QUANTITATIVE STUDIES ON ENZYMES IN STRUCTURES IN STRIATED MUSCLES BY LABELED INHIBITOR METHODS

II. Confirmation of Radioautographic Measurement by Liquid–Scintillation Counting

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ABSTRACT

Fragments of mouse diaphragm and sternomastoid muscles were incubated in diisopropylfluorophosphate (DFP)-^{3}H in conditions known to saturate all the available DFP-sensitive reaction sites. After being extensively washed, the enzyme acetylcholinesterase (AChase) was specifically reactivated by treatment with pyridine-2-aldoxime methiodide (2-PAM). The radioactive DP-groups released into solution by 2-PAM were measured by liquid scintillation counting, and related to the known number of motor endplates present. Considerable difficulty was encountered in reducing the excess, adsorbed radioactivity to acceptable levels: long washing routines, extraction with organic solvents, and removing excess muscle fiber by microdissection were necessary. Six experiments gave a mean value of 2.4×10^{7} molecules AChase per sternomastoid endplate, in reasonable agreement with the previously reported measurements by radioautography.

INTRODUCTION

In the preceding paper of this series (Rogers et al., 1969), radioautographic methods were used for determining the number of acetylcholinesterase (AChase) molecules present at motor endplates of the diaphragm and sternomastoid muscles of the mouse. These experiments involved two stages. In the first, fragments of muscle were reacted with diisopropylfluorophosphate(DFP)-^{32}P in conditions which were shown to saturate all available reactive sites: beta track radioautographs were then prepared from individual microdissected endplates, and the total number of DFP-reactive sites per endplate was determined. The second stage involved reagents highly specific for AChase, in experiments designed to measure the contribution made by this enzyme to the total number of DFP-reactive sites. Combining both stages, we obtained mean values of 3.1×10^{7} and 1.1×10^{7} molecules of AChase for sternomastoid and diaphragm endplates, respectively.

Since these experiments employed quantitative methods of radioautography which are not in

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common use, means were sought to confirm by liquid scintillation counting the values obtained, so as to provide an independent check on the radioautographic technique. Small fragments of muscle were treated with DFP-^{3}H at high specific activity, and thoroughly washed. They were then incubated with pyridine-2-aldoxime (2-PAM), which combines with the diisopropylphosphoryl-(DP-) AChase conjugate, removing the DP-group and reactivating the enzyme (Wilson et al., 1958). In the conditions used, this reaction is highly specific for AChase (Rogers et al., 1969). It releases into solution the labeled DP-groups from AChase alone, which can then be sampled and counted by liquid-scintillation techniques, and related to the number of endplates in the fragment.

MATERIALS AND METHODS

Animals and reagents used were those described in the previous paper (Rogers et al., 1969).

Labeling Procedures

Unless otherwise noted, the labeling, washing, and treatment with 2-PAM followed the routine described in the first column of Table V in the previous paper (Rogers et al., 1969), with DFP-^{3}H replacing DFP-^{32}P. Aliquots of solutions 7 and 8 in that Table (2-PAM and the subsequent two buffer washes) were combined for liquid-scintillation counting.

Experimental Design

Two types of experiment were carried out. In the first, small fragments of muscle were obtained from areas rich in endplates. These fragments were fixed in cold, buffered formalin either before or after labeling: Rogers et al. (1969) have shown that identical quantitative results can be obtained radioautographically with these two methods of fixation. After elution of labeled DP-groups and reactivation of AChase by 2-PAM, the fragments were stained by the Koelle acetylthiocholine method (Koelle and Friedenwald, 1949), as modified by Pearse (1961), to make the endplates visible. The muscle fragments were then squashed, and the endplates were counted under the microscope. In this type of experiment, control fragments of muscle without endplates were processed at the same time. All fragments were weighed, with a standardized procedure, immediately after removal from the animal. The scintillation counts from the fragments containing endplates were corrected for nonspecific, adsorbed radioactivity, and for the possible presence of AChase in the muscle fibers themselves (together forming the "muscle background") by subtraction of the counts that would have been observed from control fragments of identical wet weight.

In the second experimental pattern, fragments of muscle rich in endplates were fixed immediately on removal from the animal, and stained by the Koelle method. Following labeling with DFP-^{3}H and washing, tiny fragments carrying several endplates, or, in some experiments, individual lengths of muscle fibers with an endplate, were dissected out under the microscope. These fragments and individual fiber lengths were then collected, counted, and treated with 2-PAM in the usual way. In these experiments, micro-dissected fragments or individual fiber lengths without endplates were collected for control measurements of muscle background.

Liquid-Scintillation Counting

A Packard Tri-Carb Scintillation Spectrometer, Model 3224, was used with the counting chamber maintained at 7°. The scintillation fluid (15 ml) consisted of PPO and dimethyl-POPOP (Packard Instrument Co., Downers Grove, Ill.) with naphthalene in dioxane/toluene (9:1, v/v) (all chemicals used being scintillation grade). The counting efficiency for tritium was 30–33%, as estimated on the counting rates observed from weighed amounts of standardized tritiated hexadecane (Radiochemical Centre, Amersham, England), determined with each experiment. Corrections for quenching were based on the technique of channels' ratio in the early experiments, and on the addition of 20–100 µl of toluene-^{3}H (Packard Instrument Co.) as an internal standard in all the remaining experiments (Rogers and Moran, 1966). 10X Hyamine was obtained from Packard Instrument Co.

RESULTS

The Development of a Satisfactory Technique

INVESTIGATIONS INTO THE REMOVAL OF ADSORBED RADIOACTIVITY

In the radioautographic experiments described in the previous paper, a relatively simple washing procedure after labeling was sufficient for reducing the muscle background to levels at which reaction of DFP-^{32}P at endplates produced a recognizable track count, which could be completely abolished by pretreatment with nonradioactive DFP (see Table III, Rogers et al., 1969). Initial experiments with muscle fragments and liquid-scintillation counting, however, showed that the muscle background was so high with simple washing that it completely concealed the expected counts from labeling at endplates.

Fig. 1 illustrates the rate of removal of radioactivity from two such fragments, both containing

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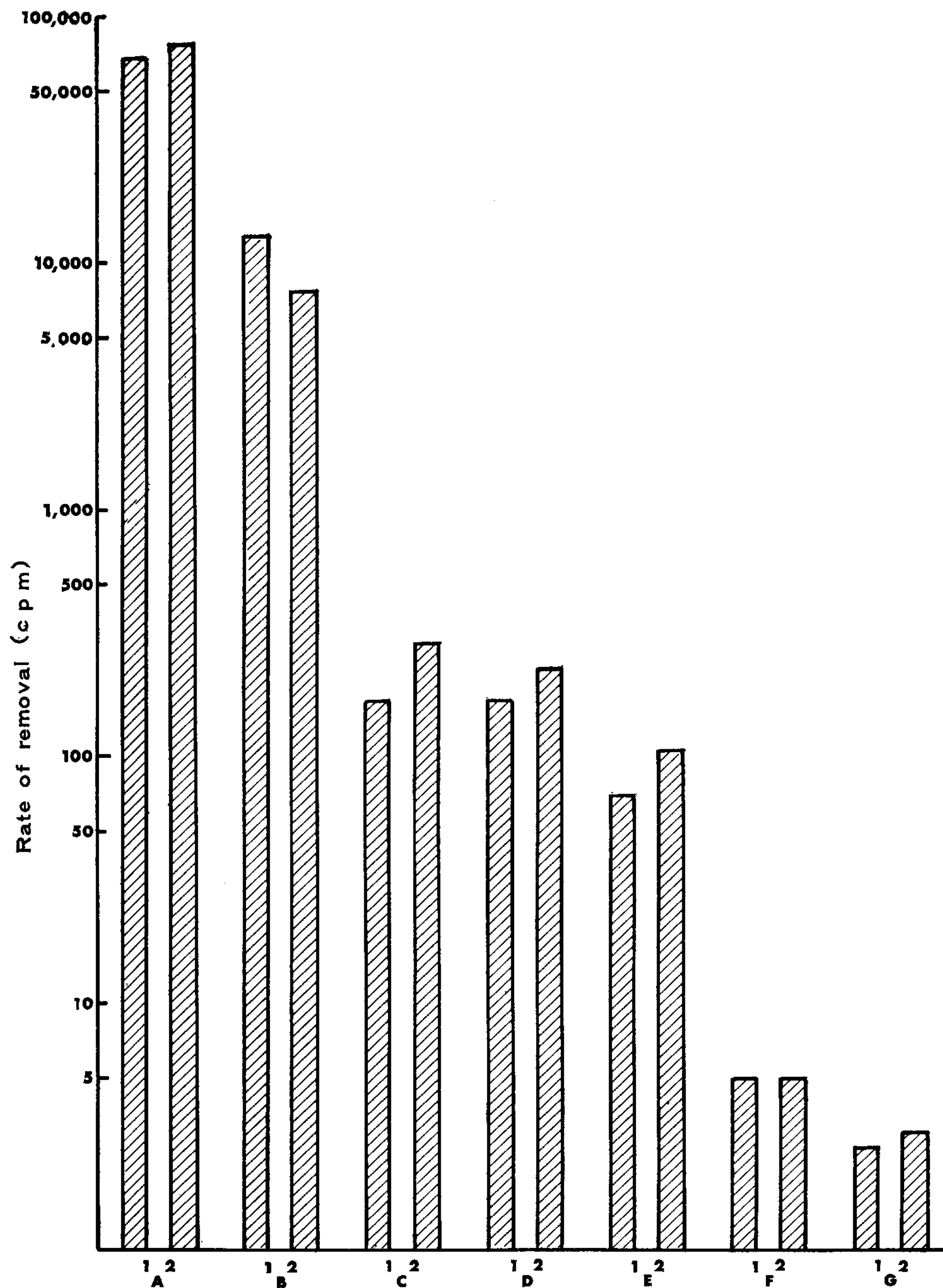

FIGURE 1 The radioactivity removed, in various washes, from fragments of sternomastoid muscle, preblocked with unlabeled (10^{-2} M) DFP (specimen 1) or not (specimen 2), and labeled with DFP-^{3}H. The isotope removed is expressed (on a logarithmic scale) as cpm per min of washing time in each solution. The washing solutions used, in turn, were *A*, unlabeled DFP at 10^{-2} M for 2 min; *B*, unlabeled DFP at 10^{-2} M for 10 min; *C*, unlabeled DFP at 10^{-3} M for 1 hr; *D*, phosphate buffer for 2 min; *E*, phosphate buffer for 10 min; *F*, buffered formalin-sucrose for 3 hr; *G*, phosphate buffer for 18 hr.

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TABLE I

Extractions of Radioactivity from Labeled Diaphragm Strips

	Sample A		Sample B	
Wet weight (mg)	3.04		3.91	
Estimated number of endplates	3,000		0	
Radioactivity in last buffer wash after labeling	186		294	
Radioactivity extracted by 2-PAM	638	870	446	628
Radioactivity in subsequent buffer rinses	231		182	
Radioactivity remaining in muscle	11,805		17,164	

Estimated "muscle background" for A $= \frac{3.04}{3.91} \times 628 = 488$ dpm.*

Radioactivity due to AChase at endplates $= 870 - 488 = 382$ dpm.

Strips A and B of formalin-fixed diaphragm were treated with unlabeled DFP, extracted with 2-PAM, labeled (for AChase) with ^{3}H-DFP, and washed with DFP and buffer, with use of the procedures of the second column of Table IV of Rogers et al. (1969). 2-PAM was applied for a second time (10^{-3} M, 30 min, 20°), and the total radioactivity (recorded in dpm) extracted in this and two subsequent buffer washes was determined. The radioactivity finally remaining in the muscle was then determined by digesting the fragments in hyamine and preparing counting samples from the neutralized digest.

* Disintegrations per min.

endplates, but one of them being pretreated with unlabeled DFP. In the final change of buffer, the rate of removal of radioactivity from each fragment was 2×10^4 times slower than in the first change of unlabeled DFP. Nevertheless, even at this greatly reduced rate, 2.5–3.0 cpm were being removed from each fragment every minute. From our beta track measurements (Rogers et al., 1969), this radioactivity is equivalent to the DFP reacted with AChase at 30 endplates. Clearly, even with such extensive washing, sufficient adsorbed DFP-^{3}H persists in small muscle fragments to make direct measurements of the DP-groups removed by 2-PAM from endplates almost impossible. This adsorbed isotope must be very diffusely spread through the muscle, so that it does not interfere with the radioautography of endplates.

In a second scintillation-counting experiment, the radioactivity eluted by 2-PAM was compared in two thin strips of diaphragm, one with endplates, the other without endplates (Table I). After treatment with 2-PAM, each strip was digested in hyamine so that the radioactivity remaining in the muscle could be counted. The radioactivity apparently released from AChase represented 3.25% of the total radioactivity present in the strip with endplates, after the washing procedure: in the strip without endplates, the comparable figure was 0.5%.

In a series of five experiments on sternomastoid fragments containing a total of 34,424 endplates, in which this simple washing procedure was employed, the values obtained for the apparent number of AChase molecules per sternomastoid endplate ranged from 5×10^7 to <0 (i.e. less than the value of the muscle background control). Clearly, variations in the level of muscle background were large relative to the radioactivity released by 2-PAM. It was obvious that further measures would have to be taken to improve the washing out of adsorbed radioactivity, before acceptable results could be obtained.

Three procedures, in addition to the aqueous washes listed in Materials and Methods, were investigated in an attempt to bring down the levels of radioactivity due to muscle background. These procedures were: (*a*) physically reducing the volume of muscle accompanying each sample of endplates, by microdissection; (*b*) very prolonged washing in aqueous solutions; and (*c*) extraction of the fragments with organic solvents.

MICRODISSECTION: Some degree of microdissection was used in all subsequent experiments. Either the fragment that had been labeled was carefully trimmed into very small fragments immediately along the course of the motor nerve, or, in the case of muscle fragments that had been stained, individual endplates and groups of end-

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plates were dissected out, as with the material prepared for radioautography (Rogers et al., 1969).

THE USE OF PROLONGED AQUEOUS WASHES: In one experiment (Table II), fragments of sternomastoid muscle, two of which had been pretreated with nonradioactive DFP, were labeled and then incubated for extremely long periods in stirred phosphate buffer at 0°. The total washing time for one fragment was 78 hr before the fragment was taken for microdissection of single endplates. The remaining fragments were trimmed, and washing was continued for a total of 96 hr. The rate of removal of radioactivity from these fragments in the last buffer solution before treatment with 2-PAM was less than 1 cpm per min of washing time, a value which was lower than that in previous experiments. The calculated number of DP groups removed by 2-PAM per endplate was 1.0–2.0 × 10^7 (Table III).

TABLE II

Procedure Used in Exhaustive Washing Method for Removal of Excess Isotope from Labeled Sternomastoid Muscle

Treatment	Volume	Temp.	Time
	ml	*°C*	*min*
Groups I and III only			
1. Unlabeled DFP at 10^{-2} M	2	20	20
2. Buffer	20	0	10
All Groups			
3. DFP-^{3}H at 10^{-4} M	0.5	20	20
4. Buffer	2	0	2
5. Unlabeled DFP at 10^{-2}M	2	20	2
6. Unlabeled DFP at 10^{-3} M	5	20	10
7. Unlabeled DFP at 10^{-3} M	10	20	60
8. Buffer	2	0	2
9. Buffer	10	0	10
10. Formalin	10	0	1,140
11. Buffer (2 baths)	1,000	0	1,440
12. Extraction procedure*			

Groups I and II were prestained by incubation (10 min) in the Koelle medium.
Groups III and IV were unstained. All solutions were made up in 0.02 M phosphate buffer (pH 7.4). Treatments 1–12 were applied in sequence.
* At this stage, one fragment from Group II was taken for microdissection and extraction with 2-PAM in the usual manner. The remaining fragments had one further buffer wash at 0° and then underwent the 2-PAM extraction.

TABLE III

The Radioactivity Extracted by 2-PAM from Exhaustively Washed Fragments of Labeled Sternomastoid Muscle

Material	No. of endplates	DP-groups per plate*
Group II, microdissected	230	1.0 × 10^7‡
Group II, fragment	205	2.0 × 10^7
Group IV, fragment	540	1.2 × 10^7
Group IV, fragment	412	1.2 × 10^7

The material used is specified in Table II. The radioactivity extracted is expressed as the equivalent number of DP-groups per endplate, i.e. the number of AChase molecules reacting. Group II material was prestained by the Koelle reaction, while Group IV was unstained.
* To deduce the values for AChase molecules per endplate, these values must be corrected for the effect of the stain (see Table VIII for the corrected values).
‡ The actual counts measured were 5038 for fibers with endplates and 4400 for the control fibers, measured over 220 min.

EXTRACTION WITH ORGANIC SOLVENTS: Since DFP is lipophilic, it is not surprising that washing in aqueous media should be a relatively inefficient method of removing free DFP from a multiphase system such as a fragment of muscle. It seemed likely that a brief wash in an organic solvent such as chloroform would be highly effective in removing the loosely bound DFP detected here.

For staining endplates by the Koelle method when an organic solvent extraction step was used, it was found necessary to apply the formalin fixation before, and not after, the labeling reaction. Small fragments of diaphragm with and without endplates were fixed in cold formalin, stained by the Koelle reaction, labeled with DFP-^{3}H, and washed, according to the original procedure (Table I of Rogers et al., 1969). After the third buffer wash (step 5), one-half of the fragments was taken through 50 and 95% alcohol to a 1:1 mixture of 95% alcohol and chloroform, then back through 95 and 50% alcohol to buffer (step 6). Aliquots were taken from these solutions for scintillation counting. The remaining fragments

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TABLE IV

Studies on Washing Procedures Using Organic Solvents for Removal of Excess Isotope

Specimen	Muscle		Washing procedure	Radioactivity		
	Weight	Endplates		Final wash*	2-PAM extract*	Hyamine digest
	mg			*cpm*	*cpm*	*cpm*
A	5.9	Present	Standard	12	82	940
B	3.3	Present	Standard + organic solvents	971	18	417
C	5.7	Absent	Standard	11	64	1,105
D	2.2	Absent	Standard + organic solvents	596	9	467

For details of the procedures used, see text. Each specimen was a strip of diaphragm, either with or without the band of endplates. The total radioactivity removed in each case is recorded.

* The final wash after labeling was step 6 of Table I for specimens A and C, and the organic solvent extractions for B and D.

were washed as described in Table I of Rogers et al. (1969) and were placed in buffer (pH 7.9) while the first fragments were in alcohol and chloroform. Table IV presents the observed count rates in the alcohol and chloroform washes, or the equivalent buffer wash, and in a Hyamine digest of the muscle fragments, made after treatment with 2-PAM. It can be seen that the organic solvent removed more than 50% of the radioactivity in the fragments, whereas the buffer wash removed only about 1%. In fact, the majority of the radioactivity removed by the organic solvents came out in the first tube of 50% alcohol.

In the experiments of Table V, sternomastoid fragments rich in endplates were stained with the Koelle reaction (10-min incubation), fixed in cold buffer formalin, labeled with DFP-^{3}H, and washed as described above, with the addition of 70 and 90% alcohol washes. After microdissection, 2-PAM extracts of the endplates were counted. Similar groups of muscle fibers without endplates, treated in parallel, were extracted by 2-PAM as controls. The counting rate of specimens with endplates was very nearly double that attributed to the muscle background in this material (Table V), and the mean number of DP-groups removed by 2-PAM per endplate was 1.8×10^7.

TABLE V

Extraction of Radioactivity by 2-PAM from Microdissected Endplates

Specimen	No. of endplates	cpm	DP-groups per endplate*
Group A. Microdissected	211	42.3	1.1×10^7
	0	28.8	
Group B. Microdissected	136	41.6	2.4×10^7
	0	22.5	

Two groups of microdissected sternomastoid endplates were used, and with each an equal control group of muscle fibers without endplates was treated similarly. The muscle had been stained by the Koelle method before labeling. The washing procedure included extraction with alcohol (see text). The radioactivity extractable from the endplates by 2-PAM was corrected by subtraction of that radioactivity coming from the same number of similar fibers of muscle above, in calculating the groups per endplate.

* These values must be corrected for the effect of staining (see Table VIII).

The Rate of "Ageing" of the DP-AChase Complex

The DP-AChase complex has been shown to "age," with loss of one isopropyl group by hydrolysis: after ageing, the inhibited AChase can no longer be reactivated by 2-PAM (Behrends et al., 1959; see discussion in Rogers et al., 1969). Since this process would reduce the radioactivity liberated by 2-PAM in our experiments, an attempt was made to measure the rate of ageing in our conditions of work. From three mice, the strip of endplates from the entire diaphragm was

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TABLE VI

Ageing of DP-AChase in Endplates

Incubation period	Radioactivity extracted
min	*cpm*
50	61
440	55
1250	16

Three similar, labeled strips of diaphragm were incubated in pH 7.4 phosphate buffer at 22°-25° for the times shown. They were then treated with 2-PAM (10^{-3} M, 30 min), and the extracts were counted.

dissected out, with a margin of muscle on either side of it; a similar strip of muscle without endplates was also obtained. After fixation in cold formalin, these strips were labeled with DFP-^{3}H, washed according to the basic procedure (with the addition of washes in 70 and 90% alcohol), and incubated at pH 7.4 at room temperature (22–25°C) for varying times. The strips were then treated with 2-PAM in the usual way. From the radioactivity removed by 2-PAM (Table VI), it is clear that ageing was a relatively slow process at this temperature; a rough estimate of 10–12 hr for its half-life can be made. Recent, more extensive determinations of ageing *in situ* in such material, which have been made by radioautography (J. Wieckowski and E. A. Barnard, 1969, in preparation), give values consistent with this value. A value of 10–12 hr at this temperature is also compatible with the biochemical results of Hobbiger (1956) and of Davies and Green (1956). The material described in Table V was kept at room temperature for no more than 35 min between the start of incubation in DFP-^{3}H and the treatment with 2-PAM. The material described in Table III was kept at room temperature for about 95 min. The rest of the washing procedure was carried out at 0–4°, at which temperature the rate of ageing of DP-AChase is enormously slower (Davies and Green, 1956; Latki and Erdmann, 1961). With a half-life for ageing of 10 hr, the correction factor applicable to our results would be 5–10%. In view of the much greater errors from other sources, this correction has been ignored.

The Effect of Staining on the Phosphorylation of AChase by DFP

Rogers et al. (1969) have presented evidence that the period of staining of endplates in the Koelle acetylthiocholine-staining medium prior to treatment with DFP proportionately reduces the number of sites that can be phosphorylated per endplate. After 10 min of incubation, 40-50% of the sites that could be labeled after 2 min of incubation were no longer available for phosphorylation.

In the radioautographic experiment, it was not possible to examine unstained material without some doubt as to the position and extent of the endplates. An experiment was, therefore, carried out in which we measured, by liquid scintillation counting, the reduction in labeling caused by 10 min of incubation in the Koelle medium, in comparison with the labeling in unstained material, with use of strips of diaphragm (Table VII). With practice, it is possible to dissect quite reproducible strips of diaphragm, weighing about 3 mg and containing 2000–2500 endplates each. The 2-PAM extracts from stained strips had 55% of the isotope that was present in 2-PAM extracts from similar, unstained strips. This finding is in very close agreement with the radioautographic evidence quoted above. Clearly, incubation in the Koelle medium reduces the number of AChase sites available for phosphorylation by DFP. All the results obtained in those scintillation-counting

TABLE VII

Effect of the Acetylthiocholine Staining Reaction on the Reaction of DFP-^{3}H with AChase in Endplates

Sample	^{3}H from stained strips	Sample	^{3}H from unstained strips
	dpm		*dpm*
A	155	E	215
B	198	F	416
C	135	G	199
D	122		
Mean	153	Mean	277

The radioactivity extracted by the 2-PAM treatment from similar endplate-bearing strips (two in each sample) of diaphragm was measured in each case. Stained strips were incubated in the Koelle medium for 10 min prior to labeling with DFP-^{3}H. The mean value for the stained strips is 55% of that of the unstained strips.

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TABLE VIII

Number of Molecules of AChase per Endplate of the Mouse Sternomastoid Muscle

Material	No. of endplates	Molecules AChase per endplate*
(Table III, group II) Microdissected endplates	230	1.8×10^7
(Table III, group II) Stained fragment	205	3.5×10^7
(Table III, group IV) Unstained fragment	540	1.2×10^7
(Table III, group IV) Unstained fragment	412	1.2×10^7
(Table V, group A) Microdissected endplates	211	2.0×10^7
(Table V, group B) Microdissected endplates	136	4.4×10^7
	Mean =	2.4×10^7

* Correction has been made (see text) for the effects of prestaining, where appropriate.

experiments which involved prior staining were, therefore, corrected, by use of the same correction factors applied to the radioautographic data (Rogers et al., 1969).

Determination of the Number of Molecules of AChase per Endplate

Six experiments that were technically satisfactory were carried out, involving a total of 1,734 endplates from mouse sternomastoid muscle. The experiments are those listed in Tables III and V. They are summarized in Table VIII, in which corrections for prior staining have been applied, converting the number of DP-groups liberated per endplate by treatment with 2-PAM into the number of molecules of AChase per endplate. The values range from 1.2×10^7 to 4.4×10^7. For comparison, the values obtained by radioautography range from 2.2×10^7 to 4.0×10^7 (Rogers et al., 1969).

DISCUSSION

Bulk Extraction Measurements of Endplate Radioactivity

At first sight, this experimental approach to the measurement of AChase at motor endplates, the employment of selective extraction of DP-groups with 2-PAM and of liquid-scintillation counting, appears more direct than the method employing beta track radioautography. The latter involves the measurement, by track counting, of the total number of sites per endplate that are available for phosphorylation of DFP, and then the determination of the percentage of these sites that are active centers of AChase (Rogers et al., 1969). But this direct and apparently simpler approach with liquid-scintillation counting is complicated by two technical problems.

The first of these problems is the loss of spatial discrimination in preparing samples for liquid scintillation counting. In the radioautographic experiments referred to above, it was possible to confine counting to the stained endplates themselves, and to ignore beta tracks which started from nearby muscle. This is obviously impossible in measurements made on 2-PAM eluates of fragments of muscle. In the scintillation-counting experiments, the high concentration of AChase in the endplates was accompanied by radioactivity derived from a much larger volume of muscle. Inevitably, the muscle background in these experiments was higher relative to the signal obtained from the endplates than it was in the radioautographs.

The second complicating factor is the comparatively small number of labeled DP-AChase molecules to be determined. The background from the Packard spectrometer itself was relatively large when compared to the small amounts of radioactivity in the DP-groups extracted. Even with the use of plastic counting vials, this background was around 20 cpm (which is normal for such an instrument, and is not significantly improved at lower usable temperatures). At the highest specific activity of DFP-^{3}H available (4.3 c/mmole), it required 4×10^9 molecules of DFP-^{3}H to give an equivalent counting rate. In other words, the DFP-^{3}H eluted from 200 unstained sternomastoid endplates, or from 350–400 stained sternomastoid endplates, was needed to produce a counting rate equal to the irreducible background count of the technique itself. By contrast, the background in beta track radioautography was practically zero

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at exposure times of 24 hr. In spite of the factor of approximately 50 by which the specific activity of the DFP-^{32}P was less than that of the DFP-^{3}H, the counting times of 24 hr and the insignificant background of the beta track material enabled useful counts to be obtained from single endplates, whereas hundreds of endplates were needed for each sample for scintillation counting.

Steps to Reduce the Muscle Background

The majority of scintillation-counting experiments were directed towards reducing the muscle background, i.e. those counts originating from DFP-^{3}H adsorbed nonspecifically to the tissues, and the radioactivity eluted by 2-PAM from structures other than motor endplates. It was clear initially that nonspecific adsorption was responsible for practically all the muscle background observed, a conclusion supported by the radioautographic data presented. For instance, Fig. 1 demonstrates that there is little difference in the counting rate observed from two similar muscle fragments, in one of which all the available sites had been phosphorylated with nonradioactive DFP prior to labeling with DFP-^{3}H.

Two approaches were then used to reduce this muscle background. The first approach involved a reduction in the amount of muscle accompanying the endplates. Muscle fragments were kept as small as possible, and, in several experiments, resort was made to microdissection of individual endplates, in order to produce a physical reduction in the muscle background. This approach alone was not adequate. The second approach, more adequate washing, was effective when an organic solvent was used to extract the adsorbed DFP. This kind of extraction is preferable to very long extraction in aqueous solutions, in order to avoid ageing. Useful results were obtained (Table V) only when the technique for giving a high ratio of endplates to muscle was combined with the procedure for reducing the level of nonspecific radioactivity.

The Estimation of Muscle Background

The muscle background was always appreciable, in relation to the counting rates attributable to radioactivity removed from AChase by 2-PAM. The methods used to estimate this background, therefore, require careful examination.

In the case of small muscle fragments, muscle background was estimated from control blocks of muscle that did not contain endplates. The wet weights of experimental and control blocks were determined, and corrections were made on the assumption that the muscle background was directly proportional to the weight of the fragment. It is difficult to believe that these wet weights were very accurate for fragments averaging 3 mg. Unfortunately, the fragments were required for subsequent staining and microscopy, in order to determine the number of endplates present. For this reason, more accurate methods of finding the mass of these fragments, such as drying to constant weight or digestion and chemical estimation of the protein present, were not possible.

It is probably not correct to assume that the level of muscle background is directly proportional to the weight of the fragment. The washing out of radioactivity from a block of tissue is likely to be a complex function involving not only the total mass of the tissue but also its shape. Although every effort was made to provide control fragments that matched the experimental ones as closely as possible, it is likely that this method of estimating the muscle background introduced appreciable variation into the results.

Control fragments of muscle were obtained from regions of the same muscle that were well away from the path of the nerve of supply. They differed from the experimental fragments in features other than the absence of endplates, therefore. We have noted, however, that significant labeling above muscle background cannot be detected radioautographically in peripheral nerve in our conditions. It is unlikely that the absence of nerve fibers in the control fragments introduced an appreciable error.

In the experiments involving microdissection of individual endplates, several of these sources of error were eliminated. Control observations were based on lengths of muscle fiber without endplates which were dissected from the same fragment of muscle as the endplates themselves. Indeed, these lengths were, in many cases, from the same muscle fiber. Differences in the rate of washing out of radioactivity between control and experimental samples can, therefore, be ruled out. Matching of control and experimental fiber lengths was done under the dissecting microscope: it is unlikely that a significant bias was introduced at this stage.

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The Components of the Muscle Background

It is clear from the data presented in Fig. 1 and in Tables I and IV that adsorbed radioactivity formed a very large part of the muscle background in the early experiments. But after prolonged aqueous washing or extraction with organic solvents, which reduced the rate of washing-out of adsorbed radioactivity from the muscle fragments to very low levels, relatively large amounts of radioactive DFP remained in the muscle (Table IV). Much of this radioactivity can be attributed to DFP-sensitive enzymes other than AChase. Rogers et al. (1969) have demonstrated the very heavy labeling with DFP of mast cells lying between the muscle fibers. The enzyme that is responsible is a protease, from which 2-PAM does not liberate DP-groups (Darzynkiewicz and Barnard, 1967). Other proteases and esterases which are sensitive to DFP occur in the muscle fibers themselves.

In all the experiments with scintillation counting that were technically satisfactory (Tables III and V), 2-PAM eluted significant amounts of radioactivity from muscle which lacked endplates. Histochemically, it is clear that AChase is present at very high concentration in the motor endplates, and is at or below the level of detection in the muscle fibers themselves. But in these counting samples, the volume of muscle present, even after microdissection, was probably several hundred times greater than the volume of endplate material. It is thus quite reasonable that the very low concentrations of AChase in the muscle fibers should give a recognizable counting rate, of the same order of magnitude as that attributable to the endplates.

Conclusions on the Number of AChase Molecules per Endplate

The agreement obtained between the results of the liquid-scintillation counting (a range of 1.2–4.4 $\times$ 10^7 molecules AChase per endplate) and those obtained previously by beta track radioautography (2.2–4.0 $\times$ 10^7 molecules per endplate), for the sternomastoid muscle of the mouse, is satisfactory. Both methods involve the labeling of AChase with radioactive DFP and the subsequent measurement of this radioactivity, with the use of 2-PAM for defining the specificity. But the variables and possible sources of error in the liquid scintillation-counting method are distinct from those in beta track radioautography. The advantage of employing a bulk extraction procedure is the independent means it offers of checking a result arrived at by *in situ* study. The over-all correlation thus established between the radioactivity per endplate measured *in situ* by track radioautography, on the one hand, and that measured by extraction and scintillation counting, on the other, is, therefore, taken as a confirmation of the validity of our values for the absolute number of these AChase active centers per endplate.

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