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ACETYLCHOLINE ACTIVITY IN THE SCIATIC NERVE*

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Abstract—As measured on both the guinea pig ileum and the frog rectus abdominis muscle, acetylcholine (Ach) is uniformly distributed along the sciatic nerve. Measured on the ileum, about 75 per cent of the Ach activity is found in the soluble portion of the cell, while on the rectus abdominis, the corresponding figure is about 35 per cent. Total Ach equivalents in sciatic nerve are higher when measured on the rectus abdominis muscle than on the ileum. Evidence is presented that the activity measured on the ileum is attributable to either Ach or a very similar substance. It is suggested that sciatic nerve contains a substance other than Ach to which the frog rectus abdominis muscle is especially sensitive.

MOST of the acetylcholine-like activity of brain is associated with particulate material that appears to contain synaptic vesicles.[1,2] Comparable studies on peripheral nerve are lacking. In this paper are described observations on the acetylcholine (Ach) activity in fractions obtained after centrifugation of homogenates of the sciatic nerve.

Also presented are measurements of the distribution of Ach activity along segments of the sciatic nerve. These measurements were prompted by previous studies showing a non-uniform distribution of several substances along the axon. Thus, norephinephrine is present in higher concentration in nerve endings than in the nerve trunk[3] and, conversely, the concentration of homarine decreases from the proximal to the distal portion of the squid axon.[4]

A preliminary account of this work has been published.[5]

METHODS

Rabbits that had been anesthetized with an intraperitoneal injection of sodium pentobarbital (30 mg/kg body weight), unless stated otherwise, were injected intraperitoneally with physostigmine (1 mg/kg body weight). After about 10 min, when the animals were salivating, air (10 ml) was injected into the marginal ear vein; the animals died without convulsing. In a refrigerated room both sciatic nerves were removed. The nerve was cut on its emergence from the spinal cord, and the main branches (the peroneal and tibial nerves) were cut just before they entered the foot. The length of the nerves varied from 15 to 18 cm, and each weighed about 0·5 g. For the study of the regional distribution of Ach, the nerve was divided into three pieces of equal length, the proximal, medial, and distal portions.

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Homogenization and fractionation of nerves

In a refrigerated room the whole nerve or its portions was homogenized with a Teflon pestle and glass homogenizing tube in 3 to 5 ml of 0·3 M sucrose containing, in most experiments, physostigmine (0·35 or 14 μg/ml). For the studies of the subcellular distribution of Ach activity, the homogenates were submitted to centrifugation at 1,200 $\times$ *g* for 20 min; the resultant sediment is referred to herein as nuclei and debris (*N*). The supernatant suspension was centrifuged at 100,000 $\times$ *g* for 60 min. The sediment thus obtained, called the particulate fraction (*P*), was separated from the clear supernatant solution (*S*).

Extraction of Ach activity

In experiments in which the sucrose solution contained 0·35 μg or less of physostigmine/ml, the fractions were tested either directly in suspension or after extraction with acetone. The fraction was suspended in 20 volumes of acetone at room temperature for 1 hr. After centrifugation the supernatant material was collected and the acetone evaporated under a stream of nitrogen; the resultant residue was suspended in 1 to 3 ml of Tyrode's solution and assayed. Control experiments showed that recovery of known Ach by this method ranged from 76 to 92 per cent. Extraction of the acetone — precipitable material with 5% trichloroacetic acid (TCA) did not yield additional Ach activity.

In some experiments a solution of TCA was used to extract Ach from the sciatic nerve. After the nerve was homogenized as described in isotonic sucrose containing 14 μg physostigmine/ml, 0·1 μc of radioactive acetylcholine-methyl-^{14}C iodide (specific activity 5·18 mc/mmole; New England Nuclear Corp.) and an equal volume of 10% TCA were added to the suspension, which was then centrifuged. As a control, the same quantity of radioactive Ach was dissolved in an identical mixture of sucrose and TCA, but without sciatic nerve tissue. The solutions were extracted three times with two volumes of diethyl ether to remove TCA. The aqueous extracts were reduced to a volume of 0·1 ml by a stream of nitrogen, and the residue was suspended in 0·2 ml of water and chromatographed, as described. Corresponding acetone extracts of radioactive Ach, with and without sciatic nerve tissue, were carried through the same procedure.

Paper chromatography

In experiments in which the sucrose solution contained 14 μg of physostigmine/ml, the extract was submitted to descending paper chromatography on Whatman 3MM paper, in a solvent system of water-saturated *n*-butanol. In this system Ach has an R_f value of 0·12 and physostigmine an R_f of 0·73. Routinely, after drying the chromatogram, a portion of the chromatogram, R_f 0·00 to 0·20, was eluted with 4 ml of Tyrode's solution and the eluate assayed; known amounts of Ach were run routinely as controls in these procedures; an average recovery of 75 per cent of added Ach was obtained. In some experiments the paper chromatogram was cut into strips of 6 cm each, which were eluted with 4 ml of Tyrode's solution and assayed.

Radioactivity was measured with a Vanguard automatic chromatogram scanner.

Bioassay

Unless otherwise stated, Ach activity was measured on the isolated guinea pig ileum. In some experiments the frog rectus abdominis muscle was used. The tissues were suspended in a 5-ml bath. The ileum was maintained at 35° in a medium containing aerated Tyrode's solution to which pyrilamine maleate (5 ng/ml), and lysergic acid diethylamide (50 ng/ml), had been added.[6] The frog rectus abdominis muscle was used at room temperature in a bath containing an aerated solution appropriate for frog tissue; each liter contained 6·4 g NaCl, 0·3 g KCl, 0·17 g $CaCl_2$, 0·35 g $NaHCO_3$, 0·7 g glucose, and 10 mg physostigmine. Each dose of the extract and of known Ach was allowed to remain in contact with the frog muscle for 2 min, after which the bathing fluid was changed twice; the interval between doses was 4 min. Contractions were recorded with a model M5P Mini-Polygraph (Gilson Medical Electronics) and a force-displacement transducer, F3–03 (Grass Instrument Co.). The amount of Ach was determined by the method of approximation[7] and expressed as equivalents of Ach, as free base, corrected for the losses occurring during extraction.

Other methods

Protein was measured by the method of Lowry *et al.*[8] and radioactivity of extracts was measured in a liquid scintillation counter.

RESULTS

Paper chromatography of Ach extracted with acetone and with TCA

Table 1 shows that Ach-^{14}C extracted with acetone, either in the presence or absence of homogenates of nerve tissue, showed an R_f value of 0·12. The Ach-^{14}C treated with

TABLE 1. PAPER CHROMATOGRAPHY IN WATER-SATURATED *n*-BUTANOL OF ACETYLCHOLINE-^{14}C AND OF NERVE EXTRACTED WITH EITHER ACETONE OR TRICHLOROACETIC ACID

Extract	R_f	Radioactivity (%)	Biological activity*
Acetone			
Ach-^{14}C alone	0·12	100	100
Ach-^{14}C and sciatic nerve	0·12	100	100
Trichloroacetic acid			
Ach-^{14}C alone	0·12	72	78
	0·52	28	22
Ach-^{14}C and sciatic nerve	0·52	100	100

* Measured on the guinea pig ileum.

trichloroacetic acid showed an additional zone of radioactivity at R_f 0·52; added to homogenates of nerve before extraction, Ach-^{14}C showed an area of radioactivity only at R_f 0·52. In all instances biological activity, as measured on the guinea pig ileum, was associated with radioactivity, and zones without radioactivity were inactive on this preparation. These observations suggest that the ileum is responding to material in nerve which is identical with or very similar to Ach.

In all the following experiments, acetone was used to extract Ach activity from nerve.

Ach activity in sciatic nerve as measured on the guinea pig ileum (Table 2)

When physostigmine was not administered to the animal and, concomitantly, physostigmine was omitted from the homogenizing medium, no Ach activity was detected in the nerve extract. Treatment of the rabbit with physostigmine resulted in

TABLE 2. ACETYLCHOLINE EQUIVALENTS IN NERVE EXTRACTED IN THE PRESENCE OF VARYING CONCENTRATIONS OF PHYSOSTIGMINE

Physostigmine			Ach equivalents*	
Injected (mg/kg)	0·3 M sucrose (μg/ml)	Method of extraction	(μg/g wet weight)	(μg/g protein)
0	0	None	0	0
1	0	None	0·17	3·0
1	0·35	None	0·46 ± 0·07	8·6
1	0·35	Acetone	0·44 ± 0·04	8·0
1	14·0	Acetone, paper chromatog.	1·53 ± 0·43	28·0
1	28·0	Acetone, paper chromatog.	1·62	29·0

* Measured on guinea pig ileum. Values are means ± standard deviation.

the recovery of a small amount of Ach activity—0·17 μg/g of wet weight or 3·0 μg/g of protein. The recovery was increased when the nerves, removed from physostigmine-treated animals, were homogenized and extracted in an isotonic sucrose solution containing 0·35 μg physostigmine/ml. It is noteworthy that the level of Ach activity, 0·46 μg/g wet weight, obtained by directly testing the sucrose homogenates, did not differ significantly from the value obtained after extracting the sucrose homogenates with acetone—0·44 μg/g of wet weight.

When the concentration of physostigmine in the sucrose solution was increased to 14 μg/ml, it was necessary to subject the acetone extract to paper chromatography in order to separate physostigmine from Ach activity, because this concentration of physostigmine interfered with the assay of Ach activity on the ileum by causing strong spontaneous contractions and an increased tone. With this concentration of physostigmine, which has been used by Whittaker,[9] the yield of Ach activity was increased to 1·53 μg/g wet weight or 28·0 μg/g protein. A further increase in the concentration of physostigmine did not produce a significant increase in the yield of Ach activity.

The similarity of action on the guinea pig ileum of nerve Ach activity and known Ach

Known Ach was compared with the Ach activity extracted from the nerves of physostigmine-treated animals. Sucrose homogenates containing 0·35 μg physostigmine/ml were tested both directly and after acetone extraction. The acetone extracts of nerves homogenized in the presence of 14 μg physostigmine/ml were chromatographed, and the material that was eluted in the area of R_f 0·00 to 0·20 was tested.

The slopes of the dose–response curve for the extracts and for known Ach were identical. The contractions induced by the extracts and by known Ach were prevented by the same dose of atropine, 6 ng/ml. The time of recovery of the ileum after atropine

was the same whether extracts or known Ach was tested. Both the extracts and known Ach were inactivated by heating at pH 10 for 5 min in a boiling-water bath. The extracts and known Ach were inactivated by incubation with acetylcholinesterase from bovine erythrocytes (Fig. 1).

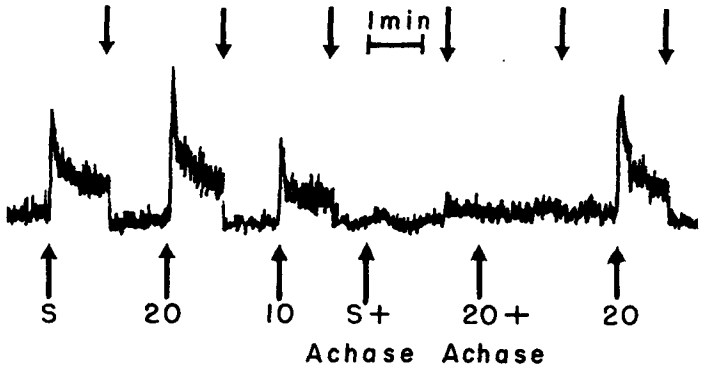

FIG. 1. The inactivation by acetylcholinesterase of Ach activity in the suprnatant fraction of sciatic nerve. The supernatant fraction or known Ach in 0·3 M sucrose containing 0·35 μg physostigmine/ml was incubated for 1 hr at 37° with 500 ng of acetylcholinesterase from bovine erythrocytes. S: Supernatant fraction without incubation; S + Achase: supernatant fraction after incubation. Numbers: ng of acetylcholine; 20 + Achase: 20 ng of acetylcholine after incubation with acetylcholinesterase.

Regional distribution of Ach activity as measured on the guinea pig ileum

No striking differences in the concentration of Ach activity were seen in the proximal, medial, and distal segments of the nerve, despite the variation in total content because of differences in the concentration of physostigmine (Table 3).

TABLE 3. REGIONAL DISTRIBUTION OF ACETYLCHOLINE ACTIVITY* IN NERVE TREATED WITH DIFFERENT CONCENTRATIONS OF PHYSOSTIGMINE

Physostigmine in 0·3 M sucrose	Method of extraction	Ach equivalent Wet wt. (μg/g)	Ach equivalent Protein (μg/g)	Region	Distribution Wet wt. (%)	Distribution Protein (%)
0·35	None	0·48	8·7	Prox.	28·0	28·6
				Med.	39·1	31·5
				Dist.	32·0	39·9
14·0	Acetone, paper chromatog.	1·57 ± 0·19	28·5 ± 4·1	Prox.	35·1 ± 6·8	33·6 ± 5·9
				Med.	33·4 ± 4·8	26·4 ± 6·1
				Dist.	31·5 ± 9·2	40·0 ± 8·9

* Measured on the guinea pig ileum. Values are means ± standard deviation.

Subcellular distribution of Ach activity as measured on the guinea pig ileum

When whole nerves were fractionated, the bulk of Ach activity was found in the supernatant fraction, whether the nerves were treated with a low or high concentration of physostigmine (Table 4).

This pattern of subcellular distribution in whole nerve also was encountered in the nerve segments. Table 5 shows that most of the Ach activity is found in the supernatant fraction of the proximal, medial, and distal segments.

TABLE 4. SUBCELLULAR DISTRIBUTION OF ACETYLCHOLINE ACTIVITY* IN NERVE TREATED WITH DIFFERENT CONCENTRATIONS OF PHYSOSTIGMINE

Physostigmine in 0·3 M sucrose	Method of extraction	Ach equivalents Wet wt. (μg/g)	Ach equivalents Protein (μg/g)	Distribution (%) Wet wt. $N+P$†	Wet wt. S†	Protein $N+P$	Protein S
0·35	Acetone	0·50 ± 0·08	9·0 ± 1·7	20·7 ± 3·1	79·3 ± 4·7	13·6 ± 3·0	86·4 ± 4·8
14·0	Acetone, paper chromatog.	1·54	28·0	30·9	69·1	16·7	83·8

* Measured on the guinea pig ileum. Values are means ± standard deviation.
† N, nuclei and debris; P, particulate fraction; S, supernatant solution.

TABLE 5. SUBCELLULAR DISTRIBUTION OF ACETYCHOLINE ACTIVITY IN NERVE SEGMENTS TREATED WITH DIFFERENT CONCENTRATIONS OF PHYSOSTIGMINE

Physostigmine in 0·3 M sucrose	Method of extraction	Ach equivalents* (μg/g wet weight)	Region	Regional distribution (%)	Subcellular distribution (%) N	P	S
0·35	Acetone	0·34 ± 0·06	Prox.	31·4 ± 5·1	6·4	4·7	20·2
			Med.	39·5 ± 7·1	5·1	8·4	26·0
			Dist.	29·1 ± 8·5	2·2	2·5	24·4
14	Acetone, paper chromatog.	1·33 ± 0·23	Prox.	32·1 ± 11·3	5·2	2·5	24·4
			Med.	40·7 ± 3·6	7·9	2·9	29·9
			Dist.	27·2 ± 10·2	3·3	2·1	21·8

* Measured on the guinea pig ileum. Values are means ± standard deviation.

To test the possibility that Ach activity is present in the particulate fractions in a strongly bound form that is not released by acetone and therefore inactive upon the ileum, the particulate fractions were subjected to treatments that are known to release particle-bound substances.[10] Freezing and thawing five times, incubation in 0·1 N H_2SO_4 for 10 min at room temperature, incubation in water for 1 hr at 37°, shaking for 5 min with two volumes of diethyl ether (which was removed under nitrogen), and extraction in 5% TCA failed to increase the Ach activity of the particulate fractions.

The total particulate fraction was tested for its capacity to sequester soluble Ach by adding 0·1 μc radioactive Ach to homogenates of nerve in sucrose containing 14 μg physostigmine/ml. Acetone extracts of the total particulate fraction, N and P, contained 9·8 per cent of the radioactive Ach.

Subcellular distribution of Ach activity as measured on the guinea pig ileum and frog rectus abdominis muscle

The distribution of Ach in subcellular fractions of nerve segments homogenized in the presence of 14 μg physostigmine/ml of 0·3 M sucrose was determined with both the guinea pig ileum and the frog rectus abdominis muscle (Table 6). Measured with the guinea pig ileum, Ach equivalents were 1·57 μg/g weight, while measurements with

TABLE 6. REGIONAL AND SUBCELLULAR DISTRIBUTION OF ACETYLCHOLINE ACTIVITY AS MEASURED ON THE GUINEA PIG ILEUM AND THE FROG RECTUS ABDOMINUS MUSCLE

	Guinea pig ileum	Frog rectus abdominis
Acetylcholine equivalents* (μg/g wet weight)	1·57 ± 0·41	2·55 ± 0·43
Distribution (percentage)*		
Proximal portion		
Nuclei and debris	5·5 ± 1·2	10·8 ± 7·1
Particles	2·8 ± 1·0	10·2 ± 3·3
Soluble fraction	26·8 ± 2·2	15·7 ± 6·3
Medial portion		
Nuclei and debris	6·6 ± 3·7	11·4 ± 3·8
Particles	2·3 ± 1·9	12·4 ± 3·0
Soluble fraction	24·5 ± 5·8	11·1 ± 5·2
Distal portion		
Nuclei and debris	2·8 ± 1·0	14·4 ± 6·6
Particles	2·5 ± 1·2	8·4 ± 4·0
Soluble fraction	26·2 ± 2·6	5·6 ± 3·1

* Values are means ± standard deviations.

the frog muscle gave a value of 2·55 μg/g wet weight. Although the regional distribution was uniform when measured with either preparation, the subcellular distribution obtained from measurements with the guinea pig ileum differed from those obtained with the frog rectus abdominis muscle: as measured with the ileum, over 75 per cent of the total Ach activity was present in the soluble portion, whereas the assays with frog muscle indicated that less than 35 per cent was present in the soluble fraction.

To determine whether this discrepancy was attributable to the presence of physostigmine in the extracts tested on the frog muscle, 0·5 mg physostigmine was added to the bath (in addition to that already present in the Tyrode's solution). This concentration of physostigmine did not itself affect the muscle nor did it influence the action of the extract or of known Ach.

In order to examine the extracts for material that might have affected the response of the muscles to the extracts or to known Ach, the following experiments were performed. Untreated extracts and extracts heated at pH 10 for 5 min in a boiling-water bath (a procedure that inactivated known Ach) were tested on both the ileum and the rectus abdominis muscle. The contraction induced by the untreated extract when tested alone was additive to the contraction induced by the same extract to which known Ach was added. The alkaline-heated extract to which known Ach was added caused the same height of contraction as did the identical amount of Ach without the extract.

When Ach activity was extracted from nerve homogenized in sucrose devoid of physostigmine, no Ach-like activity was detectable on the frog rectus abdominis muscle.

DISCUSSION

Acetone quantitatively extracted Ach activity from nerve homogenates. It was used in this work because on paper chromatography, acetone extracts gave a single spot for Ach activity and for known Ach. TCA extracts, on the other hand, caused the formation of a second spot (Table 1). The finding of this artifact, recently reported by others,[11] complements previous work showing differences in R_f values of the chloride, bromide, sulfate, and perchlorate of choline esters, including acetylcholine.[12, 13] TCA also causes artifacts in the paper chromatography of catecholamines, tryptamines, and imidazoles.[14]

Ach activity was uniformly distributed along the course of the nerves, whether these were homogenized in low or high concentrations of physostigmine (Tables 3 and 5). The uniform distribution of Ach activity in the nerve containing significant cholinesterase activity indirectly supports the finding[15, 16] that cholinesterase activity also is uniformly distributed along the nerve.

As measured on the guinea pig ileum, over 75 per cent of the Ach activity in nerve was found in the soluble fraction (Tables 4, 5 and 6). This value may in fact be even higher since the particulate fraction sequestered some of the added soluble Ach. Earlier work showed that 70 per cent of the Ach activity (measured on the clam heart) in rat sciatic nerve is extracted by Locke's solution,[17] and 80 per cent of Ach activity (measured on the frog rectus abdominis muscle) in the thoracic nerve of the cockroach is extracted with Ringer's solution.[18]

As measured on the frog rectus abdominis muscle, only about 35 per cent of the Ach activity in sciatic nerve is found in the supernatant fraction (Table 6). A further difference in the results obtained with the ileum and the rectus abdominis was the estimate of Ach activity in whole nerve; the ileum recorded 1·57, the rectus abdominis 2·55 μg/g wet weight (Table 6); the latter value is similar to that obtained in experiments in which Ach activity was measured on the leech muscle[19] and frog heart.[20]

The differences obtained with the frog rectus abdominis muscle and the guinea pig ileum cannot be attributed to obvious sources of artifact that have been ruled out in these experiments. It is reasonable to suggest, therefore, that a substance or substances is present in this nerve tissue that has potent action on the rectus abdominis muscle and relatively little action on the ileum. The rectus abdominis is known to be especially sensitive to many choline esters, in contrast to the guinea pig ileum which is relatively insensitive to choline esters other than Ach.[21]

The following observations are consistent with the idea that the guinea pig ileum responded to Ach or a very similar substance in the nerve extract. The slopes of the dose–response curves for the extract and for known Ach were identical. The effect of both the extract and known Ach were prevented by the same dose of atropine, and the atropinized ileum regained its sensitivity to Ach and the extract concomitantly. The actions of both the extract and the known Ach were destroyed by heating in mild alkali and by acetylcholinesterase. Finally, when the extract was chromatographed on paper, all material with activity on the ileum was eluted in the area R_f 0·00 to 0·20, which included known Ach (also see Table 1). Accordingly, a substance other than

Ach is probably responsible for the additional activity that was measured on the rectus abdominis muscle.

Direct evidence has been obtained[22] to support the idea that sciatic nerve contains a substance in addition to Ach, probably a choline ester, to which the rectus abdominis muscle is especially sensitive. First, the action of the nerve extract is less sensitive to D-tubocurarine than is Ach. Second, only 66 per cent of the total activity measured with the frog rectus abdominis muscle is recoverable in the material that is eluted with an R_f of 0·00 to 0·20. That it is a choline ester is further indicated by the necessity of using physostigmine in the extraction procedure to obtain active material. This hypothetical choline ester may be confined to peripheral nerve, for extracts of brain[23] and skeletal muscle[24] showed the same Ach activity when measured on the ileum and rectus abdominis muscle.

The presence of a compound with a potent action of the nicotinic type and only slight muscarinic activity would have important physiological and pharmacological implications. Among them is the likelihood that *some* of the actions traditionally ascribed to Ach may in fact be attributable to another substance. Since this substance is especially active on skeletal muscle and relatively inactive on the ileum, it may be a transmitter at the neuromuscular junction. The relative resistance of this substance (as compared with Ach) to D-tubocurarine parallels the discrepancy sometimes observed between the effectiveness of an agent in blocking the endogenous transmitter and in blocking Ach.[25] This discrepancy has been ascribed to the relative proximity of the endogenous transmitter to the receptor, but it may instead rest on the liberation at the nerve terminal of another choline ester either alone or in combination with Ach. It is also possible that Ach releases the unknown substance at the nerve terminals, in accordance with a presynaptic site of action of Ach, as proposed by Riker[26] and others.[27, 28]

The distribution of Ach in sciatic nerve differs markedly from the distribution in brain, in which most of the Ach is found in particulate material.[1, 2] It is possible that in brain the particle-bound material with Ach activity may not be Ach but another substance.[23] Also, in sciatic nerve the particle-bound Ach (if present at all) may be restricted to the synaptic vesicles that are found at nerve terminals,[2] in apposition to an intermingled with muscle fibers; few, if any, nerve terminals would be present in the portion of the sciatic nerve used in these experiments. It is worthy of note that the subcellular distribution of biogenic amines differs among cells and organs.[6, 10]

REFERENCES

1. V. P. WHITTAKER, *Biochem. Soc. Sympos.* no. 23, 109 (1963).
2. E. DE ROBERTIS, G. RODRIGUEZ DE LORES ARNAIZ, L. SALGANICOFF, A. PELLEGRINO DE IRALDI and L. M. ZIEHER, *J. Neurochem.* **10**, 225 (1963).
3. U. S. VON EULER, *Acta physiol. scand.* **43**, 155 (1958).
4. E. L. GASTEIGER, P. C. HAAKE and J. A. GERGON, *Ann. N. Y. Acad. Sci.* **90**, 622 (1960).
5. E. A. CARLINI and J. P. GREEN, *Fed. Proc.* **22**, 170 (1963).
6. E. A. CARLINI and J. P. GREEN, *Brit. J. Pharmacol.* **20**, 264 (1963).
7. J. H. BURN, *Biological Standardization*, p. 211. London, Oxford Univ. Press (1950).
8. O. H. LOWRY, N. J. ROSENBROUGH, A. L. FARR and R. J. RANDALL, *J. biol. Chem.* **193**, 265 (1951).
9. V. P. WHITTAKER, *Biochem. J.* **72**, 694 (1959).
10. J. P. GREEN, *Advanc. Pharmacol.* **1**, 349 (1962).
11. G. PEPEU, K. F. SCHMIDT and N. J. GIARMAN, *Biochem. Pharmacol.* **12**, 385 (1963).
12. V. P. WHITTAKER and S. WIJESUNDERA, *Biochem. J.* **51**, 348 (1952).

13. E. HEILBRONN, *Acta chem. scand.* **12**, 1481 (1958).
14. B. ROBINSON and D. M. SHEPHERD, *J. Pharm. Pharmacol.* **13**, 374 (1961).
15. E. KOENIG and G. B. KOELLE, *J. Neurochem.* **8**, 169 (1961).
16. D. H. CLOUET and H. WAELSCH, *J. Neurochem.* **8**, 201 (1961).
17. M. PRAJMOVSKY and J. H. WELSH, *J. Neurophysiol.* **11**, 1 (1948).
18. J. M. TOBIAS, J. J. KOLLROS and J. SAVIT, *J. cell. comp. Physiol.* **28**, 159 (1946).
19. G. S. BARSOUM, *J. Physiol. (Lond.)*, **84**, 259 (1935).
20. K. LISSÁK, *Amer. J. Physiol.* **125**, 778 (1939).
21. V. P. WHITTAKER, *Handbuch der experimentellen Pharmakologie* **15**, 1 (1963).
22. E. A. CARLINI and J. P. GREEN, *Science.* **141**, 901 (1963).
23. J. CROSSLAND and P. H. REDFERN, *Life Sci.* **10**, 711 (1963).
24. C. O. HEBB, *J. Physiol. (Lond.)* **163**, 294 (1962).
25. H. H. DALE and J. H. GADDUM, *J. Physiol. (Lond.)* **70**, 109 (1930).
26. W. F. RIKER, *Arch. Neurol. (Chic.)* **3**, 488 (1960).
27. G. WERNER and A. S. KUPERMAN, *Handbuch der experimentellen Pharmakologie* **15**, 570 (1963).
28. G. B. KOELLE, *J. Pharm. Pharmacol.* **14**, 65 (1962).