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

I. The Number of Acetylcholinesterase Molecules and of Other DFP-Reactive Sites at Motor Endplates, Measured by Radioautography

A. W. ROGERS, Z. DARZYNKIEWICZ, M. M. SALPETER, K. OSTROWSKI, and E. A. BARNARD

From the Molecular Enzymology Unit, Departments of Biochemistry and Biochemical Pharmacology State University of New York, Buffalo, New York, 14214, and the Section of Neurobiology and Behavior, Division of Biology, Cornell University, Ithaca, New York, 14850. Dr. Rogers' present address is M.R.C. Neuroendocrinology Research Unit, Department of Human Anatomy, Oxford, England. Dr. Darzynkiewicz's and Dr. Ostrowski's present address is Department of Histology, The Medical Academy, Warsaw, Poland. Reprint requests and inquiries should be addressed to Dr. E. A. Barnard, Molecular Enzymology Unit, State University of New York, Buffalo, N.Y. 14214

ABSTRACT

Di-isopropylfluorophosphate (DFP) labeled with phosphorus-32 was applied to fragments of the diaphragm and sternomastoid muscles of the mouse, in conditions in which it saturated all available sites at the motor endplates. After adequate washing and exchange with unlabeled DFP, single endplates were obtained by microdissection and their radioactivity was found by beta track radioautography. The number of sites phosphorylated by DFP-^{32}P per endplate was relatively constant for each muscle: in the sternomastoid, about 9×10^7 sites per endplate, in the diaphragm, about 3×10^7. Reaction with DFP-^{32}P was abolished by prior treatment with unlabeled DFP. Labeling was unaffected by prior fixation in formaldehyde, but was inversely proportional to the time of incubation in the Koelle staining medium, when this preceded labeling. The contribution of acetylcholinesterase (AChase) to this total number of DFP-reactive sites was determined by three methods. The first involved reactivation of the phosphorylated AChase by pyridine-2-aldoxime methiodide (2-PAM), in conditions in which the reactivation of other enzymes would be insignificant. The other two methods involved protection of the active centers of AChase from phosphorylation by labeled DFP by use of 284C51, an inhibitor highly specific for this enzyme, or by use of eserine. Each of these methods indicated that about 35% of the DFP-reactive sites at endplates of the sternomastoid and diaphragm are AChase. The mean number of AChase molecules was thus found to be 3.1×10^7 and 1.1×10^7 per endplate in sternomastoid and diaphragm, respectively. No significant reaction of labeled DFP with muscle and nerve was observed. Mast cells in the muscle had a concentration of DFP-reactive sites far higher than the endplates.

INTRODUCTION

The number and distribution of acetylcholinesterase (AChase) molecules at individual motor endplates must be known for any analysis, at the molecular level, of the transmission processes at these junctions. This number is considered here for two mouse muscles; the distribution of AChase at endplates at the ultrastructural level in the material described here has recently been reported upon (Salpeter, 1967).

For the study of various enzymes, including AChase, at the single cell level, valuable information has previously been obtained by enzyme histochemistry, with either the light or electron microscope. This involves incubation of the tissue in a medium containing a suitable substrate, and precipitation of one of the products of enzyme action as a detectable deposit. It is almost impossible, however, to deduce the amount of enzyme present in a given structure from measurements made on the deposited product. Ostrowski and Barnard (1961) have suggested the use of isotopically labeled enzyme inhibitors as reagents, in an alternative approach which is based on radioautography, to demonstrate the distribution of radioactivity—and hence of the enzyme—in the tissue. In this method, measurement of the amount of inhibitor bound by a given structure leads directly to the number of enzyme sites present. One reagent used for this purpose is di-isopropylfluorophosphate (DFP), a known inhibitor of AChase. This reaction is irreversible, resulting in the formation of a covalent bond with a serine residue at the active center of the enzyme (see review by Cohen and Oosterbaan, 1963). It, therefore, takes place in a 1:1 ratio. DFP also phosphorylates a number of other enzymes, including pseudocholinesterase (Chase), chymotrypsin, a number of nonspecific esterases, and a few other "active serine" enzymes. It can only be made the basis for the measurement of AChase in a given structure if its specificity is adequately controlled by the parallel use of other reagents specific for this enzyme. In this paper, we report the measurement, firstly, of the total number of sites phosphorylated by DFP in motor endplates of two muscles of the mouse. Secondly, we determine the proportion of these sites that represent AChase.

Experimental Approaches

Previous work on the localization of DFP-reacted enzymes by radioautographic techniques has been based on the use of tritiated DFP and grain density radioautographs (Ostrowski et al. 1963; Ostrowski et al., 1964; Darzynkiewicz et al., 1967). While the distribution and relative numbers of enzymic sites phosphorylated by DFP can be conveniently studied in this way, several factors combine to make this method impractical for measuring the absolute number of DFP-reactive sites present in structures the size of motor endplates. DFP labeled with phosphorus-32 was therefore used here for the latter purpose, and its uptake into single endplates was measured by beta track radioautography. Levinthal and Thomas (1957) have demonstrated the quantitative precision of this technique in an elegant series of experiments on the number of atoms of phosphorus in single virus particles, and its potentialities have been further explored by Levi et al. (1963).

For the separation, from all the DFP-reactive sites, of those sites that represent AChase, two methods have been adopted. The first involves the use of the compound pyridine-2-aldoxime methiodide (2-PAM), developed in biochemical studies as a specific reactivator of phosphorylated AChase (Wilson and Ginsburg, 1955; Davies and Green, 1956). This nucleophilic oxime attaches itself to the DP-AChase complex, removing the DP-group and restoring activity to the enzyme. (For recent reviews of the properties of 2-PAM, see Koelle, 1963). All the evidence available upon 2-PAM shows that it is highly specific for AChase, restoring activity to pseudocholinesterase (Chase) inhibited with organophosphorus compounds at less than $1/1000$ of the rate seen with AChase (Wilson et al., 1958). The fraction of the labeled endplate sites that could be reactivated by 2-PAM was, therefore, taken to be the AChase contribution.

The second approach has involved protection of true AChase with inhibitors more specific for this enzyme than DFP itself. Thus, the Burroughs-Wellcome compound 284C51 (1,5-bis(4-allyl-dimethyl-ammoniumphenyl) pentane-3-one dibromide) is claimed to be a highly specific inhibitor for AChase (Austin and Berry, 1953; Fulton and Mogey, 1954; Bayliss and Todrick, 1956). Eserine

TABLE I

The Basic Labeling and Washing Procedure Applied to Muscle Fragments

Solution	Vol.	Time	Temp.
	ml	*min*	°C
1. DFP-^{32}P at 10^{-4} M	1.0	20	20
2. Phosphate buffer (× 2)	2.0	2	20
3. Unlabeled DFP at 10^{-3} M	5.0	10	20
4. Unlabeled DFP at 10^{-4} M	10.0	20	0–4
5. Phosphate buffer (× 3)	20.0	20	0–4

Treatments 1–5 were applied in sequence, after Koelle staining, with continuous agitation in each. All solutions were in sodium phosphate buffer (0.02 M, pH 7.4). In later experiments, a 6th step, a wash in 10 ml of 70% ethanol at 20° for 2 min, was added.

is specific for both AChase and Chase (Augustinsson, 1963). AChase-active centers should be protected from reaction with DFP by the presence of the specific inhibitor. Protection by such inhibitors from labeling with DFP, and (independently) reactivation of DFP-reacted sites by 2-PAM, have been shown here to occur at the endplate sites, and the results are demonstrated to be consistent within themselves and with other evidence. The results permitted the number of AChase molecules at the endplate of mouse sternomastoid and diaphragm muscles to be deduced.

A preliminary report of part of this work has been published (Rogers et al., 1966).

MATERIALS AND METHODS

DFP-^{32}P and DFP-^{3}H were obtained from the Radiochemical Center, Amersham, England, and from New England Nuclear Corporation, Boston, at specific activities of 40–90 mc/mmole (^{32}P) and 1–4.3 c/mmole (^{3}H). Nonradioactive DFP was obtained from Sigma Chemical Co., St. Louis (and was handled with the precautions due for its great toxicity). DFP was stored in dried propane-diol in sealed containers at −35°C, and was used within 2 hr of dilution in aqueous media.

Eserine and 2-PAM were obtained from Sigma. A sample of 284C51 (mp 194–196°) was kindly provided by Dr. H. T. Openshaw, Wellcome Research Laboratories, Beckenham, Kent, England. Ethopropazine hydrochloride ("Parsidol"®) was kindly provided by the Warner-Lambert Research Institute, Morris Plains, N. J., research affiliate of Warner-Chilcott Laboratories. The tissue fixative was always 4% (w/v) formaldehyde in 0.027 M phosphate, pH 7.4, used at 4°C.

Labeling with DFP-^{32}P and Beta Track Radioautography

^{32}P-LABELING PROCEDURES: Male Swiss mice (18–34 g) of unknown age were killed by a blow on the head. Small fragments of the sternomastoid and diaphragm muscles, from regions rich in endplates, were immediately dissected and placed in phosphate buffer (0.02 M, pH 7.4) at 4°C. The endplates in each fragment were then lightly stained by using an acetylthiocholine technique (Pearse, 1961, p. 890) modified from that of Koelle and Friedenwald (1949), with incubation times of from 2 to 20 min. Following staining, the muscle was fixed in formalin for 5–18 hr in most of the experiments. In the remainder of the experiments, fixation was delayed until after the labeling and washing procedures were completed.

The labeling and washing procedures used (except where noted) were those described in Table I. Barnard and Ostrowski (1964) have shown that labeling in striated muscle reaches a maximum at

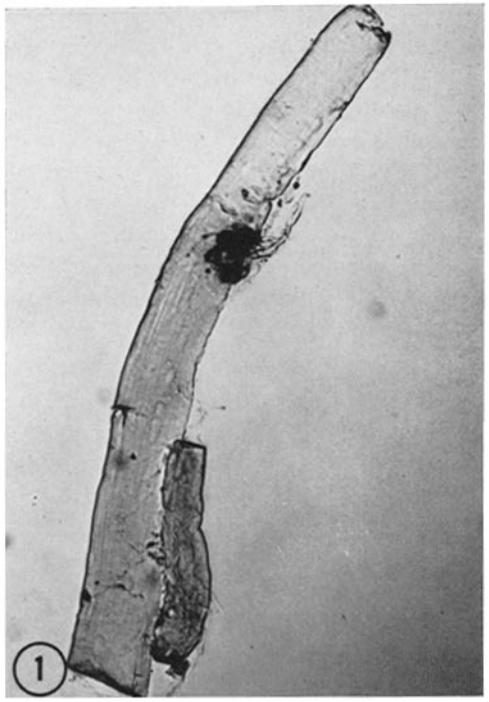

FIGURE 1 A microdissected length of muscle fiber, showing the stained endplate on it. This material is the type that was used in preparing the track radioautographs. In the majority of cases, the terminal axon could be seen at the time of isolating the endplate for radioautography, and the endplate always appeared to be structurally complete. × 120.

15–20 min, on incubation of small fragments in radioactive DFP at 10^{-4} M at room temperature.

BETA TRACK RADIOAUTOGRAPHY: After labeling, the fragments of muscle were transferred to distilled water in an ice-cooled dish. Individual lengths of muscle fiber were microdissected, some bearing stained endplates (Fig. 1) and some without endplates. Approximately 40 lengths of muscle fiber were placed, by means of a pipette, on each gelatinized slide, which was then allowed to dry. The techniques we have employed for the track radioautography, with Ilford G5 emulsion, are described in detail by Rogers (1967).

The mounted radioautographs were examined with a Leitz Ortholux microscope, with either the KS × 53 or KS × 100 oil immersion objective. All the track counts were made by two observers (A.W.R. and K.O.): there was no significant difference between counts made by either observer on the same material.

Endplates were accepted for counting only if they appeared histologically to be complete and undamaged. In addition, only those endplates on the surface of a muscle fiber which faced towards the emulsion were counted. In other words, if any part of a stained endplate was seen to be in contact with the microscope slide, and separated from the emulsion by its muscle fiber, that endplate was rejected.

The appearance of a beta track in Ilford G5 emulsion, and the grain spacing within these tracks, are well documented (Levi et al., 1963; Rogers, 1967). A track was recorded only when it could be clearly traced back to the area of tissue under examination. In view of the wide separation of the muscle fibers on the slides, the assumption was made that any track that could be traced to the area of tissue under observation in fact entered the emulsion, rather than left it, at that point.

For the case of ^{32}P, self-absorption within the single muscle fibers can be ignored (Roger, 1967). It follows that the beta tracks seen to enter the emulsion at a stained endplate may, in fact, have originated in the muscle fiber beneath that endplate. This muscle background was estimated by counting the number of beta tracks entering the emulsion from randomly selected areas on the fiber lengths without endplates. With a micrometer net in one eyepiece, the surface area of each stained endplate was measured, and the observed track count from that endplate was corrected by subtracting the appropriate background value from an equivalent area of muscle fiber alone.

THE CALCULATION OF THE NUMBER OF SITES PHOSPHORYLATED PER ENDPLATE: For a small source at the interface between glass and nuclear emulsion, emitting beta particles in the energy range of ^{32}P, the tracks entering the emulsion directly above the source represent one half of the radioactive decays taking place during exposure (Rogers, 1967). Having excluded endplates that were buried beneath their muscle fiber or otherwise shielded from direct contact with the nuclear emulsion, we made the assumption, in our calculations, that this same factor relates the observed track counts from the areas occupied by stained endplates (after correction for muscle background) to the disintegrations within the endplate during exposure. For beta particles from ^{32}P, less than 1% will fail to produce recognizable tracks (Rogers, 1967). The disintegration rate per hour per endplate (D) can, therefore, be calculated from the track count observed at the endplate (T_E) and that found for an equal area of muscle fiber alone (T_M) by the equation:

$$D = (T_E - T_M)\frac{2}{h}$$

where h is the exposure time in hours. After correcting for radioactive decay, calculated to the midpoint of exposure, we can readily convert the disintegration rate into the number of DP-^{32}P groups that have been introduced, since the specific activity of the DFP-^{32}P is known.

Labeling with DFP-^3H and Grain-Density Radioautography

Small fragments of muscle were fixed in formalin, and then labeled by one of the procedures specified in the text. Sections of muscle were then cut at 5 μ on a cryostat (American Optical Co., Buffalo), mounted on gelatinized slides, and radioautographed either by the stripping film technique, with Kodak AR-10 film (Pelc, 1956), or by dipping the slides in Ilford K2 nuclear emulsion (Rogers, 1967). Exposure times averaged 2 wk. Following development, fixation, and washing, the slides were immersed in a solution of 2-PAM at 10^{-2} M for 20–30 min, and then stained for the presence of AChase by the method of Karnovsky and Roots (1964). In spite of the radioautographic procedures, it was possible to produce sufficient staining in these sections to enable endplates to be positively identified (Fig. 2).

In one experiment, the muscle was stained by the α-naphthyl acetate technique for demonstrating nonspecific esterase (Pearse, 1961), which also enables endplates to be positively identified (see Hopsu and Pontinen, 1964). This material was labeled after staining. The cryostat sections were mounted on gelatinized slides, and then covered with a thin but impermeable membrane of polyvinyl chloride (PVC), according to the technique of Sawicki and Darzynkiewicz (1964). The radioautographic emulsion was applied over this membrane.

The grain counts in the material labeled with DFP-^3H were all performed by one observer (Z. D.)

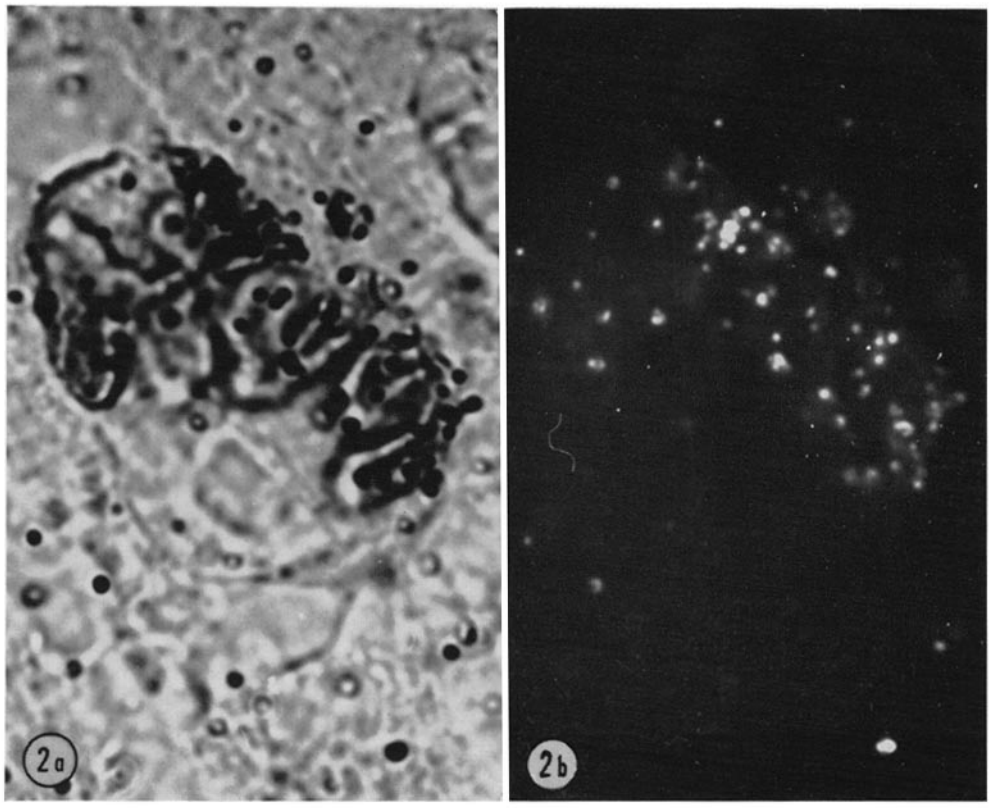

FIGURE 2 Photomicrographs of a section of diaphragm after labeling with DFP-^{3}H, to show a stained endplate: (*a*) by transmitted light, (*b*) by dark-field incident illumination. It can be seen that the silver grains in the radioautograph (reflecting, in *b*) are at a higher density over the endplate than over surrounding muscle fibers. The endplate is positively identified by staining (after 2-PAM treatment), by the Karnovsky and Roots (1964) method, through the emulsion after radioautography. Radioautograph prepared with Ilford K2 emulsion. × 1,200.

using a Leitz Ortholux microscope equipped with a Pl. Apo. × 100 objective and × 10 eyepieces. The grains were viewed by transmitted and vertical incident light alternately (Rogers, 1967), for distinguishing them with certainty from the deposited stain. Corrections for background were made on the basis of grain counts over adjacent areas of muscle fiber.

Labeling with DFP-^{3}H and Electron Microscope Radioautography

The sternomastoid muscles of one mouse were fixed *in situ* by treatment with cold 1% glutaraldehyde in phosphate buffer. Small fragments rich in endplates were dissected out, and fixation in cold glutaraldehyde continued for a further 3 hr. After being washed in buffer, this material was labeled by using the procedures "DFP-^{3}H-2-PAM" or "DFP-2-PAM-DFP-^{3}H" (Table IV). After postfixation in OsO_4, electron microscope sections were prepared and radioautographed with Ilford L4 and Kodak NTE emulsions, by the techniques described by Salpeter (1967).

Staining Procedures for Demonstration of Mast Cells

Small muscle fragments were fixed in formalin in the usual way, and then treated with ethylene glycol monoethyl ether, according to the technique of Guidotti and Spinelli-Rossi (1963). These fragments were labeled (Table I) with DFP-^{3}H, and then sectoned on a cryostat at 5 μ and radioautographed. After photographic processing, the sections were stained with toluidine blue (0.025% in acetate buffer at pH 4.5) for 30 min at 0°C. Mast cells showed clear metachromatic staining.

RESULTS

Measurement of the Total Number of DFP-Reactive Sites per Endplate

Diaphragm and sternomastoid muscle specimens were treated with DFP-^{32}P to label endplates. Radioautographs of microdissected single endplates were then prepared (Fig. 1). Beta tracks numbering not more than 10 per endplate could be clearly seen entering the emulsion from the endplate, at the exposure times used (Figs. 3 and 4).

Table II lists the calculated values for the number of molecules of DFP-^{32}P bound per endplate for all the available experiments. In addition, a number of factors which differed, from one experiment to another, are listed. With the sternomastoid muscle in particular, a wide range of values was found, 0.78–8.6 $\times$ 10^7 molecules per endplate. This variation is large relative to that occurring within each experiment (Table II, last column). The factors that varied among the experiments were, therefore, examined to identify the source of this variation.

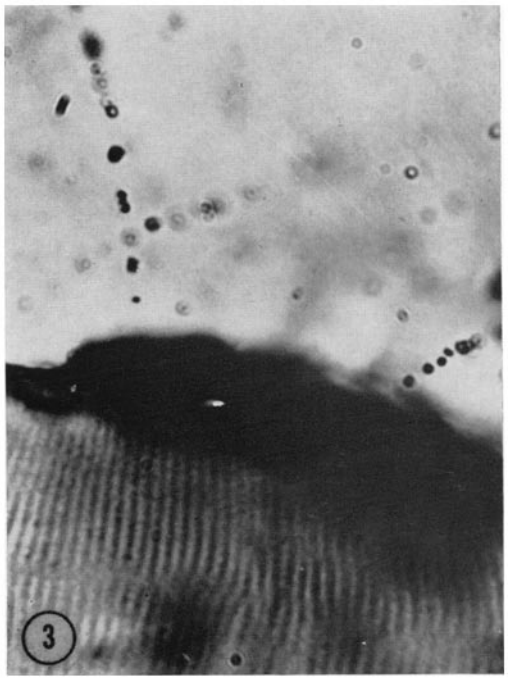

FIGURE 3 A beta track radioautograph, showing two tracks of silver grains formed by beta particles entering the emulsion from the stained endplate. $\times$ 1,200.

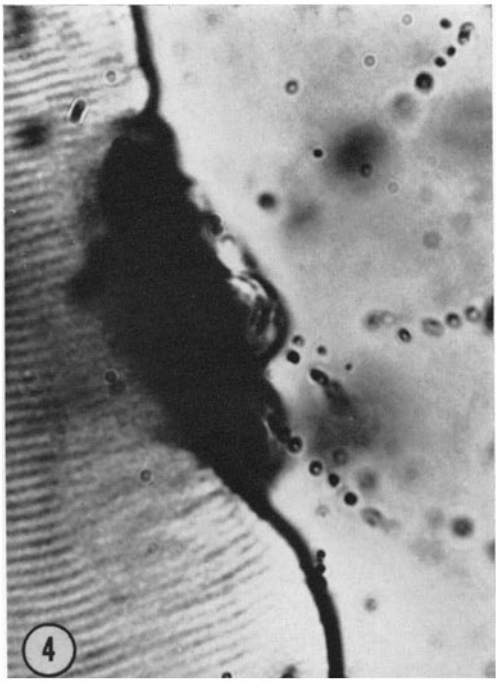

FIGURE 4 A beta track radioautograph showing several tracks in the vicinity of a stained endplate. When the focus was changed, these tracks could all be traced back to the endplate itself. $\times$ 1,200.

THE EFFECT OF PRIOR TREATMENT WITH UNLABELED DFP: In Exp. 4, the fragments of sternomastoid muscle, with and without endplates, were separated into two groups. One group was preincubated in unlabeled DFP at 10^{-4} M for 20 min at room temperature, and both groups were then labeled by the standard (Table I) procedure. Without the preblocking, the endplates showed a track count significantly above the level in muscle fibers alone. No such difference was seen in the other group, however, in which all the sites of phosphorylation had presumably been blocked by the initial treatment with unlabeled DFP (Table III). It would seem reasonable to infer that differences in track density between endplates and muscle fibers without endplates reflect differences in the numbers of sites available for phosphorylation by DFP, and are independent of nonspecific adsorption of DFP-^{32}P. It is interesting to note that the muscle background was actually higher in the material preblocked with unlabeled DFP. The value for muscle background represents adsorbed radioactivity not removed by the wash-

TABLE II

Variation with Conditions of Labeling of the Apparent Numbers of DP-Groups per Endplate

Experiment	DFP-^{32}P labeling time	Fixation	Staining time	Exposure	DP-groups ($\times 10^{-7}$) per endplate	
					Apparent	Corrected
	min		*min*	*hr*		
A. *Sternomastoid muscle endplates*						
4	20	After	10	66	2.6	
5	20	After	10	18	3.4	6.2 ± 1.1 (18)
6	20*	After	10	18	4.0	7.3 ± 0.8 (18)
7	20	After	10	17.5	5.2	9.5 ± 1.6 (13)
8	30	Before	5	20	4.9	6.5 ± 0.7 (15)
9	30	Before	5	20	6.9	9.2 ± 1.6 (28)
12	20	After	10	18	4.4	8.0 ± 1.2 (47)
14	20	Before	5	45	1.9	
15 *a*	20	Before	2	21	8.6	9.5 ± 3.6 (13)
b			5		8.6	11.4 ± 1.9 (9)
c			10		6.0	10.9 ± 2.4 (12)
d			20		0.8	10.0 ± 0.9 (22)
16	20	Before	5	44.5	1.9	
17	16	Before	10	21	2.2	
18 *a*	20	Before	10	15	4.7	8.5 ± 1.2 (45)
b				39	3.6	
B. *Diaphragm muscle endplates*						
7	20	After	10	17.5	2.0	3.6 ± 0.4 (37)
8	30	Before	5	20	1.9	2.5 ± 0.3 (23)
9	30	Before	5	20	2.3	3.1 ± 0.8 (17)
11	20	After	10	18	1.6	2.9 ± 0.7 (38)
12	20	After	10	18	1.6	2.9 ± 0.4 (73)

The experimental variables which might have contributed to the relatively wide scatter of apparent values are listed. The values in the last column are regarded as acceptable values, obtained by correcting (see text) the apparent values for the effects of prior staining: Each value is given as the mean and its standard error, and the number of endplates used is noted in parentheses. Exps. 4, 14, 16, and 18 *b* have been ignored there because of the native chemography associated with the longer exposure periods; Exp. 17 is ignored because of the short labeling time used.

* All were labeled as shown in Table I except for those in this experiment, in which DFP-^{32}P at 10^{-3} M was used.

ing procedure in both groups of material. Small differences in the size and shape of the individual fragments could influence the efficiency of washing. In all the material presented, therefore, measurements of muscle background are taken on fibers from the same fragment as the endplates.

THE SATURATION OF AVAILABLE REACTIVE SITES BY DFP-^{32}P: Two methods were used to determine whether the standard exposure of 20 min, at room temperature, to DFP-^{32}P at 10^{-4} M was sufficient to saturate all available reactive sites in the endplates. In Exps. 8 and 9 (Table II), the time of exposure was extended to 30 min. These experiments were comparable in all other respects with Exp. 15 *b* for sternomastoid: the results were very close. With the diaphragm, no complete matching of these experiments was available, but all the values obtained with this muscle are reasonably close.

In Exp. 6, DFP-^{32}P was applied at 10^{-3} M, and a value of 4.0×10^7 molecules DFP reacted per sternomastoid endplate was obtained. Exps. 5, 7, and 12 matched Exp. 6 in every respect except for the lower concentration of DFP-^{32}P, and gave values of $3.4–5.2 \times 10^7$ molecules per endplate.

It is reasonable to assume that all available sites

TABLE III

Labeling Seen on Muscle Fibers Bearing Endplates and on Fiber Lengths without Endplates

Material	Endplate fiber	Background fiber
Group I: DFP-^{32}P labeling		
a. Tracks per endplate or equivalent area	6.3	1.4
b. Tracks per unit length of fiber	1.7	1.1
Group II: Pretreatment with unlabeled DFP		
c. Tracks per endplate or equivalent area	1.0	0.7
d. Tracks per unit length of fiber	1.6	1.6

There were 22 endplates or fibers in each group. After the usual labeling procedure, endplates showed a highly significant track count relative to background fibers (*a*). In material treated with nonradioactive DFP before labeling, there was no significant difference between endplates and background fibers (*c*). Even when the counts were averaged over the whole length of the fiber in question, there is still a significant difference from background due to the presence of an endplate (*b*); this is not so when the material is preblocked (*d*).

at the endplate are phosphorylated in the conditions listed in Table I, as suggested by Barnard and Ostrowski (1964).

THE EFFECT OF PRIOR FIXATION ON PHOSPHORYLATION WITH DFP-^{32}P In the majority of experiments listed in Table II, the muscle fragments were fixed with formalin before phosphorylation with DFP-^{32}P. It is possible that fixation could inactivate enzymes present at the endplate, reducing the apparent uptake of radioactivity. Therefore, in a series of experiments (Nos. 4, 5, 6, 7, 11, and 12), fixation was postponed until after the labeling with DFP-^{32}P. If we consider the sternomastoid results, Exps. 5, 6, 7, and 12 form a group which is directly comparable in every respect with Exps. 15 *e*, 17, and 18 *a*.

The mean value from the group of experiments in which the muscle was fixed prior to labeling was 4.28×10^7 molecules per endplate. The mean value from the groups in which fixation followed labeling was 4.25×10^7 molecules per endplate. There is thus no evidence for loss of enzymic sites through prefixation in formalin.

LATENT IMAGE FADING: Fading of the latent image in the silver halide crystal (see Rogers, 1967) may occur (particularly in the presence of moisture and of atmospheric oxygen). It may be troublesome with relatively long exposure times, of the order of several weeks, but the longest exposure in these experiments was 66 hr.

Latent image fading affects track radioautographs by reducing the density of silver grains along the course of those tracks formed early in exposure. There is a stage at which these tracks with widely spaced grains can, nevertheless, be recognized, whereas more severe fading will make it impossible to link up the remaining grains into a continuous track. The tracks seen throughout the emulsion were carefully examined with the possibility of fading in mind. The grain spacing observed was fully consistent with that described by Levi et al. (1963). It is, therefore, assumed that generalized latent image fading did not occur in this material, a result that would be predicted from the efforts made to control the humidity and, in later experiments, to exclude atmospheric oxygen during the short exposure periods.

NEGATIVE CHEMOGRAPHY: This is (see Rogers, 1967) an artifactual, chemically induced decrease in developable grains, in the immediate vicinity of the tissue responsible. It was found in several experiments in which the exposure time exceeded 24 hr. This effect persisted, in spite of efforts to dry the emulsion and to exclude atmospheric oxygen during exposure. In Exp. 18, identical material was exposed for periods of 15 and 39 hr. From the 45 endplates examined at the shorter exposure time, a value of 4.67×10^7 molecules per endplate was obtained; 27 endplates at the longer time gave 3.64×10^7 molecules per endplate.

Of the experiments in Table II in which the staining time was 10 min (excluding Exp. 17, in which the labeling time was only 16 min), eight experiments are comparable in every respect, save the length of radioautographic exposure. The values obtained from exposures of 39 and 66 hr are lower than the values in the other experiments, in all of which the exposures were for less than 24 hr. Similarly, with 5-min staining, there are five experiments which form another comparable group. Here, the reduction in the calculated number of molecules per endplate is even more marked in the material exposed for more than 24 hr.

This negative chemography affected the track counts from the muscle fibers without endplates

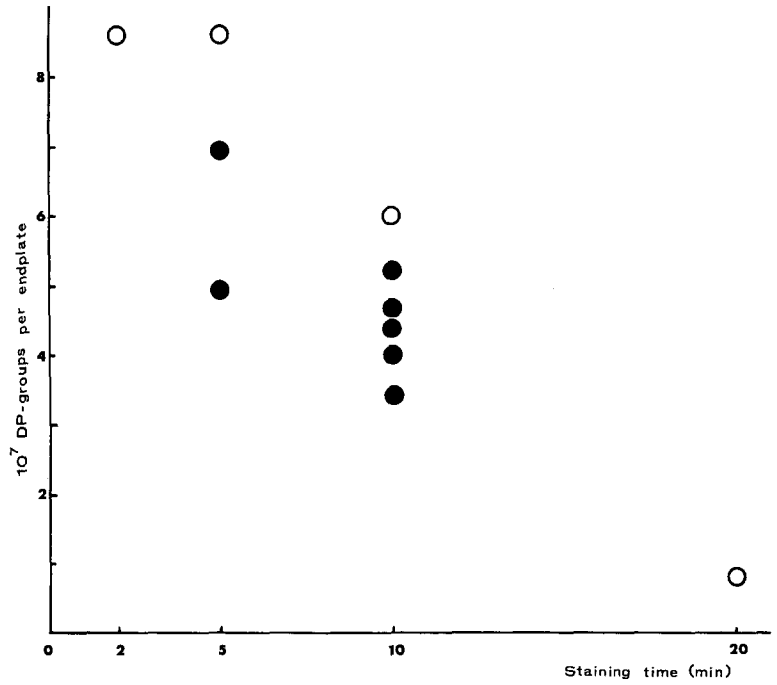

FIGURE 5 A graph to illustrate the effects of prestaining the endplate by the Koelle reaction for various periods on the observed uptake of DFP at the endplate. ○, mean values from Experiment 15; ●, the mean values from each of the other experiments on sternomastoid endplates (after excluding those experiments with exposure times longer than 24 hr or incubation times in DFP-^{32}P of less than 20 min).

just as much as the counts from endplates themselves. It is unlikely to be due to the colored precipitate of the Koelle reaction, therefore.

In view of this artifact, all experiments with exposure times longer than 24 hr should be excluded from calculations of the absolute number of molecules of DFP reacting per endplate (Table II). These experiments are, nevertheless, valid for the estimation of the relative numbers of sites phosphorylated under different conditions. There is no correlation between the exposure time and the calculated number of DP-^{32}P groups per endplate for exposures of less than 24 hr. This supports the qualitative observation that the grain spacing in tracks immediately adjacent to the tissue seemed normal in this material.

THE EFFECT OF THE KOELLE REACTION ON REACTION WITH DFP-^{32}P: All the material examined was stained by the Koelle reaction for AChase in order to identify the motor endplates. This was necessary both at the stage of microdissection and in scanning the completed radioautographs. This staining had to be carried out prior to labeling with DFP-^{32}P, as phosphorylation inhibits the enzyme and abolishes the color reaction. No other staining method is known for motor endplates which is not also abolished by treatment with DFP. To use endplates in unstained material was not desirable, because of the risk of overlap between the track densities from the least heavily labeled endplates and from the most highly labeled areas of muscle fiber.

The investigation of the effects of staining was, therefore, limited to the comparison of results after incubation for different periods of time in the medium containing acetylthiocholine. In Exp. 15, material which was otherwise identical was incubated in the Koelle medium for 2, 5, 10, and 20 min. The apparent values thus obtained for the number of sites phosphorylated by DFP-^{32}P per sternomastoid endplate, together with the mean values obtained from all the other experiments in which the exposure times were less than 24 hr, are plotted in Fig. 5.

There is an obvious reduction in the number of labeled molecules per endplate in the sternomastoid muscle with increasing incubation time in the staining medium. This effect is similar but less

noticeable in the fewer results from the diaphragm. The decrease with time appears to be approximately linear, and extrapolation to zero time gives a value of about 9.0×10^7 DFP-sensitive sites per endplate for the sternomastoid, and about 3.0×10^7 sites per endplate for the diaphragm.[1]

THE NUMBER OF DFP-REACTIVE SITES PER ENDPLATE: In calculating this number, we excluded all experiments with radioautographic exposures longer than 24 hr, in view of the negative chemography experienced with this material. Exp. 17 was also excluded, since the time of incubation in DFP-^{32}P was only 16 min. This time is too short in our conditions to give saturation of all available DFP-reactive sites (Barnard and Ostrowski, 1964).

The values of the apparent number of DP-groups per endplate for all the remaining experiments were corrected for the effects of prior staining. These corrected results are listed in the final column of Table II. They suggest that each sternomastoid endplate contains $6.2–11.4 \times 10^7$ sites that can be phosphorylated with DFP. Diaphragm endplates contain $2.5–3.6 \times 10^7$ sites.

[1] In recent experiments of Dr. J. Wieckowski (to be published) in this laboratory, it has been shown that, with improved procedures, small fibers each bearing one endplate can be obtained from rodent sternomastoid muscle without any staining treatment whatsoever. These endplates have been identified, after the dissection, by the presence of the afferent terminal nerve fiber. Tracks can be unambiguously counted for these endplates, removing the difficulty discussed here. A modified version of the acetylthiocholine-staining reaction has also been employed which, with short incubations, has been found to permit the localization of the endplates, as in the present work, but does so without retardation of the DFP reaction. The value for the number of DFP-reactive sites per mouse sternomastoid obtained by track radioautography with either of these new methods is not significantly different from the value already obtained by the extrapolation (Fig. 5) in our stained material. Hence, the assumption used in the present work has been independently validated.

Measurement of the Contribution of AChase to DFP-Reactive Sites at Endplates

Experiments to determine the percentage of DFP-reactive sites that can be considered active centers of AChase were carried out by beta track radioautography, following labeling with DFP-^{32}P, and by grain-density techniques after DFP-^{3}H.

TRITIUM LABELING OF ENDPLATES: Diaphragm muscle specimens were treated with DFP-^{3}H and radioautographs were prepared which showed that grain densities over the sectioned endplates (Fig. 2) were well above those over muscle fibers alone. If we take into account the specific activity of the DFP-^{3}H and the length

TABLE IV

The Percentage of DFP-Reactive Sites per Endplate that Could be Reactivated by 2-PAM

Muscle	Treatment	Radioautographic technique	Sites sensitive to 2-PAM
			%
Sternomastoid	DFP-^{32}P–2-PAM	Beta track	33
			37
Diaphragm	DFP-^{32}P–2-PAM	Beta track	37
	DFP-^{3}H–2-PAM	Grain density	36
			33
			46*
	DFP–2-PAM–DFP-^{3}H	Grain density	31*

In each experiment, a group of endplates was treated by the sequence indicated, and a parallel group by the DFP-^{32}P (or DFP-^{3}H) basic procedure. The measurement for the reactivated sites (determined by subtraction, except in the experiment of the last line) is expressed as a percentage of that for the total sites, per endplate. In the beta track material, each group comprised 12–48 endplates; in the grain density material, grain counts were taken from 50 or 100 sectioned endplates in each group.

* Material stained by the α-naphthyl acetate reaction.

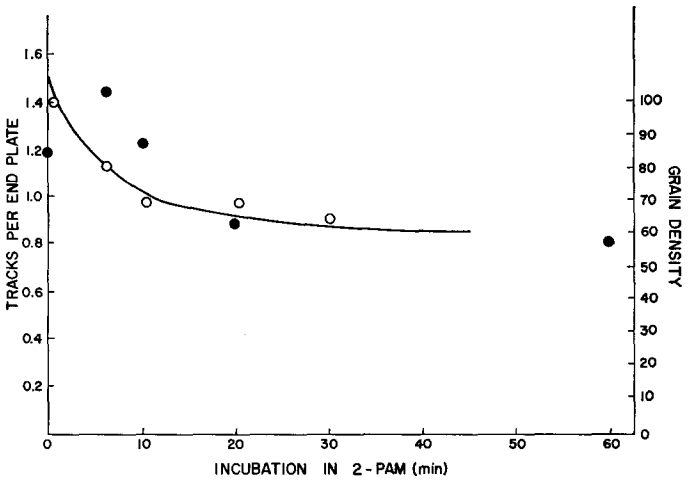

FIGURE 6 A graph to show the time-course of reactivation of DFP-labeled endplates by 2-PAM. The solid circles represent track counts on material labeled with DFP-^{32}P: each group comprised 14–24 endplates, giving a standard deviation which was between 0.18 and 0.34. The open circles represent grain counts on material labeled with DFP-^{3}H: each group comprised 100 sectioned endplates.

of exposure in each experiment, the grain densities were compatible with the densities predicted on the basis of the number of sites of phosphorylation by DFP (as measured above) and with the estimates of grain yield for tritium radioautographs available in the literature (Barnard and Marbrook, 1961; Kisieleski et al., 1961; Falk and King, 1963). In the absence of a firm estimate of the grain yield from tritium in our experimental conditions, no attempt has been made to calculate the absolute number of sites from the ^{3}H-labeled material.

In one experiment, the α-naphthyl acetate color reaction was used to stain endplates in fragments of diaphragm, before labeling with DFP-^{3}H. This method for nonspecific esterases gives noticeably more intense staining at endplates than in muscle generally, an effect that is abolished by treatment with DFP prior to staining. The cryostat sections of muscle stained in this way were covered with a protective membrane of PVC before coating with liquid emulsion, for preventing possible chemography from the stain and protecting the stain during photographic processing. The unknown thickness of the membranes introduced a source of variation into this experiment which was not present in those cases in which staining took place through the emulsion after processing. Differences in thickness in membranes covering different slides were randomized by counting from several slides in each group; the results of this experiment are included in Table IV, where they can be seen to be in line with those of the other experiments (see below).

REACTIVATION OF PHOSPHORYLATED ACHASE BY 2-PAM: Two complementary procedures were used in these experiments: in both, the labeling over an experimental group of endplates was compared with that over endplates labeled directly with radioactive DFP. The first procedure involved labeling with radioactive DFP, followed by incubation in 2-PAM to remove labeled DP-groups from AChase alone, and is designated DFP-^{3}H–2-PAM. The second involved treatment with nonradioactive DFP, followed by 2-PAM and radioactive DFP, and will be referred to as DFP–2-PAM–DFP-^{3}H. In either procedure, DFP-^{3}H may be replaced by DFP-^{32}P. The first procedure labels all DFP-reactive sites except AChase, whereas the second procedure labels AChase alone.

The time-course of reactivation by 2-PAM was first determined (Fig. 6) with the routine DFP-^{3}H– at room temperature, the radioactivity initially present at the endplate decreases for the first 15–20 min, and then remains relatively constant, even after incubation in 2-PAM for 60 min. Approximately one-third of the radioactivity at the endplate is removed in incubation times of 20 min or more.

Table IV summarizes the results obtained by the use of 2-PAM. In endplates of both the sterno-

TABLE V

Labeling Procedures Employing Reactivation by 2-PAM or Protection by Inhibitor

DFP-^{32}P–2-PAM	DFP–2-PAM—DFP-^{32}P	Inhibitor protection
1–5. Labeling and washing as in Table I 6. Phosphate buffer* 7. 2-PAM at 10^{-3} M* 8. Phosphate buffer (× 2)*	1. Unlabeled DFP at 10^{-4} M 2. Phosphate buffer (× 2) 3. Phosphate buffer* 4. 2-PAM at 10^{-3} M* 5. Phosphate buffer (× 2) 6–10. As in Table I (1–5.)	1. Inhibitor 2. DFP-^{32}P at 10^{-4} M + inhibitor 3. Phosphate buffer (× 2) + inhibitor 4. Unlabeled DFP at 10^{-3} M + inhibitor 5. Unlabeled DFP at 10^{-4} M + inhibitor 6. Phosphate buffer (× 3) + inhibitor
Labeled: All sites except AChase	AChase only	(284C51) All sites except AChase (Eserine) All sites except (AChase + Chase) (Ethopropazine) All sites except Chase

In some experiments, DFP-^{3}H was substituted for DFP-^{32}P (with the Koelle staining then postponed: see *Methods*), and grain density techniques were substituted for beta track radioautography. All solutions were made up in sodium phosphate buffer (0.02 M at pH 7.4) except those marked *, which were at pH 7.9 (0.02 M phosphate buffer). The 2-PAM treatment, and the two subsequent rinses in phosphate buffer, were each for 20 min at 20°. In later experiments, brief extraction in 70% ethanol followed the final wash in phosphate buffer. The headings are used in the text to designate these entire procedures (with DFP-^{3}H replacing DFP-^{32}P, where used). The concentrations of inhibitor were always 3×10^{-5} M (for 284C51 or ethopropazine) or 10^{-5} M (eserine).

mastoid and the diaphragm, the proportion of sites sensitive to 2-PAM was about 35%. This value was independent of the isotope, the labeling sequence, and the radioautographic method. It was not altered when the identity of the labeled endplate was checked by the naphthyl acetate staining method. For these reasons, therefore, it is concluded that about one-third of the sites phosphorylated by DFP at motor endplates is AChase.

PROTECTION OF CHOLINESTERASE BY SPECIFIC INHIBITORS: The proportion of AChase sites among the DFP-reacted sites was also determined by protection of the latter by inhibitors more specific for AChase than DFP itself. Those inhibitors used were the Burroughs-Wellcome compound 284C51, and eserine (see Introduction); the inhibitor was present before, during, and after the labeled DFP reaction (Table V). With either DFP-^{3}H or DFP-^{32}P, each of two different experiments with the anti-AChase 284C51 showed (Table VI) protection of about one-third of the total DFP-reactive sites at each endplate, while one experiment showed protection of about one-half of these sites. Eserine, which inhibits both AChase and Chase, again protected one-third of the sites in the diaphragm endplate that are reactive to labeled DFP (Table VI).

Ethopropazine (lysivane) is specific (see Discussion), at the concentration used here, for Chase. The results, after protection by this compound (Table VI), for the cases of the sternomastoid and the diaphragm endplates are not in very close agreement, but all of the three determinations made show that only low amounts of Chase are present. More experiments would be needed to determine these figures accurately. If we take into account the failure of eserine to protect more sites from reaction with DFP than 284C51 protected, it is clear that relatively few of these sites can be attributed to Chase, a conclusion supported by these experiments with ethopropazine.

DFP-Reactive Sites in Structures Other than Motor Endplates

The labeling seen in striated muscle in these experiments was variable, and it was low relative to that in endplates. We attribute most of this

TABLE VI

The Proportion of Sites per Endplate Protected from Phosphorylation by Specific Enzyme Inhibitors

Muscle	Inhibitor	Radioautographic technique	Sites protected	Enzyme protected
			%	
Sternomastoid	284C51	Beta track	51*	AChase
Diaphragm	284C51	Beta track	31	AChase
		Grain density	34	AChase
Diaphragm	Eserine	Grain density	34	AChase + Chase
Sternomastoid	Ethopropazine	Beta track	5*	Chase
Diaphragm	Ethopropazine	Grain density	16	Chase
			12	Chase

In each experiment, a group of endplates was treated by the sequence indicated (see Table IV, last column) and a parallel group by the basic DFP-^{32}P (or DFP-^{3}H) procedure. The measurement for the former (protected) group is expressed as a percentage of that for the latter. In the beta track material, each group comprised sections of 50 or 100 endplates. In the experiments marked *, the incubation period of the material in DFP-^{32}P was 16 min, instead of the usual 20 min.

radioactivity to adsorbed DFP rather than to sites that are phosphorylated irreversibly. The variation seen could be related to the extent and nature of the washing process after labeling. When a wash in organic solvents (Rogers and Barnard, 1969) was given, the muscle background was reduced, but some persisted. There are almost certainly esterase sites in muscle fibers at which DFP reacts, but they must be at a very low concentration since it was not possible to measure them in this material.

Similarly, no appreciable labeling above muscle background was observed in the peripheral nerves of this material. Salpeter (1967) has found only very low levels of labeling in muscle and in nerve in similar specimens, prepared in parallel to these, for electron microscope radioautography: the labeling over the terminal axon region was not above 2% of that in the junctional fold region.

REACTION OF DFP WITH ENZYMES IN MAST CELLS: In grain-density radioautographs of muscle sections following labeling with DFP-^{3}H, scattered but discrete areas which were highly radioactive were often encountered. These areas did not correspond in position or number with the distribution of endplates (Fig. 7). The labeling did, however, reflect phosphorylation with DFP, since it was abolished by pretreatment with nonradioactive DFP. This labeling was absent in control radioautographs of unlabeled muscle, but was present, again distinctly stronger than at the stained endplates, in radioautographs in which a thin PVC film (Sawicki and Darzynkiewicz, 1964) was interposed over the tissue, showing that no form of chemography was responsible for it. In material fixed and processed in the normal way, cytological detail in these heavily labeled structures was not well preserved, so that their identification was difficult then. These structures were eight to ten times more heavily labeled than endplates, after the direct treatment with DFP-^{3}H.

In material viewed by electron microscope radioautography, these highly radioactive structures were also seen (Fig. 8), and had the appearance characteristic of tissue mast cells.

Material was prepared by postfixation in ethylene glycol monomethyl ether (Guidotti and Spinelli-Rossi, 1964), which better preserves the cytoplasmic granules of mast cells. After toluidine blue treatment of this material, all the *highly* radioactive areas corresponded to granular cells with

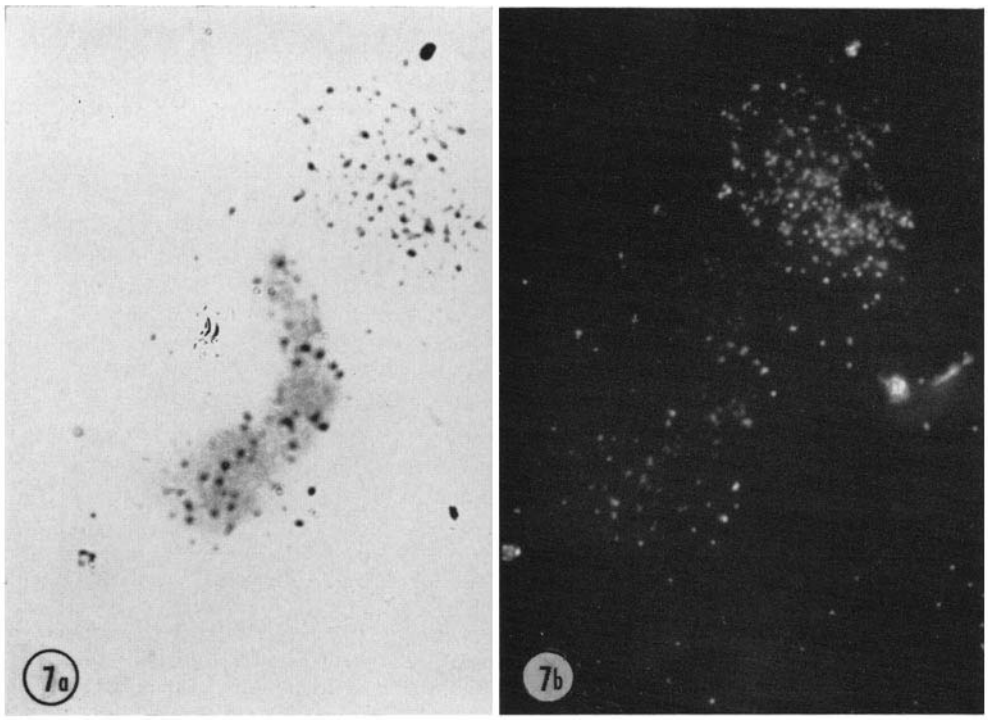

FIGURE 7 A radioautographic section of DFP-^{3}H–treated, Koelle-stained diaphragm that contains both an endplate and one of the sites of high grain density. In *a* (transmitted light), the endplate is recognized by its staining. In *b* (reflected light), only the silver grains are seen, and are shown to be heavier over the nonstainable structure.

metachromatic staining (Fig. 9), confirming that these structures were mast cells.

DISCUSSION

The Nature of the Endplate Sites Labeled by DFP

The radioactivity measured in endplates, after the subtraction of the background due to the muscle fiber itself, has well defined characteristics. It is completely absent in material treated with unlabeled DFP at 10^{-4} M for 20 min before the labeling procedure. It persists, however, through a long and thorough washing, which includes exchange with unlabeled DFP at 10^{-3} and 10^{-4} M. These features eliminate the possibility that merely adsorbed isotopic material is being measured. The absolute number of molecules of DFP-^{32}P represented by this radioactivity is reasonably constant in different animals, and in different experiments in which the background levels of radioactivity in the muscle varied considerably. It is independent of formalin fixation, and of increases in either the concentration or the time of incubation in DFP-^{32}P. The conclusion that this technique is measuring all the sites within the endplate at which the phosphorylation with DFP-^{32}P can take place is difficult to avoid.

Such irreversible phosphorylation occurs, at the low DFP concentrations and reaction times that we have used, only at the active centers of certain esterases (Cohen and Oosterbaan, 1963), and not at other proteins: the specificity of this treatment for the serine esterases has been discussed in detail elsewhere (Barnard and Rogers, 1967).

The Validity of the Quantitative Measurements

Although every effort was made to count only from endplates that appeared to be undamaged, the terminal portion of the motor nerve could not always be seen, and hence minor degrees of damage cannot be excluded. In this context, it is in-

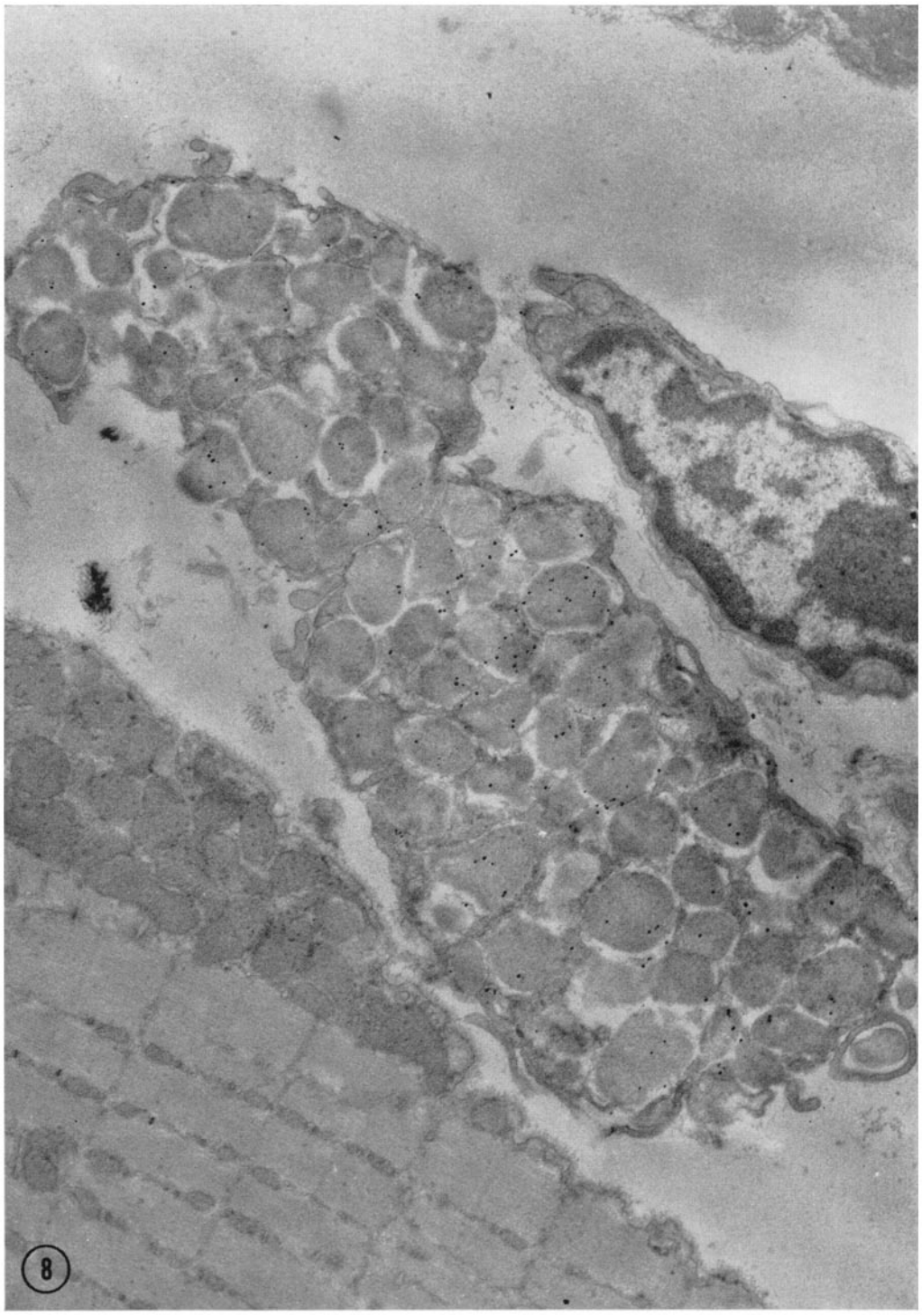

FIGURE 8 Electron microscope radioautograph of a section of sternomastoid muscle, after the DFP-^{3}H–2-PAM treatment. Coated with a monolayer of centrifuged Kodak NTE emulsion; developed with gold latensification-Elon-ascorbic acid; 11 days of exposure. Labeling is seen to be confined to granules in a mast cell, and is not over muscle fibers. × 14,000.

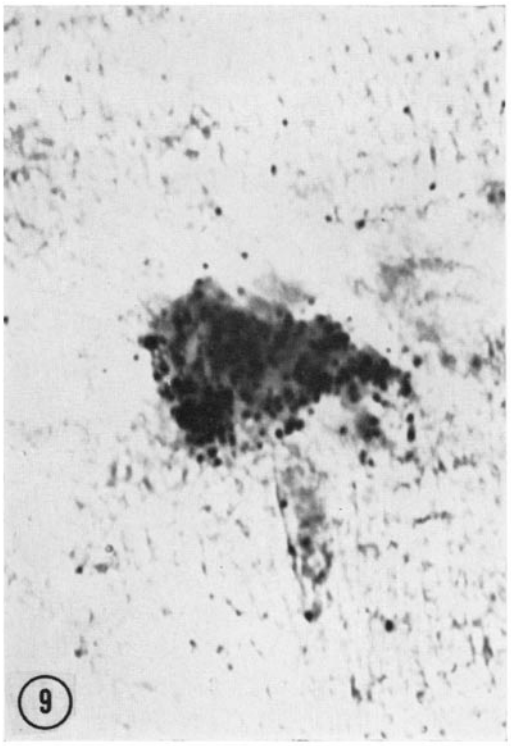

FIGURE 9 A radioautographic section of DFP-^{3}H–treated diaphragm, preserved with ethylene glycol monoethyl ether, and stained with toluidine blue. A metachromatic cell is shown, coinciding with a site of high grain density.

teresting to note that approximately 80% of the AChase at the endplate is situated in the junctional fold region, presumably in the postsynaptic membrane (Rogers et al., 1966; Salpeter, 1967). It is thus unlikely that minor degrees of damage to the terminal axon and its surrounding Schwann cells would significantly reduce the total number of sites of phosphorylation in the endplate.

The negative chemography encountered in the present experiments may have been due to the formalin fixation. Having established that formalin fixation did not inactivate any of the sites being studied, we found it preferable to control this artifact by keeping emulsion exposure periods shorter than 24 hr, rather than to investigate alternative fixatives. In one other experiment (details given by Barnard and Rogers, 1967), glutaraldehyde was found to reduce the number of sites phosphorylated by DFP by about 10%.

The Effect of Staining on the DFP Reaction

The inhibition of phosphorylation by DFP, produced by the Koelle reaction, is remarkable. It may be that the precipitate accumulating at the enzyme sites physically impedes the access of DFP. This might be expected to give the approximately linear type of relationship seen in Fig. 5. If this is the correct explanation, the extrapolation of this curve to zero time would be an acceptable method of estimating the number of sites that would have been phosphorylated in the absence of staining. Since these measurements were made, the validity of this estimate obtained by extrapolation has been confirmed by track measurements (Wieckowski and Barnard, in preparation) on sternomastoid endplates labeled without staining (see Footnote 1). It will be shown in the succeeding paper (Rogers and Barnard, 1969) that liquid scintillation counting of isotope from DFP-^{3}H–reacted diaphragm strips, stained and unstained, gives results that provide an independent confirmation of the results of the extrapolation in Fig. 5.

This inhibiting effect of the Koelle reaction has interesting consequences for histochemical techniques based thereon. It is clear that sites of high activity would soon become inhibited, due to the initially rapid buildup of deposit. Sites at which the enzyme is less concentrated, however, might be expected to continue to accumulate end-product. Any comparisons that are made between the amounts of enzyme present in different sites from the amount of deposit seen should take account of this, and should obviously be based on the shortest possible incubation times.

Variability and Reproducibility

The fairly wide scatter of mean values found in the various experiments conducted under apparently similar conditions, and the variation found within each experiment, can, in part, be attributed to this effect of staining. At microdissection, the endplates at the surface of the fragment were often more heavily stained than the endplates within the fragment. Presumably, diffusion of acetylthiocholine into the fragment limited the rate of deposition of end-product at more centrally placed endplates. Although endplates that were clearly stained were usually dissected from the surface of the block, no deliberate attempt was made to restrict dissection to this region. We have not seen, however, in any

of the work reported in this series, any evidence for difficulty of diffusion of DFP itself into muscle specimens of the size we have used: the charged substrate (and the metal) can be expected to differ from the lipophilic DFP molecule in this respect.

A further cause of variability can probably be identified in the mice. In Exp. 18, sternomastoid endplates from two mice were processed separately instead of being pooled, as in previous experiments. The track counts in 35 endplates counted from the first mouse were approximately one-half the track counts seen in the 37 endplates from the second mouse, in conditions that were identical. This individual variation is interesting and deserves further investigation.

The data permit, nevertheless, the estimation of the total number of DFP-reactive sites per endplate in the two muscle types studied, at the levels shown in Table II. Since the fraction of these sites that is AChase has been determined (Tables IV and VI), the absolute numbers of AChase molecules per endplate in these muscles are now available (Table VII). These are, strictly, the numbers of active centers of AChase. AChase purified from other sources can exist as aggregates of varying numbers of active units (Kremzner and Wilson, 1964; Changeux, 1966). Since the quaternary structure of the endplate AChase is unknown, we use "AChase molecule" for the unit with one active center, since this is the entity of the most direct physiological interest.

TABLE VII

Numbers of DFP-Reactive Sites and of AChase Molecules at Endplates

Muscle	Sites	No. of molecules per endplate	
		Mean	$\times 10^{-7}$ *Range*
Sternomastoid	Total DFP-reactive sites	8.8	(6.2–11.4)*
	AChase‡	3.1	(2.2–4.0)
Diaphragm	Total DFP-reactive sites	3.0	(2.5–3.6)*
	AChase‡	1.1	(0.9–1.2)

* From results of all the experiments of Table II (last column).

‡ On the basis of a mean of 35% for the proportion of AChase in the DFP-reactive sites.

Reactivation with 2-PAM and "Ageing"

When DFP is reacted with AChase, the diisopropylphosphoryl (DP-) portion of the DFP molecule becomes covalently bound to the serine residue at the active center of the enzyme. With time, a process known as "ageing" occurs, in which one isopropyl group becomes hydrolyzed from the molecule (Behrends et al., 1959). The inhibited AChase cannot be reactivated by 2-PAM after "ageing" has taken place. (For a full discussion of this phenomenon, see Hobbiger, 1963).

It is clear that "ageing" of the DP-AChase would reduce the proportion of sites apparently sensitive to 2-PAM in these experiments. The information available in the literature suggests that the half-time of "ageing" of DP-AChase is 2–3 hr at 37°, 10–12 hr at 20°, and some days at 0° (Hobbiger, 1956; Davies and Green, 1956; Latki and Erdmann, 1961). Evidence from our material that is consistent with this evidence is reported in the succeeding paper (Rogers and Barnard, 1969).

In the experiments reported here, the tissues were held at room temperature for about 35 min, and were then held at 0–4° for a further 3–4 hr, from the beginning of treatment with labeled DFP to the start of reactivation with 2-PAM. It is, therefore, unlikely that "ageing" significantly affected these measurements. It is interesting also that the results obtained by reactivation with 2-PAM should be in such close agreement with those obtained in the protection experiments with 284C51 and eserine. In the case of the inhibitors, "ageing" could not have influenced the results.

Protection Experiments with the Specific Inhibitors

284C51 is a reversible inhibitor of AChase, while DFP is irreversible. In these conditions, it is apparent that, with increasing incubation times in a solution containing both compounds, an increasing proportion of the AChase will become phosphorylated by DFP. The rate of this increase in phosphorylation with DFP is dependent on the intrinsic rate of reaction of DFP with the enzyme, and on the binding affinity of the inhibitor. In the case of 284C51, Austin and Berry (953) have demonstrated that this compound is very tightly bound to AChase, and for rat brain AChase the I_{50} value (concentration for 50% inhibition) is about 10^{-8} M under comparable conditions (Fulton and Mogey, 1954).

It has been shown (Barnard and Ostrowski, 1964; Wieckowski and Barnard, in preparation) that, in the conditions of these experiments, phosphorylation by DFP alone is not complete at 16 min, but reaches saturation by 20 min. The experiments involving protection by 284C51 were carried out with either a 16- or 20-min incubation in labeled DFP. These times are short for reaction of the extremely depleted free AChase, and yet they are only just long enough to enable DFP to phosphorylate all the available sites in the endplate in the absence of competition with 284C51. When incubation in labeled DFP starts with every molecule of AChase inhibited by 284C51, and the incubation medium contains in addition a suitable concentration of the latter, the probability of phosphorylation of AChase by DFP in 16–20 min must be very small.

With eserine, the position is slightly different. Wilson, Harrison, and Ginsburg (1961), working with AChase purified from eel electric organ, have demonstrated that carbamylation, a covalent attachment at the active center, is produced by reaction with eserine, in addition to the formation of a noncovalent, reversible complex. Carbamylation is slowly reversed by hydrolysis, a reactivation with a half-time of about 40 min at 25°. Earlier evidence on mammalian erythrocyte AChase (Goldstein, 1944) is also consistent with such a carbamylation. After a period of pretreatment with eserine, as we have used, all the AChase would presumably be in the carbamyl form, and the chance of phosphorylation of this enzyme with DFP would be remote.

The Proportion of Phosphorylated Sites Represented by AChase

It is interesting that the three methods of estimating this contribution of AChase to the total sites phosphorylated by DFP at the endplate, by using 2-PAM, 284C51, and eserine, respectively, each of which has different possible sources of error, should, nevertheless, give about the same result. In a similar study on the esterases in megakaryocytes of the rat (Darzynkiewicz et al., 1966 *a*), excellent agreement has again been obtained between the results of reactivation with 2-PAM and the results of protection with these same specific inhibitors. Both methods indicated that approximately 30–35% of the sites of phosphorylation by DFP in these cells were AChase. It seems reasonable to conclude that a proportion close to 35% of the DFP-reactive sites measured in the present work is, in fact, AChase.

The specificity for AChase of the methods used for this specificity here is based not only upon the known biochemical evidence obtained on the enzyme from erythrocytes or brain cited earlier, but also upon results that have been reported on rat and mouse motor endplates. The AChase there has been shown histochemically (and, therefore, only qualitatively) to be inhibited by 284C51, and reactivated (after DFP blockade) by 2-PAM (Koelle, 1963; Denz, 1953; Holmstedt, 1957; Pearse, 1961). Quantitative evidence obtained by Giacobini and Holmstedt (1960) on rat muscle endplates studied by the micro-diver technique shows that the activity therein on the AChase-specific substrate methacholine is abolished by 284C51.

One possible source of error common to the three methods used for determining the proportion of DFP-reactive sites that are AChase is the polar nature of the agents used for establishing specificity. DFP, which by contrast is lipophilic, might penetrate to AChase sites within the endplate which are inaccessible to 2-PAM, 284C51, and eserine. These experiments were carried out on fixed material, however, in which permeability barriers should have been greatly reduced. Two recent lines of evidence confirm that this type of error was not present in these experiments. First, Darzynkiewicz, Wieckowski, and Barnard (unpublished data), by using benzoyl-2-PAM (phenyl-1-methyl pyridinium-2-ketoxime iodide) have obtained results on mouse diaphragm labeled with DFP-^{3}H which are identical with the results reported here: this compound of Dr. I. B. Wilson, benzoyl-2-PAM, has a much higher lipid solubility than 2-PAM itself. Secondly, in electron microscope radioautographs of sternomastoid endplates labeled by the two methods "DFP-^{3}H–2-PAM" and "DFP–2-PAM–DFP-^{3}H," it has recently been shown by M. M. Salpeter (Salpeter, in preparation) that the distribution of the sites we have taken as AChase is identical with the distribution of the phosphorylated sites resistant to treatment with 2-PAM.

The conclusion that 35% of the sites labeled represent AChase is not in agreement with brief reports by Waser and Reller (1965) and Waser (1967). Working with mouse diaphragm, those authors claim to abolish phosphorylation with DFP in motor endplates by pretreatment with Mipafox

(*N*,*N*-di-isopropylphosphorodiamidic fluoride). From their results, they conclude that all the sites available for phosphorylation must be AChase. But Mipafox is similar in general structure and in reactivity to DFP itself, and it is known to inhibit Chase, for instance, at concentrations lower than the concentrations at which it inhibits AChase (Holmstedt, 1957). Its reactivity with other esterases has not been evaluated biochemically, but is likely to be considerable. Its value for indicating the specificity of action of DFP on AChase is, therefore, considerably less than the value of any of the agents used in the present work, all of which are more specific for AChase.

It is interesting that Waser's (1967) value for the total number of DFP-reactive sites per endplate in the mouse diaphragm is $2.5 \pm 0.5 \times 10^7$, a value very close to the values we report here and have noted previously (Rogers et al., 1966), although Waser has used a radioautographic technique considerably different from ours.

In earlier grain-counting experiments with DFP-^{3}H–treated mouse diaphragm, it was also concluded (Barnard and Ostrowski, 1964) that all the sites of reaction with DFP in the endplates were AChase. This view is no longer tenable in view of the evidence presented in this paper and the succeeding paper (Rogers and Barnard, 1969).

The Contribution of Chase and Other Esterases to the Sites of Phosphorylation by DFP

The proportion of Chase present at the motor endplate is not very accurately determined in this material. Ethopropazine has been shown to be highly specific for Chase at the concentrations used here (Bayliss and Todrick, 1956; Todrick, 1954). The experiments in which it was used showed 5% (sternomastoid) and 14% (diaphragm) protection against phosphorylation by labeled DFP (Table VI). Eserine, however, which should inhibit both AChase and Chase, gave protection which was quantitatively indistinguishable from that seen with 284C51, suggesting that little, if any, Chase was present in the endplate.

The presence of Chase at motor endplates has been demonstrated histochemically (Denz, 1953), though the techniques used do not permit any firm statement about the relative amounts of AChase and Chase.

On the basis of the evidence as a whole, it seems reasonable to assume that there is relatively little Chase at the endplates studied. The Chase probably amounts to less than 10% of the total number of sites phosphorylated by DFP.

It is interesting to speculate on the nature of the other DFP-reactive sites at the motor endplate which are not AChase or Chase. These other sites form not less than 55% of the total sites sensitive to DFP. The evidence presented here on the persistence of labeling at those sites through an extensive washing procedure, which includes exchange with nonradioactive DFP, indicates that phosphorylation, rather than any noncovalent binding, is taking place there. It is highly probable that phosphorylation of serine residues at the active centers of hydrolases is involved. There is evidence from cytochemical color reactions that nonspecific esterases are present at motor endplates (Denz, 1953; Hopsu and Pontinen, 1964), and we have confirmed, in the course of these investigations, that the α-naphthyl acetate reaction for esterases is given by these endplates and is abolished by prior treatment with DFP. It is now clear, from the evidence noted above, that relatively little of this reactivity to DFP can be attributed to Chase. These unidentified esterases are present at motor endplates in higher concentration than AChase itself, and their nature and role, if any, in neuromuscular transmission clearly invite further analysis.

AChase at Endplates of Sternomastoid and Diaphragm

The values for the numbers of molecules of AChase at endplates of mouse sternomastoid and diaphragm are listed in Table VII. The succeeding paper (Rogers and Barnard, 1969) reports measurements made, by liquid scintillation counting, of the radioactivity eluted from sternomastoid endplates by 2-PAM, after labeling with DFP-^{3}H. The range of values in these experiments was $1.2–4.4 \times 10^7$ molecules AChase per endplate, with a mean of 2.4×10^7. This value is in good agreement with the values reported here.

Electron microscope radioautographs of the labeled sternomastoid material, obtained by M. M. Salpeter, have yielded a value of $1.0–2.0 \times 10^7$ molecules AChase per endplate, which is again within the range of values reported here (Rogers et al., 1966). It was possible to show (Salpeter, 1967) that over 85% of the AChase molecules lie in the region of the junctional folds of the postsynaptic membrane. It may be presumed that these molecules are associated with the postsynaptic mem-

brane itself. On this basis, it has been possible to calculate from the electron microscope radioautographic data the density of AChase molecules per unit area of postsynaptic membrane (Salpeter, 1967).

DFP-Reactive Sites in Mast Cells

The labeling observed in mast cells is not due to the presence of AChase. Cholinesterases in mast cells are not detectable by the usual methods. Barnard et al. (1968) have shown that, in mast cells from both rat and mouse, all the sites that react with DFP are proteases or other esterases: in the rat, 85% of the sites are active centers of a chymotrypsin-like enzyme (Darzynkiewicz and Barnard, 1967).

The presence of these cells, which have a concentration of DFP-reactive sites far in excess of the concentration found at endplates, is a potential source of error in any technique that does not limit measurement to histologically recognizable endplates. It is now apparent that the early work on DFP-^{3}H uptake by mouse diaphragm (Ostrowski et al., 1963; Barnard and Ostrowski, 1964) did not differentiate mast cells from endplates. Staining of endplates was avoided in making the radioautographs in that early work, but, as a consequence, many of the labeled areas encountered will have been mast cells and not endplates. Those earlier results should, therefore, be disregarded in favor of the results with the present material, in which the endplates were always clearly demonstrated. In spite of the difficulties introduced into the measurement of the total number of DFP-reactive sites per endplate by the prior staining of the tissue by the Koelle acetylthiocholine method, this or some similar technique to enable endplates to be positively identified is essential if acceptable results are to be obtained with striated muscle. Radioautographic measurements from relatively wide areas of tissue, as in the densitometric method of Waser and Reller (1965), will also inevitably include some radioactivity from mast cells.

Conclusion on the Technique Employed

It is clear from the results presented here that it is possible, with relatively simple control experiments, to apply an irreversibly binding inhibitor to tissue in conditions which saturate the available reactive sites, and to measure these sites and differentiate them from sites of reversible adsorption.

Only rarely will this total of reactive sites in itself give a direct measure of any particular enzyme or receptor, since only a very few of the reagents available at present are sufficiently specific for one molecule of biological interest. (One such reagent may be amethopterin-^{3}H: see Darzynkiewicz et al., 1966 *b*). Even where a compound appears to be highly specific for a particular biological system when examined, for example, by pharmacological tests for activity, its application to tissues after labeling will often result in binding of radioactivity to many sites which are apparently irrelevant to its site of action. In these circumstances, measurement of all available reaction sites must be accompanied by use of some other specific properties (e.g. by protection with an agent of high affinity) for indicating the proportion of these sites that correspond to the molecule under investigation. This approach is adopted in our present and previous work with DFP, and similarly in work on measurements made of the numbers of molecules of AChase in megakaryocytes of the rat (Darzynkiewicz et al., 1966 *a*, 1967).

Subject to these considerations, the present work indicates that, where a suitable inhibitor is available or can be made, the labeled inhibitor approach can provide valid measurements of the number of enzyme sites in biological sources of cellular dimensions, particularly when it is used in conjunction with the sensitive and precise techniques of beta track radioautography.

This work was supported by grants GM-11754 (to Dr. Barnard) and GM-10422 and Career Development Award K3-NB-3738 (to Dr. Salpeter), from the National Institutes of Health, United States Public Health Service. The technical assistance of Miss S. Homer, Mrs. N. Holyer, and Mr. S. Twal is gratefully acknowledged. We would like to thank Dr. G. M. Lehrer (Mount Sinai Hospital, New York) for helpful discussion on the application of ethopropazine.

Received for publication 28 November 1967, and in revised form 9 December 1968.

REFERENCES

AUGUSTINSSON, K. B. 1963. *In* Handbuch der Experimentellen Pharmakologie. G. B. Koelle, editor. Springer-Verlag, Berlin. **15**:89.

AUSTIN, L., and W. K. BERRY. 1953. *Biochem. J.* **54**:695.

BARNARD, E. A., and J. MARBROOK. 1961. *Nature.* **189**:412.

BARNARD E. A., and K. OSTROWSKI. 1964. *Exp. Cell, Res.* **36**:28.

BARNARD, E. A., and A. W. ROGERS. 1967. *Ann. New York Acad. Sci.* **144**:584.

BARNARD, E. A., J. KAWIAK, J. KOMENDER, and M. DERECHIN. 1968. *Fed. Proc.* **27**:641.

BAYLISS, B., and A. TODRICK. 1956. *Biochem. J.* **62**:62.

BEHRENDS, F., C. H. POSTHUMUS, I. v. D. SLUYS, and F. A. DEIERKAUF. 1959. *Biochim. Biophys. Acta.* **34**: 576.

CHANGEUX, J. P. 1966. *Mol. Pharmacol.* **2**:369.

COHEN, J. A., and R. A. OOSTERBAAN. 1963. *In* Handbuch der Experimentellen Pharmakologie, G. B. Koelle, editor. Springer-Verlag, Berlin. **15**: 299.

DARZYNKIEWICZ, Z., A. W. ROGERS, and E. A. BARNARD. 1966 *a. J. Histochem. Cytochem.* **14**:379.

DARZYNKIEWICZ, Z., A. W. ROGERS, E. A. BARNARD, D. H. WANG, and W. C. WERKHEISER. 1966 *b. Science.* **151**:1528.

DARZYNKIEWICZ, Z., and E. A. BARNARD. 1967. *Nature.* **213**:1198.

DARZYNKIEWICZ, Z., A. W. ROGERS, and E. A. BARNARD. 1967. *J. Histochem. Cytochem.* **14**:915.

DAVIES, D. R., and A. L. GREEN. 1956. *Biochem. J.* **63**:529.

DENZ, F. A. 1953. *Brit. J. Exp. Pathol.* **34**:329.

FALK, G. J., and R. C. KING. 1963. *Radiat. Res.* **20**: 466.

FULTON, M. R., and G. A. MOGEY. 1954. *Brit. J. Pharmacol.* **9**:138.

GIOCOBINI, E., and B. HOLMSTEDT. 1960. *Acta Pharm. Toxicol.* **17**:94.

GOLDSTEIN, A. 1944. *J. Gen. Physiol.* **27**:529.

GUIDOTTI, G. G., and F. SPINELLI-ROSSI. 1964. *Exp. Cell Res.* **33**:254.

HOBBIGER, F. 1956. *Brit. J. Pharmacol.* **11**:295.

HOBBIGER, F. 1963. *In* Handbuch der Experimentellen Pharmakologie. G. B. Koelle, editor. Springer-Verlag, Berlin. **15**:921.

HOLMSTEDT, B. 1957. *Acta Physiol. Scand.* **40**:322.

HOPSU, V. K., and J. PONTINEN. 1964. *J. Histochem. Cytochem.* **12**:853.

KARNOVSKY, M. J., and L. ROOTS. 1964. *J. Histochem. Cytochem.* **12**:219.

KISIELESKI, W. E., R. BASERGA, and J. VAUPOTIC. 1961. *Radiat. Res.* **15**:341.

KOELLE, G. B., and J. S. L. FRIEDENWALD. 1949. *Proc. Soc. Exp. Biol. Med.* **70**:617.

KOELLE, G. B. 1963. *In* Handbuch der Experimentellen Pharmakologie. G. B. Koelle, editor. Springer-Verlag, Berlin. **15**:187.

KREMZNER, L. T., and I. B. WILSON. 1964. *Biochemistry.* **3**:1902.

LATKI, O., and W. D. ERDMANN. 1961. *Arch. Exp. Pathol. Pharmakol. (Naunyn-Schmiederbergs).* **240**:514.

LEVI, H., A. W. ROGERS, M. WEISS BENTZON, and A. NIELSON. 1963. *Kgl. Danske Videnskab. Selskab, Mat.-Fys. Medd.* **33**: No. 11.

LEVINTHAL, C., and C. A. THOMAS. 1957. *Biochim. Biophys. Acta.* **23**:453.

OSTROWSKI, K., and E. A. BARNARD. 1961. *Exp. Cell Res.* **25**:465.

OSTROWSKI, K., E. A. BARNARD, Z. STOCKA, and Z. DARZYNKIEWICZ. 1963. *Exp. Cell Res.* **31**:89.

OSTROWSKI, K., Z. DARZYNKIEWICZ, T. RYMASZEWSKA, and E. A. BARNARD. 1964. *Exp. Cell Res.* **36**: 43.

PEARSE, A. G. E. 1961. Histochemistry, Theoretical and Applied. Churchill, London.

PELC, S. R. 1956. *Intern. J. Appl. Rad. Isotopes.* **1**:172.

ROGERS, A. W. 1967. Techniques of Autoradiography. Elsevier, London.

ROGERS, A. W., Z. DARZYNKIEWICZ, E. A. BARNARD, and M. M. SALPETER. 1966. *Nature.* **210**:1003.

ROGERS, A. W., and E. A. BARNARD. 1969. *J. Cell Biol.* **41**:686.

SALPETER, M. M. 1967. *J. Cell Biol.* **32**:379.

SAWICKI, W., and Z. DARZYNKIEWICZ. 1964. *Folia Histochem. Cytochem.* **1**:283.

TODRICK, A. 1954. *Brit. J. Pharmacol.* **9**:76.

WASER, P. G., and J. RELLER. 1965. *Experientia.* **21**: 402.

WASER, P. G. 1967. *Ann. New York Acad. Sci.* **144**: 737.

WILSON, I. B., and S. GINSBURG. 1955. *Biochim. Biophys. Acta.* **18**:168.

WILSON, I. B., S. GINSBURG, and C. QUAN. 1958. *Arch. Biochem.* **77**:286.

WILSON, I. B., M. A. HARRISON, and S. GINSBURG. 1961. *J. Biol. Chem.* **236**:1498.