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CHOLINESTERASE CONTENT OF NORMAL AND DENERVATED SKELETAL MUSCLE IN THE GUINEA-PIG

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The increased sensitivity to applied acetylcholine (ACh) of effectors deprived of their cholinergic innervation has often been attributed to a decrease of the enzyme cholinesterase (ChE) in these tissues. Different organs exhibit various degrees of hypersensitivity. The greatest increase in response to applied ACh occurs in the case of skeletal muscle. Smooth muscle, glands, autonomic ganglion cells and neurones of the spinal cord and brain all develop less intense sensitization. Comparison of the post-denervation changes in ACh sensitivity and ChE activity, in such different structures, soon reveals a serious lack of quantitative correlation between the two. For instance, as Cannon & Rosenblueth (1949) point out, the decline of ChE content in denervated ganglia, or spinal motor neurones, exceeds that in denervated skeletal muscle.

In the case of muscle itself, marked differences between species are reported, but differences of the same magnitude are reported for the same species by different authors. In toad sartorius, Feng & Ting (1938) found ChE concentration decreased by 30% one month after denervation, but did not consider that a significant fall. Rat gastrocnemius has been studied repeatedly. Martini & Torda (1937) found a fall of ChE to less than one-half in 2 weeks. According to Stoerk & Morpeth (1944) the ChE falls by 30% in 4 weeks, and Meng (1940) described a slight fall, which he, however, did not consider significant. Marnay & Nachmansohn (1937) described a rise of ChE concentration in guinea-pig gastrocnemius to 150% of the normal level during the first post-operative month. Leibson (1939) did not observe the rise until the second week in rabbit soleus. From her summary, it appears that the concentration increases up to the sixth week. The level of ChE in rabbit gastrocnemius was measured

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3 months after denervation by Rjabinowskaja (1940). She concluded that the enzyme concentration had increased, but the values cited do not support this statement. For guinea-pig skeletal muscle, Couteaux & Nachmansohn (1940), using the m. gastrocnemius, and Couteaux (1942), using the m. vastus lateralis and m. plantaris, found that the concentration of ChE rose during the first 3 weeks after denervation but during this period of rapid muscle atrophy, the total enzyme content of the muscle was reduced to less than 50% of that present in normal muscle. A preliminary report by Sawyer, Davenport & Alexander (1950) cites an increase of ChE activity in denervated skeletal muscles of rats, rabbits and guinea-pigs.

Unfortunately, none of the above data were reported in a manner amenable to statistical analysis, and it is therefore impossible to assess the significance of the changes. Thus, the work in this field to date has failed either to establish or to rule out a causal relationship between the fall of cholinesterase in a denervated muscle and the concomitant increase in its sensitivity to ACh. We have therefore re-investigated these changes.

METHODS

Guinea-pigs weighing between 250 and 500 g. were used. The m. serratus anterior was chosen for these tests, because experiments on its ACh sensitivity were in progress at our laboratory and it seemed desirable to have information on enzyme changes in the same structure. After the animals were killed by a blow on the head, the tissue was dissected out rapidly, blotted between filter papers and weighed. The thin muscle is suitable for micro-application work (Brooks, 1951), but is sometimes hard to remove entire because of its short tendinous insertions. The resulting sampling error contributes heavily to the standard error (S.E.) of the mean fresh weights, as listed in Table 1. It was minimized by always using the same, easily distinguishable, second and third digitations. Furthermore, paired muscles from the same animals were used in each comparison. The choice of side for denervation was random.

The muscle on one side was denervated by cutting the long thoracic nerve. The proximal part of the nerve was easily exposed by making a ventro-medial skin incision and dividing the underlying muscles. The distal nerve segment was avulsed for a length of 1 cm. to prevent re-innervation.

After removal from the carcass, the muscle was comminuted with scissors and the macerated material was then ground in an ice-cooled Potter homogenizer. Each muscle sample was diluted to 3 ml. with distilled water before grinding and further distilled water was added after the grinding to bring the mixture to a total volume of 5 ml.

The manometric assay was carried out on aliquots of homogenate either immediately or on the next day, the material having been kept in stoppered test-tubes at 0° C. The main chamber of each manometric Warburg vessel contained 1·0 ml. homogenate and 4·0 ml. M/40-bicarbonate buffer (pH 7·4) with the appropriate type and amount of substrate solution in the side-arm as described below. After thermal equilibration, the substrate was tipped into the chamber and the manometers read at intervals of 10 min. for about 1 hr., while the vessels were shaken continuously at 37° C. bath temperature. Vessels containing substrate and bicarbonate buffer only were run with each group as controls. The substrates employed were Mecholyl (DL-acetyl-β-methylcholine chloride) for true ChE, benzoylcholine chloride for pseudo-ChE (Mendel, Mundell & Rudney, 1943), and tributryin for tributyrin ali-esterase. The use of 0·33 ml. of 9% mecholyl provided a final concentration in the reaction mixture of 0·03 M.; 0·17 ml. of 4·5% benzoylcholine yielded a final solution of 0·006 M.; and 0·3 ml. of 5% tributyrin produced 0·01 M. final concentration in the vessel.

RESULTS

Expression of data

For the purpose of this study the most important measurement was that of the total enzyme in each sample of muscle homogenate. This has been expressed as microlitres of CO_2 evolved per hour per total muscle. Normal and denervated samples were compared both as differences and as ratios. In many earlier studies ChE content has been expressed as 'Q' ratio, which represents the number of μl. CO_2 evolved per unit weight of sample. This index is useful where differences between various tissue regions are being studied, but is misleading in the present case because the proportions of water, fat and protein change during atrophy of the muscle. It is, however, of interest to note the effect of expressing the results presented below in terms of the dry weight of the tissue rather than its fresh weight. This was tested on the serratus muscle homogenates by drying aliquots overnight at 80° C. and weighing after five days in the desiccator. A comparison of the fresh and dried weights is given in Table 1.

Tables 2 and 3 contain mean values of enzyme activities, and mean ratios of such quantities. Some ratios have been evaluated as the means of the individual fractions in each of the five groups, and are marked by an asterisk. These means of ratios differ in value from the ratios of the mean values listed in the preceding columns of the tables.

The standard errors of all means (S.E.) were evaluated according to the formula:

$$\text{S.E.} = \sqrt{\frac{S\,(d^2)}{n\,(n-1)}}.$$

Response of m. serratus anterior to denervation

Before the experimental variant, change of ChE activity, was examined, it was thought important to establish that the m. serratus anterior of the guinea-pig underwent the classical changes after denervation. Terminal nerve twigs disappeared during the 10 days following nerve section. The nerve trunk became vacuolated, and its component fibres were no longer clearly distinguishable through the microscope. Fibrillation could be observed under the microscope by reflected light when the muscle was exposed in animals lightly anaesthetized with urethane. Fibrillation was present by the third day in guinea-pigs weighing 250–300 g.; in larger specimens onset was delayed by several days. This confirms the findings of Feinstein, Pattle & Weddell (1945).

Since endplates are easily visible in the exposed muscle, sensitivity to applied acetylcholine chloride was tested in several specimens. Volumes of 0·01 ml. were applied to the muscle surface with a fine pipette, held in a micro-manipulator; the fluid was easily dropped on the endplate regions after removal of the overlying fascia. Twitch responses were observed through the microscope in

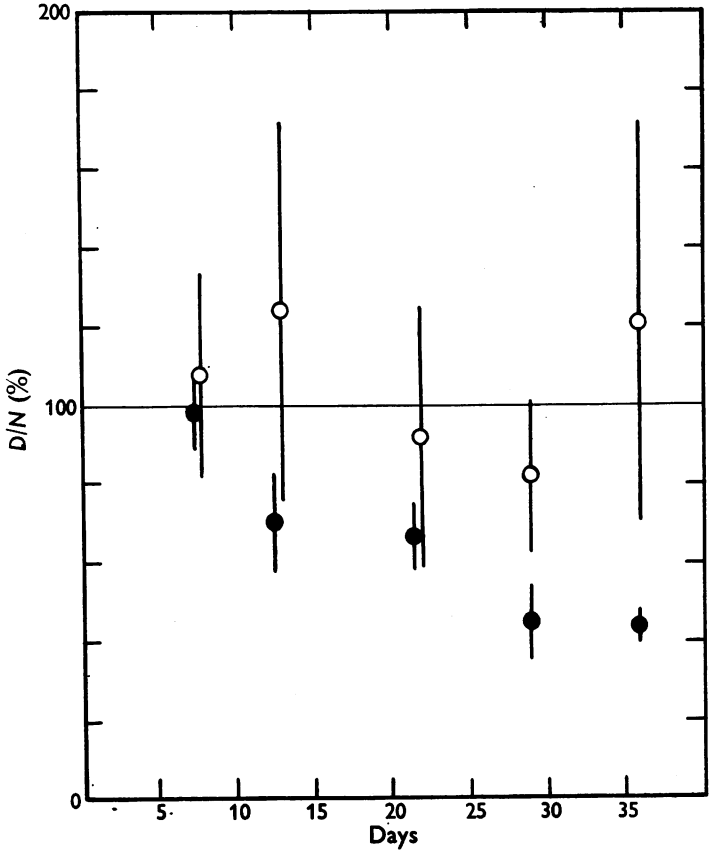

Fig. 1. Changes in weight and total true cholinesterase content following the denervation of skeletal muscle (guinea-pig). Abscissa: days after denervation. Ordinate: values for denervated muscle expressed as percentage of normal values. ●, change of fresh weight; ○, change of ChE content. All data are plotted ±2 S.E. The values at 8, 13 and 22 days have been separated along the time scale for the sake of clarity.

TABLE 1. Mean values of weights of guinea-pig muscles

Days post-operative	No. of animals	Fresh weight (mg.) N.M.	Fresh weight (mg.) D.M.	Fresh weight (mg.) $\frac{D.M.}{N.M.} \times 100 \pm$ S.E.	Fresh weight (mg.) P	Dried weight (mg.) N.M.	Dried weight (mg.) D.M.	Dried weight (mg.) $\frac{D.M.}{N.M.} \times 100$
8	10	182·5	178·4	98±4·9	0·5	54·1	57·1	105
13	8	173·0	121·6	70±5·8	<0·001	37·2	30·0	81
22	6	339·2	228·5	67±4·2	<0·001	90·2	78·2	87
29	9	242·1	107·9	45±4·5	<0·001	58·6	37·5	64
36	6	285·0	126·1	44±2·6	<0·001	67·6	45·7	68

N.M., normal muscle.
D.M., denervated muscle.
S.E., standard error.
P, probability that change of weight of denervated muscle is significant.

these preliminary experiments. The threshold of the muscle to acetylcholine fell during the first week after nerve section from 10^{-5} to 10^{-8} M., a change of about one thousand times. The increase in sensitivity can only be stated approximately, because it is not known what concentration of ACh actually reaches the receptive areas when it has been applied to the tissue surface as described.

Muscle atrophy began during the second post-operative week; this is a later onset than in rat skeletal muscle (Solandt, DeLury & Hunter, 1943). The muscle weight curve levelled off during the fifth week at about 45% of the normal. The changes are shown in Fig. 1 and are tabulated in Table 1.

From the above it is apparent that the behaviour of the m. serratus does not vary from the pattern usually observed after a skeletal muscle has had its motor supply severed.

Cholinesterase in denervated muscles

Pseudo-ChE was tested in four normal and three denervated specimens. Since its activity never amounted to more than about 5% of the true ChE activity of normal or of denervated muscles, no further attention was paid to it. The insignificance of the pseudo-ChE at the neuromuscular junction has also been demonstrated histochemically by Koelle (1950). Thus the discrepancies between reports of different authors cannot be due to their having added pseudo-ChE values to those of true ChE activity by using ACh as substrate.

The activity of true ChE in normal muscle is tabulated in Table 2. The values obtained are of the same order of magnitude as those reported for guinea-pig gastrocnemius by Marnay & Nachmansohn (1937) and for guinea-pig vastus lateralis by Couteaux & Nachmansohn (1940). The animals were obtained from litters of various ages, and as a result the fresh weights of the muscles given in Table 1 show significant differences between groups. It is interesting that the ChE content of the muscles did not vary significantly between groups, as tested by analysis of variance. The data do not suggest that true ChE content in the normal muscle is correlated with fresh weight. That ChE remains roughly constant in the growing muscles of various species has been shown by Nachmansohn (1940), who reported that after an initial rise of ChE activity early in embryonic life, the enzyme concentration per unit weight of muscle decreased during the growth of the tissue.

The most significant finding of this study is that the true ChE content of atrophying muscle remains essentially normal. Fig. 1 demonstrates graphically the contrast of decreasing muscle weight and steady total true ChE content. Table 2 contains mean values for all groups. If the enzyme is measured per unit weight of muscle, its concentration doubles as atrophy reduces the muscle weight to half of the normal. An increase of this order of magnitude, expressed in terms of the 'Q' ratio, has indeed been reported for guinea-pig muscle by Marnay & Nachmansohn (1937), Couteaux & Nachmansohn (1940), and

Couteaux (1942). These authors also commented on the fact that the changes in enzyme content observed by them took place well after the disappearance of all the intramuscular nerve twigs and inferred that ChE is located in the muscle part of the myoneural junction complex. That inference is fully borne out by the present data.

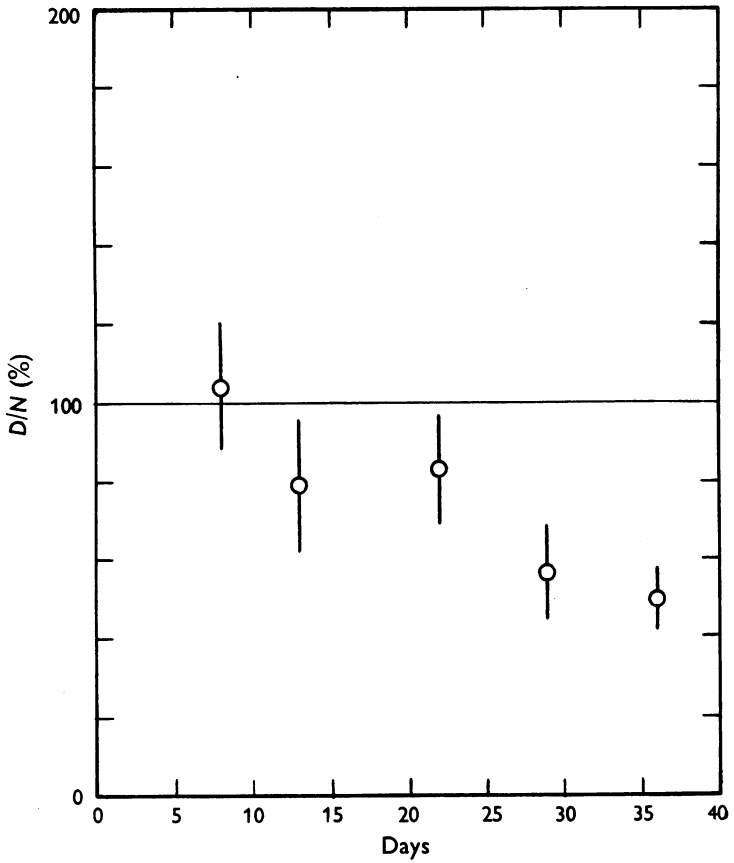

Fig. 2. Change in total content of tributyrin ali-esterase following the denervation of skeletal muscle (guinea-pig). Abscissa: days after denervation. Ordinate: values for denervated muscle expressed as percentage of normal values. All data are plotted ± 2 S.E.

An instructive comparison of enzyme distribution is afforded by examination of changes of tributyrin ali-esterase. Table 3 gives the mean values of all groups before and after denervation. These groups are identical with those listed in Tables 1 and 2. The data of Table 3 are plotted in Fig. 2. It is readily seen that the ali-esterase decreases roughly in proportion to the atrophy undergone by the muscles. This suggests that this enzyme is distributed throughout the muscle cells, rather than being found in high concentrations at specific loci as in the case of true ChE.

TABLE 2. Mean values of true cholinesterase activity of guinea-pig muscles

Days post-operative	No. of animals	Total true ChE per muscle (μl. CO_2/hr./muscle) N.M.	D.M.	$\frac{\text{D.M.}^*}{\text{N.M.}} \times 100 \pm$ S.E.	True ChE per 100 mg. fresh wt. $\frac{\text{D.M.}^*}{\text{N.M.}} \times 100 \pm$ S.E.	True ChE per mg. dry wt. $\frac{\text{D.M.}}{\text{N.M.}} \times 100$
8	10	404·0	426·5	108±12·9	110± 7·9	101
13	8	223·6	297·0	124±24·0	169±29·1	164
22	7	372·6	328·2	93±16·2	142±19·3	134
29	9	334·2	278·1	82± 9·4	183±18·8	130
36	6	267·5	313·0	121±25·1	267±51·6	172

N.M., normal muscle.
D.M., denervated muscle.
S.E., standard error.
*, ratios evaluated as means of individual ratios in each of 5 groups.

TABLE 3. Mean values of tributyrin ali-esterase activity of guinea-pig muscles

Days post-operative	No. of animals	Total ali-esterase per muscle (μl. CO_2/hr./muscle) N.M.	D.M.	$\frac{\text{D.M.}^*}{\text{N.M.}} \times 100 \pm$ S.E.	Ali-esterase per 100 mg. fresh wt. $\frac{\text{D.M.}^*}{\text{N.M.}} \times 100 \pm$ S.E.	Ali-esterase per mg. dry wt. $\frac{\text{D.M.}}{\text{N.M.}} \times 100$
8	10	549·6	570·1	104±7·8	106± 4·7	99
13	8	606·0	458·1	79±8·4	114± 7·1	94
22	8	1168·0	966·8	83±9·5	128±14·0	95
29	9	950·1	511·0	57±6·0	125± 8·2	85
36	6	1042·0	525·1	50±3·6	114± 4·3	74

N.M., normal muscle.
D.M., denervated muscle.
S.E., standard error.
*, ratios evaluated as means of individual ratios in each of 5 groups.

DISCUSSION

In the present experiments the ChE content of the serratus muscle did not decline after denervation. That the scatter of the data was not large enough to obliterate significant changes is shown by the progressive decrease of tributyrin ali-esterase content of denervated muscles, a decrease that was readily detected without any suggestion of ambiguity. It is difficult to reconcile these findings with those of Couteaux & Nachmansohn (1940) and of Couteaux (1942). They described a decrease of total ChE content to 45% of the values found in normal gastrocnemius, vastus lateralis, and plantaris muscles of the guinea-pig. Their studies, however, describe a more severe atrophy, down to 25% of the normal muscle weight; it may be that the muscle does lose ChE under these conditions.

It might be argued that the present experiments fail to detect a small region of marked decrease in that fraction of the enzyme located at or in the junction itself. Elsewhere in the muscle, the enzyme store might remain unchanged or be increased after denervation, thus hiding the small but crucial reduction in ChE at the junction. Against such an argument could be set the fact that the

concentrations of ChE found in the aneural part of the guinea-pig and the toad gastrocnemius, according to Couteaux & Nachmansohn (1940) and Feng & Ting (1938) are some twenty thousand times less than the concentrations in the myoneural complex. The histochemical evidence (Koelle, 1950), although not yet suitable for precise quantitative interpretation, likewise suggests that most of the true ChE of the muscle is located in the endplate region. In denervated rat flexor muscle, indeed, the area stained by a modification of Koelle's method usually decreases to a quarter of its normal size in 5 weeks as the muscle atrophies (Kupfer, personal communication). However, the remaining enzyme cannot be assayed on the basis of density of stain in such tissue. If most of the enzyme is located in the junctional region, it is hard to imagine how a small local reduction in its level could bring about the enormous sensitization of denervated muscle to ACh. It should in any case be noted that a muscle whose ChE has been inhibited by treatment with eserine or DFP, though hypersensitive to arterially injected ACh, does not respond with the non-propagated contracture characteristic of denervated muscle.

The sequence of events in a sympathetic ganglion after preganglionic denervation has been studied extensively and a comparison with muscle may be useful. The superior cervical ganglion of the cat is approximately eight times richer in true ChE than skeletal muscle (Sawyer & Hollinshead, 1945). Upon denervation its true ChE level drops to 20% of the normal in the first post-operative week, when nerve endings are degenerating, and stays at that level for at least five weeks (Sawyer & Hollinshead, 1945; von Brücke, 1937). These findings establish the presynaptic endings of the nerves as the carriers of the bulk of true ChE in the ganglion, in contrast to muscle where the ChE is mostly located in the muscle part of the myoneural junction. The denervated ganglion, however, although it has lost most of its true ChE, becomes only about four times more sensitive to applied ACh, a ratio much smaller than that for muscle (Rosenblueth & Cannon, 1939). No doubt a small fraction of the ChE of normal muscle is likewise located in the endings of the motor nerve and disappears when these degenerate, but there is no reason to suppose that this portion is particularly important for the inactivation of the ACh liberated at the junction, whose post myoneural component is in any case rich in ChE.

This comparison of the two tissues emphasizes the difficulties that are encountered when one attempts to explain the supersensitivity of denervated structures in terms of a loss of the enzyme that normally destroys a chemical transmitter. In skeletal muscle, at any rate, the explanation will not hold.

SUMMARY

1. The activity of cholinesterase in two digitations of the m. serratus anterior of the guinea-pig has been studied in paired normal and denervated muscles from the same animals.

2. In growing animals between 250 and 500 g. body weight there is no increase in the true cholinesterase content of the muscle.

3. After denervation, the muscle exhibits fibrillation and hypersensitivity to applied acetylcholine within three days. Atrophy begins during the second post-operative week and levels off during the fifth week at about 45% of the normal muscle weight.

4. The *total content* of true cholinesterase is unchanged by denervation. The *concentration* of the enzyme, however, doubles when the muscle weight is halved by atrophy.

5. Pseudo-cholinesterase does not contribute more than approximately 5% to the total esterase activity of normal or denervated muscle.

6. Tributyrin ali-esterase content of the muscle decreases roughly in proportion to the loss of weight of the atrophying muscle.

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Note added in proof. Since this paper was submitted for publication, the post-denervation changes of true ChE in the gastrocnemius muscles of guinea-pigs and rats were studied by one of us (V.B.B.). It was found that in both species muscle weight and total content of true ChE decreased concomitantly to about one-half of the normal values during the first three weeks after nerve section. This confirms the findings of Couteaux & Nachmansohn (1940) and of Martini & Torda (1937). The experiments with the serratus anterior muscle of the guinea-pig were repeated and the total content of true ChE was unchanged after denervation in confirmation of the results reported in this paper.

It therefore becomes impossible to make a statement about the pre- and post-synaptic distribution of true ChE in nerve-muscle junctions which covers *all* skeletal muscles. However, the unchanged content of true ChE of one denervated muscle still rules out any general theory designating ChE change as the agent responsible for supersensitivity.

The details of these experiments will be published elsewhere.

REFERENCES

Brooks, V. B. (1951). *Science*, **113**, 300.

Brücke, F. Th. von (1937). *J. Physiol.* **89**, 429.

Cannon, W. B. & Rosenblueth, A. (1949). *The Supersensitivity of Denervated Structures*, 1st ed., p. 195. New York: Macmillan.

Couteaux, R. (1942). *Bull. biol.* **76**, 14.

Couteaux, R. & Nachmansohn, D. (1940). *Proc. Soc. exp. Biol., N.Y.*, **43**, 177.

Feinstein, B., Pattle, R. E. & Weddell, G. (1945). *J. Neurol. Neurosurg. Psychiat.* **8**, 1.

Feng, T. P. & Ting, Y. C. (1938). *Chin. J. Physiol.* **13**, 141.

Koelle, G. B. (1950). *J. Pharmacol.* **100**, 158.

Leibson, R. (1939). *Bull. Biol. Méd. exp. U.R.S.S.* **7**, 514.
Marnay, A. & Nachmansohn, D. (1937). *C.R. Soc. Biol., Paris*, **126**, 785.
Martini, E. & Torda, K. (1937). *Klin. Wschr.* **16**, 824.
Mendel, B., Mundell, D. B. & Rudney, H. (1943). *Biochem. J.* **37**, 473.
Meng, C. W. (1940). *Chin. J. Physiol.* **15**, 143.
Nachmansohn, D. (1940). *J. Neurophysiol.* **3**, 396.
Rjabinowskaja, A. M. (1940). *C.R. Acad. Sci. U.R.S.S.* **27**, 97.
Rosenblueth, A. & Cannon, W. B. (1939). *Amer. J. Physiol.* **125**, 276.
Sawyer, C. H., Davenport, C. & Alexander, L. M. (1950). *Anat. Rec.* **106**, 287.
Sawyer, C. H. & Hollinshead, W. H. (1945). *J. Neurophysiol.* **8**, 137.
Solandt, D. Y., DeLury, D. B. & Hunter, J. (1943). *Amer. J. Physiol.* **140**, 247.
Stoerk, H. C. & Morpeth, E. (1944). *Proc. Soc. exp. Biol., N.Y.*, **57**, 154.