detection of sympathomimetic substances in the venous outflow from the perfused rabbit ear. The principle of running fluid containing an active substance over the surface of a tissue suspended in air had been first reported by Finkleman (1930).

The technique described by Kwiatkowski involved the injection of 0.1 ml. of drug solution into a side-arm of the tube carrying the fluid ; for histamine assay the concentration of drug required in the injected solution was in the region of 10 ng./ml. The injection of drugs into a moving stream may lead to errors due to uneven mixing, but this has been avoided in the technique recently described by Gaddum (1953). In this method (superfusion) the flow of fluid over the tissue is interrupted for a given period, the tissue is allowed to drain and the surface is then covered with the drug solution applied from a dropping pipette. The drug is allowed to act for a given period before the flow of superfusing fluid is restarted.

It is claimed that very small quantities (5 drops, 0.2 ml.) of drug solution can be used and that this amount is " a reasonable compromise between the conflicting claims of the accuracy which might be achieved with more drops and the economy which would be achieved with fewer." The concentrations of drug used for histamine assay were 1–4 ng./ml. Spencer (personal communication) has suggested that the accuracy of the assay may be improved by the use of 0.5–0.8 ml. of drug solution, this fluid being applied immediately after stopping the flow of the superfusing fluid, without any period of drainage. Test solution volumes of 0.6 ml. have been chosen by Adam, Hardwick and Spencer (1954). The absence of flowing fluid may sometimes result in a response—possibly from release of choline esters (Magnus, 1930) or from a temperature change.

However, apart from the literature quoted above, there seems to be little information concerning the relationship of the volume of the test solution to the accuracy of the assay, and no direct comparison has been made of the accuracy of the superfusion method with that of the usual bath techniques.

Because we were faced with the problem of assaying very small volumes of test solution, it was of interest to us to obtain information on these points. We have accordingly investigated them, using histamine and the guinea-pig ileum.

With the " large-bath " method (volume 2–5 ml.), it is possible to use volumes of test solution of the order of 0.2–0.5 ml. if the concentration of histamine is sufficient to give a final concentration in the bath of 10 ng./ml. If the " small-bath " method is used, the tissue is surrounded by the undiluted test solution ; the concentration required is in the region of 1–5 ng./ml., but the smallest convenient bath volume for most tissues is 1.5 ml. —a volume considerably in excess of that claimed necessary for the superfusion method. It seemed to us that the superfusion method would be advantageous, for only small volumes of test solution are required ; but it was first necessary to determine how the use of such small volumes was related to the accuracy obtained. The only estimates of precision for histamine assays appear to be those determined for the large-bath method by Schild (1942), for the small-bath method by Mongar and Schild (1950) and by Boura, Mongar and Schild (1954), and for the superfusion technique by Adam, Hardwick and Spencer (1954). No comparison of the precision of the small-bath and superfusion methods seems to be available, nor is there any analysis showing the limits of accuracy of superfusion assays using various quantities of test solution. We have accordingly studied the responses of the guinea-pig ileum to histamine, relating them both to the volume of fluid applied

(or the number of drops) and to the duration of drug contact with the tissue, and have compared the response of the superfused preparation with that of a similar tissue in a bath of the smallest convenient volume.

Furthermore, the application of the superfusion method to the assay of substances other than histamine has been studied. The rat colon and the rat uterus preparations have been used for 5-hydroxytryptamine, and the frog rectus for acetylcholine assays, although Kwiatkowski (1941) has reported that the frog rectus is unsuitable for the assay of acetylcholine by the superfusion technique.

METHODS

Histamine Assay on Guinea-pig Ileum

Superfusion Method.—The tissue was suspended in a water-jacketed "Perspex" chamber. The solutions were contained in glass Mariotte bottles, the outflow from these being controlled by electromagnetic clamps acting on small bore rubber tubes (Schild, 1946), and the rate of flow adjusted for each solution by a screw clip placed about the tube below the clamp. From these tubes the fluids ran down a water-jacketed, thin-walled, glass warming-tube and on to a glass finger leading to the thread connecting the tissue to the writing lever. The glass finger was arranged to be within the confines of the tissue chamber to reduce temperature variation from cooling of the fluids by air currents.

Bath Method.—A glass bath of 2.0 ml. capacity and water-jacketed was used. The inflow to the bath was exactly as for superfusion except that the screw clips were not used and the required amount of fluid to fill the bath was controlled by limiting the time during which the electromagnetic clamps remained open. The bath was emptied from the bottom, the suction being under control of a further clamp.

The administration of drug solutions and superfusion or bath fluid was automatic, separate timer circuits being available to control each operation independently.

A frontal writing lever was used; it gave a 13 : 1 magnification and was weighted between 0.5 and 0.75 g. excluding the weight of the tissue. Sections of the ileum within about 1 in. of the ileo-caecal junction were obtained from female virgin guinea-pigs weighing 300–400 g. The mammalian Tyrode solution, oxygenated by bubbling O_2 through the bath or Mariotte bottle, had the following composition (g./l.): NaCl 8.0, KCl 0.2, $CaCl_2$ 0.2, $MgCl_2$ 0.1, $NaHCO_3$ 1.0, NaH_2PO_4 0.05, and glucose 1.0. The temperature of all fluids running over the tissue was 27–28° C., but in the bath experiments it was 30° C. Values for histamine, used as the acid phosphate, are in terms of the base taken as 36.16% of the salt.

Experiments to investigate the relationship between the accuracy of the assay and the amount of drug given (or the duration of exposure of the tissue to the drug) were designed as follows:

(a) *Superfusion without Cessation of Fluid Flow.*—Here the tissue was exposed to 4 different concentrations of drug for different time intervals, namely 5, 10, 15, 20, 25, 30, 35, and 40 sec., the exposure being preceded and immediately followed by administration of the superfusing fluid without any cessation of flow. In this, as in all experiments, both drug concentration and times of exposure of the tissue to the drug were arranged in random block design of 4×4 for each of the 8 time intervals. Since the rate of flow of each of the solutions was the same throughout the experiment, and constant at 1 drop/sec., and since approximately 20 drops were equivalent to 1 ml., times and volumes are related.

(b) *Superfusion with Cessation of Fluid Flow.*—In this type of experiment the drug administration was preceded by a 10 sec. interval and was followed by a 30 sec. interval when no solution flowed over the surface of the tissue. Controls were performed to ascertain that no spontaneous contraction was induced by these "dry" intervals. Each drug solution was administered in different amounts, namely from 5–60 drops (see Table I), the relation between drops and volume being as above, and the same 4×4×8 block design being used.

(c) *Bath Method.*—Here the volume of fluid in the bath was always the same—1.5 ml. in the presence of the tissue. Times of exposure to the drug solutions varied in 10 sec. intervals from 10 to 60 sec., and any one administration of the drug was immediately followed by two washes with Tyrode solution. The 3 doses were given in a block design.

Acetylcholine Assay on the Frog Rectus

The superfusion method was used as in (a) and (b) above; the apparatus was the same, but the assay was conducted at room temperature. The frog rectus was prepared in the usual way and used either directly or after treatment for 30 min. with 2.0 μg./ml. eserine sulphate in Ringer solution; when so treated the superfusion fluid also contained eserine. The oxygenated amphibian Ringer solution had the following composition (g./l.): NaCl 6.5, KCl 0.14, $CaCl_2$ 0.12, $NaHCO_3$ 0.2, and NaH_2PO_4 0.01. The frontal writing lever (13 : 1) was loaded at 1.0 g. Acetylcholine was used as the bromide and the values are given in terms of the salt.

5-Hydroxytryptamine Assay on the Rat Uterus

Attempts were made to assay 5-hydroxytryptamine on the superfused rat uterus with the apparatus described for the histamine assay, but several modifications were necessary to ensure uniform responses. Very slight changes in temperature produced erratic spontaneous contractions. Although the temperature variation of about 0.5° C., obtained in the change over from superfusion fluid to drug solution, did not affect the ileum preparation, it was too great to permit uniformity of response of the uterus.

The final design to ensure constancy of temperature and a constant rate of fluid flow was as follows. The drug solutions were placed in thin-walled, stoppered tubes immersed in a water-bath. Two "polythene" tubes passed through the stoppers, one (2.0 mm. internal

diameter) being connected via an electromagnetic clamp to a source of air pressure, and the other (0.5 mm. internal diameter) reaching the bottom of the container. This latter tube conducted the warmed drug solution to the top of the thin-walled, water-jacketed, glass tube which was arranged to end well inside a large water-jacketed chamber, and the drops were conducted by a glass finger to a thread connecting the tissue to the lever. The chamber was covered with a slotted "Perspex" disc. The superfusion fluid was contained in a large reservoir and passed through a warming coil in the bath and hence to the top of the water-jacketed tube. By this means the temperature inside the bath was kept constant within 0.1° C., both in the presence and in the temporary absence of fluid, over a range of temperature extending up to 36° C. The rate of flow of solutions could be kept constant or, if required, varied within wide limits without affecting the temperature of the fluids reaching the tissue. The alteration in rate of flow of solutions was made by increasing the level of water in a simple escape valve, thus increasing the air pressure delivered to the individual drug bottles. Since all bottles were fed from a common air source such alteration affected each to the same degree.

Results and Discussion

Histamine Assay on Guinea-pig Ileum

Results, which are typical of many experiments, are shown in Table I and Figs. 1 and 2 and illustrate most of the points raised in this study.

Relationship Between the Amount of Drug and the Response.—(i) From Fig. 1, graph (a). Increasing the amount of drug applied to the tissue, i.e., increasing the volume of any one test solution, produces an increased response finally reaching a maximum. In graph (a) the dose-response curves for 20, 25, 30, 35 and 40 drops are not significantly different.

(ii) This increase in response is often accompanied by a change in the slope of the dose-response curve, and in some experiments, as illustrated here (see Table I (a)), there is a deviation from linearity of response—in all instances "convex upwards"—since the increment may not be equal for all doses.

(iii) From Fig. 2, line (a). The increased response is accompanied by a decrease in the precision coefficient (s/b). This is high when small amounts of test solution are used and reaches an approximately constant value once a certain amount of drug has been applied or once the time of application has exceeded a certain value—25 drops or 25 sec.

As the application of greater volumes of drug involves longer exposure to the drug it is necessary to determine whether the increased response is simply due to the period of drug contact or if

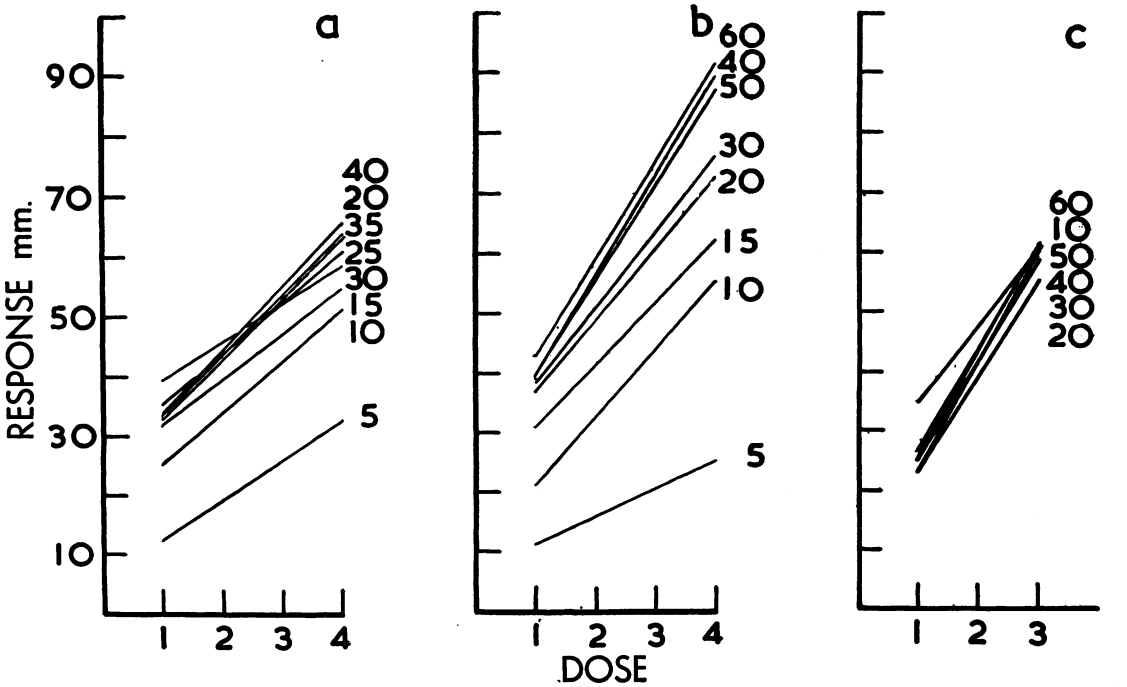

FIG. 1.—Dose-response curves for histamine assay using the guinea-pig ileum. (a) Superfusion without cessation of flow, (b) Superfusion with cessation of flow, (c) Bath method. Dose is given in the form of the dose metameter: 1 unit = 3.6 ng./ml. of histamine base. Figures to the right of graphs (a) and (c) give times of drug contact in sec.; in (a) and (b) these figures also represent the number of drops applied.

TABLE I

RESULTS OF HISTAMINE ASSAYS ON GUINEA-PIG ILEUM BY THREE METHODS; FOR DETAILS SEE TEXT

Time column refers either to time of drug contact in sec., or number of drops applied, depending upon method. Doses are on an arithmetic scale and $\bar{x}$ refers to the mean dose metameter for either 4 or 3 doses

Technique	Time in Sec. or Drops	$\bar{x}$	$\bar{y}$	b		s/b	P Deviation from Linearity	λ (s/b with Log Scale of Doses)
Superfusion without cessation of fluid flow (a)	5		22·4	6·6	6·58	0·997	0·2–0·1	0·189
	10		38·0	8·4	7·31	0·87	>0·2	0·173
	15		43·3	7·6	6·19	0·81	0·2–0·1	0·164
	20		48·2	10·4	3·95	0·38	0·05–0·01	0·072
	25	2·5	47·8	8·3	3·81	0·46	0·05–0·01	0·088
	30		49·0	6·2	2·51	0·40	=0·1	0·079
	35		48·0	9·7	4·32	0·45	>0·2	0·089
	40		49·8	10·6	3·54	0·33	0·05–0·01	0·065
Superfusion with cessation of fluid flow (b)	5		18·4	4·8	2·85	0·59	0.1–0·05	0·057
	10		38·5	11·2	4·79	0·43	>0·2	0·064
	15		48·3	10·8	2·83	0·26	>0·2	0·027
	20		54·6	12·0	3·63	0·30	=0·1	0·026
	30	2·5	57·9	12·7	3·86	0·30	>0·2	0·038
	40		64·6	16·7	5·56	0·33	>0·2	0·035
	50		64·1	16·7	3·99	0·24	>0·2	0·032
	60		67·3	16·2	4·84	0·30	>0·2	0·043
Bath (c)	10		43·8	17·6	4·04	0·23	>0·2	0·083
	20		39·5	16·5	5·51	0·33	>0·2	0·063
	30		41·8	16·4	6·19	0·38	>0·2	0·087
	40	2·0	40·9	17·6	5·00	0·28	>0·2	0·103
	50		42·1	17·0	2·83	0·17	0·2–0·1	0·085
	60		43·2	18·1	3·70	0·20	0·1–0·05	0·049

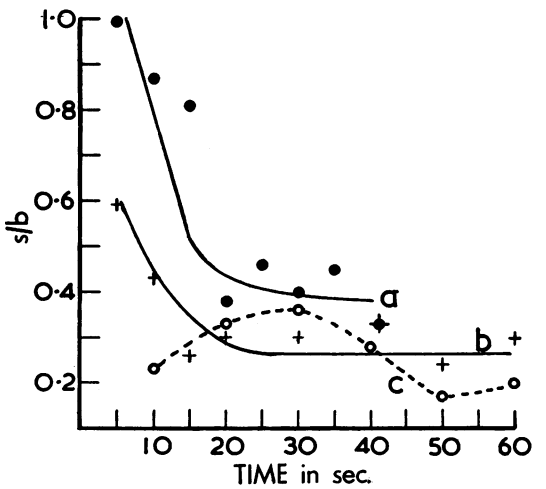

FIG. 2.—Histamine on guinea-pig ileum. Graph relating precision coefficient (s/b) to duration of drug contact in sec., or no. of drops applied (depending on method). Lines a, b, and c indicate methods referred to in text and Fig. 1.

it is only affected by the amount of drug. The response to the application of a small quantity of drug might have been larger had not the superfusion fluid been re-admitted, thus washing away the drug before it had completed its action.

Experiment (b) was designed to elucidate this point. Here, although the volumes used varied (Table I), the tissue was in contact with the drug for at least 35 sec., a period much in excess of the 20 sec. point at which the previous maximal responses were obtained. If the increased response was simply due to the time of exposure to the drug then the period of 35 sec. should allow adequate time for maximal response. Fig. 1 (b) and Fig. 2, line (b), show, however, that there was an increase in the response with increasing amounts of drug, indicating that it is this quantity which is of significance and not merely the time of drug contact. In this particular experiment deviations from linearity did not appear (Table I (b)), although the unequal increments produced by increasing the time of exposure at the different dose levels led to alterations of slope. Again the value of the precision coefficient decreases to a minimum at about the 15–20 sec. period, the quantity of drug administered being 0.75–1.0 ml.

It appears from these results that the quantity of drug administered, rather than the time interval during which the contraction was allowed to occur, was the variable which led to the increased magnitude of contraction and increased precision.

This hypothesis was confirmed, as can be seen by reference to Fig. 1 (c) and Fig. 2, line (c). In the bath method the tissue was immersed in a constant volume of drug solution, the time of contact being varied. Here there is neither increase in the magnitude of contraction with increase in time, the dose-response curves not being significantly different, nor any clear trend in the values of the precision coefficient.

In Table I the precision coefficient has been calculated on the basis of doses arithmetically spaced, since, in practice, this gradation provided

a means of using 4 doses of drug within a four-fold range of concentration, which appears to be a convenient limit for linearity of response. The s/b values are, therefore, not comparable with those calculated by Schild (1942), Boura *et al.* (1954) and Adam *et al.* (1954). We have recalculated our s/b values on the basis of log dose, the results being given in Table I, and they show precisely the same trends as those calculated on the arithmetic basis. The mean minimal value for experiment (b) is 0.033, this being comparable with those given by these authors.

(iv) It may be noted by reference to Fig. 1 that the dose range used in these experiments was from 3 to 14 ng./ml., this being 3–5 times that employed by Gaddum (1953) and Adam *et al.* (1954). It is possible that this relative insensitivity was due to the use of large guinea-pigs, as suggested by the latter authors, and to the low working range of temperature.

It is clear that the use of very small volumes of drug solution will not give as great precision as larger volumes, and this means that the degree of economy of test fluid must be weighed against the precision desired. In our experience, for histamine assay on the guinea-pig ileum by the method of superfusion with cessation of fluid flow, 0.75–1.0 ml. of fluid is required to reach optimal levels of precision.

Further Points of Experimental Technique.— From experiments of the type (b) we are of the opinion that a 10 sec. interval before, and a 30 sec. interval following, drug contact can be allowed without the occurrence of spontaneous contractions This is in agreement with the finding of Gaddum (1953), but is contrary to that of Adam *et al.* (1954), the explanation possibly being associated with the low working temperature and relative insensitivity of the preparations in our experiments. No atropine has been added to the Tyrode solution in these experiments.

The records obtained by the method of superfusion are easier to interpret than those from experiments using the bath method; movements of the recording system due to washing out are eliminated. This is of particular value when testing solutions which may contain substances giving a delayed response.

The longevity of the tissue, in so far as consistency of response is concerned, seems much greater in the superfusion method. As many as 200 separate drug administrations have been performed on several occasions without decrement or significant alteration of response.

Acetylcholine Assay on the Frog Rectus

It was of interest to see if the slow contraction of this type of muscle would influence the relationship between duration of drug contact and response. The tracing (Fig. 3), the dose-response

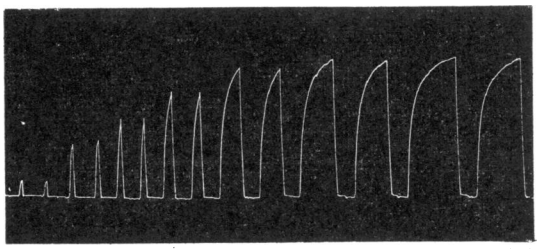

FIG. 3.—Response of superfused frog rectus preparation to 0.2 μg./ml. ACh bromide: frontal writing lever. Responses are in pairs relating to duration of drug contact. Reading from left to right —5, 10, 15, 25, 50, 75, and 100 sec., at 1 drop/sec.

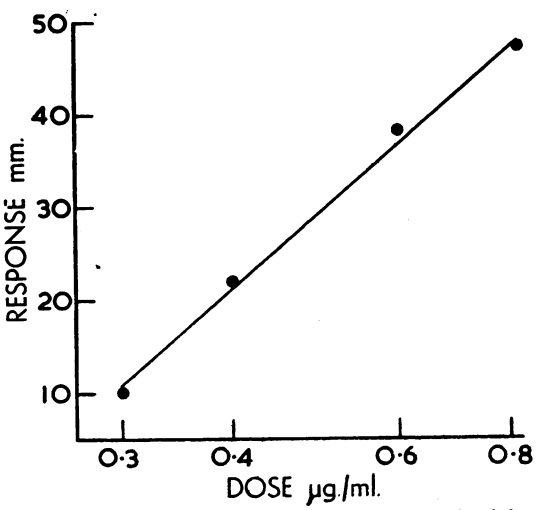

FIG. 4.—ACh dose-response curve obtained from superfused frog rectus preparation (50 sec. drug exposure). Dose is on a log. scale.

curve (Fig. 4) and the analysis of variance (Table II) show the augmentation of response with increasing time of drug contact, the linearity of response over this dose range and the stability of the preparation. The period of drug contact for optimal response appears to be about 50 sec., i.e. 2.5 ml. of fluid. The use of a frontal writing lever permits precise determination of the peak of the response as relaxation occurs promptly when the Ringer fluid is re-admitted. This preparation although requiring 2.5 ml. of test solution does give an extremely accurate and clear-cut assay.

A possible reason for the previous unsuccessful application of the superfusion method to the frog

TABLE II

ANALYSIS OF VARIANCE OF THE ACh ASSAY SHOWN IN FIG. 4

Adjustment for mean: 10384

Nature of Variance	d.f.	Sum of Squares	Mean Square	Variance Ratio	P
Regression ..	1	2,489	2,489	2,999	<0·001
Deviation from linearity ..	2	8	4	4·8	0·1–0·05
Between doses	3	2,497	832	1,002	<0·001
,, runs	2	5	2·5	3·01	0·2–0·1
Error ..	6	5	0·83		
Total ..	11	2,507			
b: 88 Vb: 2·6	Equation: Y=29·42+88 (x−0·6901)				
s/b: 0·01					

rectus emerges from these results. Injection of small quantities of drug into a side-arm of the tube carrying the superfusion fluid, as practised by Kwiatkowski (1941), did not give sufficient duration of contact between drug and tissue to obtain constancy of response.

5-Hydroxytryptamine Assay on the Rat Uterus

Following the modifications to the superfusion apparatus described under " Methods," stability of this preparation with regard to freedom from spontaneous activity on change-over of solutions, or during the period of cessation of fluid flow, has been achieved. Despite this, the rat uterus has not given as satisfactory results as either the guinea-pig ileum or the frog rectus preparations.

When using various tissues for other assays, it has been found that the temperature stabilizing modifications, together with the simple method for simultaneously changing the rates of flow from all bottles, are of great advantage, and have, therefore, been described in this paper.

SUMMARY

1. Experiences of the superfusion method for the assay of histamine, acetylcholine and 5-hydroxytryptamine are described.

2. The relationship between response and the volume of test fluid or the duration of application of test fluid to the tissue has been investigated for the histamine assay on the guinea-pig ileum and the acetylcholine assay on the frog rectus preparations.

3. Precision coefficients have been determined for these relationships in the assay of histamine. The significance of these findings together with other points of technique are discussed.

One of us (G. W. C.) is grateful to Dr. H. Cullumbine and to the Ministry of Supply for facilities afforded in the Porton laboratories, and to Mr. K. E. V. Spencer for introduction to the method of superfusion.

We also thank Dr. Merrell E. Speeter of the Upjohn Company, Kalamazoo, U.S.A., for a generous supply of 5-hydroxytryptamine creatinine sulphate.

REFERENCES

Adam, H. M., Hardwick, D. C., and Spencer, K. E. V. (1954). *Brit. J. Pharmacol.*, **9**, 360.

Boura, A., Mongar, J. L., and Schild, H. O. (1954). Ibid., **9**, 24.

Finkleman, B. (1930). *J. Physiol.*, **70**, 145.

Gaddum, J. H. (1953). *Brit. J. Pharmacol.*, **8**, 321.

—— Jang, C. S., and Kwiatkowski, H. (1939). *J. Physiol.*, **96**, 104.

Kwiatkowski, H. (1941). Ibid., **100**, 147.

Magnus, R. (1930). Lane Lectures. *Stanford Univ. Publ. med. Sci.*, **2**, 307.

Mongar, J. L., and Schild, H. O. (1950). *J. Physiol.*, **111**, 47*P*.

Schild, H. O. (1942). Ibid., **101**, 115.

—— (1946). *Brit. J. Pharmacol.*, **1**, 135.