ELECTRON MICROSCOPE RADIOAUTOGRAPHY AS A QUANTITATIVE TOOL IN ENZYME CYTOCHEMISTRY

I. The Distribution of Acetylcholinesterase at Motor End Plates of a Vertebrate Twitch Muscle

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ABSTRACT

Tritiated diisopropylfluorophosphate (DFP) was used to phosphorylate acetylcholinesterase (AChase) in the motor end plate of mouse sternomastoid muscle, and its distribution within the end plate was evaluated quantitatively by electron microscope radioautography. With the use of emulsion layers whose sensitivity to tritium had been calibrated, the density of AChase in different components of the end plate was calculated. The AChase was primarily localized (85%) in the junctional fold region. The concentration of AChase there was more than 20,000 active sites per cubic micron of tissue. The resolution of the technique was not sufficient to determine whether there was some AChase in the nerve end bulb; however, if there is any there, the concentration must be less than 10% of that at the junctional fold region.

The problem of the localization and quantitation of acetylcholinesterase at neuromuscular junctions is an old one and has been studied in a variety of ways both biochemically and histochemically. (Review of the early literature is given in Couteaux 1955; Koelle, 1963.) Most studies favored the view that the enzyme is located primarily in the subneural apparatus. A notable exception was expressed by Nachmansohn (1963). Recently, the problem of localization has been reopened with the use of electron microscope histochemical techniques (Lehrer and Ornstein, 1959; Barrnett, 1961; Zacks and Blumberg, 1961; Miledi, 1964) and the problem of quantitation has been attacked with the use of irreversible enzyme inhibitors (Ostrowski and Barnard, 1961; Barnard and Ostrowski, 1964; Waser and Reller, 1965).

The use of radioautography has been suggested by Ostrowski and Barnard (1961) for the localization of enzyme sites that had been labeled with radioactive inhibitors. These investigators point out that radioautography could be used for the determination of sites of some enzymes for which color reactions are not available; that radioautography provides a valuable alternative to, and check on, enzyme localization by color reactions, and that radioautography with enzyme inhibitors lends itself to quantitative evaluation.

These authors applied light microscope radioautography to the quantitative evaluation of acetylcholinesterase (AChase) at motor end plates using the irreversible enzyme inhibitor diisopropylfluorophosphate-^{3}H (DFP-^{3}H) (Ostrowski et al., 1963; Barnard and Ostrowski, 1964). It has subsequently been shown (Rogers et al., 1966) that some quantitative results reported in these inves-

tigations were subject to error. This was probably due to a very high uptake of DFP by muscle mast cells, which were not distinguished from end plates in the unstained material used. Nevertheless, their approach appears valid theoretically, and promises to be specific, given adequate biological controls (see Discussion). In the present study, the distribution of AChase within the motor, end plate of a vertebrate twitch muscle was evaluated quantitatively by the use of electron microscope radioautography after this enzyme was selectively labeled with DFP-^{3}H. Some of the results of this work are included in a preliminary report (Rogers et al. 1966).

MATERIALS AND METHODS

PREPARATION OF TISSUE: The mouse sternomastoid muscle was used. It was treated so as to label only active sites of acetylcholinesterase (AChase). The material was labeled by Dr. Andrew W. Rogers using the procedures worked out by him and Dr. E. A. Barnard, of the State University of New York, Buffalo. The general purpose was to phosphorylate active sites of the AChase molecule with the use of DFP-^{3}H, and to eliminate as far as possible both the radioactive labeling of other active sites as well as the background radioactivity due to nonspecific binding of DFP to various tissue components. For this reason the tissue was first incubated in nonradioactive DFP to phosphorylate all sensitive sites, then in pyridine-2-aldoxime methiodide (2-PAM), the highly selective reactivator of phosphorylated AChase (Wilson and Ginsburg, 1955; Wilson, Ginsburg, and Quan, 1958; Koelle, 1963; Nachmansohn, 1960, 240), and subsequently in DFP-^{3}H to phosphorylate with radioactive DP-groups the reactivated sites of AChase.

In detail, the tissue was treated as follows. The sternomastoid muscle of male Swiss mice was: (*a*) fixed in glutaraldehyde (1.5% in 0.06 M phosphate buffer at pH 7.4) for approximately 2 hr and then thoroughly rinsed in buffer; (*b*) incubated in unlabeled DFP (10^{-3} M in phosphate buffer pH 7.4 at room temperature) for 20 min and then washed several times in buffer for a total of 20 min; (*c*) incubated in 2-PAM (10^{-3} M at room temperature) for 40 min and then washed in cold buffer and left in buffer overnight; (*d*) incubated in DFP-^{3}H (10^{-4} M; 2.56 c/mmole) for 30 min at room temperature, rinsed in buffer and then in nonradioactive DFP (10^{-3} M for 10 min and then 10^{-4} M for 1 hr) and finally rinsed and stored in buffer overnight or longer; (*e*) postfixed in OsO_4, stained in uranyl nitrate, and embedded in Epon 812.

RADIOAUTOGRAPHIC SPECIMEN PREPARATION: Electron microscope radioautograms were prepared as described by Salpeter and Bachmann (1964, 1965).

Two groups of specimens were prepared. In group I, sections of 1000 A (light gold interference color) were coated with monolayers of Ilford L4 (purple interference color) and developed with Microdol X for 3 min. Owing to the large developed grains obtained this way ($\sim$0.3 μ), group I was used for easy grain counting over large areas of tissue at low magnification. Furthermore, the relatively thick sections were used to increase the number of developed grains obtained per radioautogram.

Group II was used for higher resolution. Sections of 600 A (silver interference color) were coated with monolayers of centrifuged Kodak NTE (silver-to-pale gold interference color) and developed by the Gold-latensification Elon ascorbic acid procedure at 24°C.

In both groups the ribbons of sections were placed on collodion-coated slides, stained with uranyl acetate (3 hr), vacuum-coated with a thin carbon layer (50–100 A), and coated with liquid emulsion. The exposure time was 15–27 wk. For the quantitative evaluation, detailed information on section and emulsion thickness is essential and was obtained as described previously (Bachmann and Salpeter, 1965). Interferometric measurements of section thickness confirmed that the thickness judgments made from the interference colors of the sections while in the sectioning trough are accurate within 10–15% (Peachey, 1958; Bachmann and Sitte, 1958).

STATISTICAL ANALYSIS OF ELECTRON MICROSCOPE RADIOAUTOGRAMS: From a preliminary examination of the material, the radioactivity at neuromuscular junctions was found to be quite low. End bulbs seen in sections approximately 600 A thick, coated with Kodak NTE emulsion (Fig. 1) and exposed for 8 wk or more, had on the average less than one developed grain. Kodak NTE emulsion is not stable with exposure periods longer than about 2 months. Group II was, therefore, not very useful for this study.

Since junctional end bulbs are small, and since they are surrounded by a large volume of muscle which itself contains some radioactivity, it is difficult to get a meaningful assessment of the distribution of radioactivity at the junction just by looking at the radioautograms. The following statistical analysis was therefore undertaken. Numerous radioautograms from Group I were thoroughly scanned and every end plate or part of an end plate seen was photographed whether it had developed grains associated with it or not. Samples of such radioautograms are shown in Figs. 2–6.

The fine structure of the sternomastoid end plate conforms to that of the typical mammalian "twitch type" neuromuscular junction which has been de-

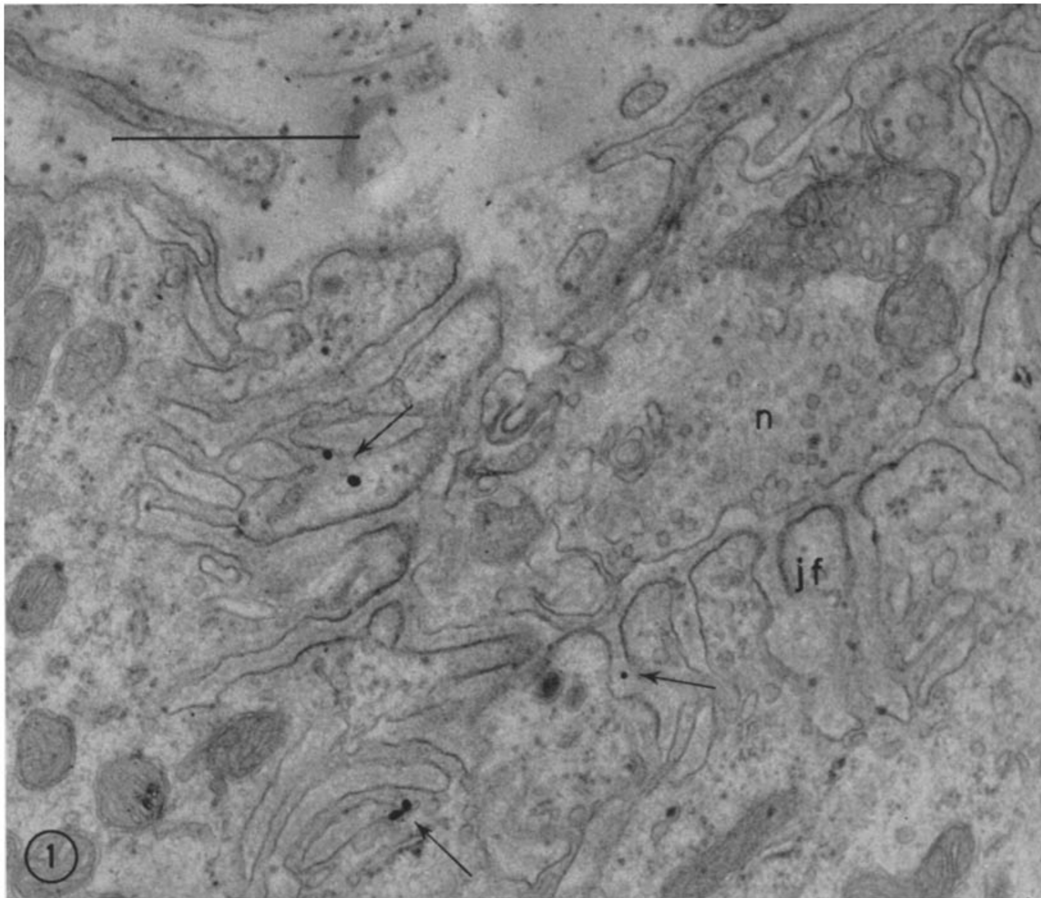
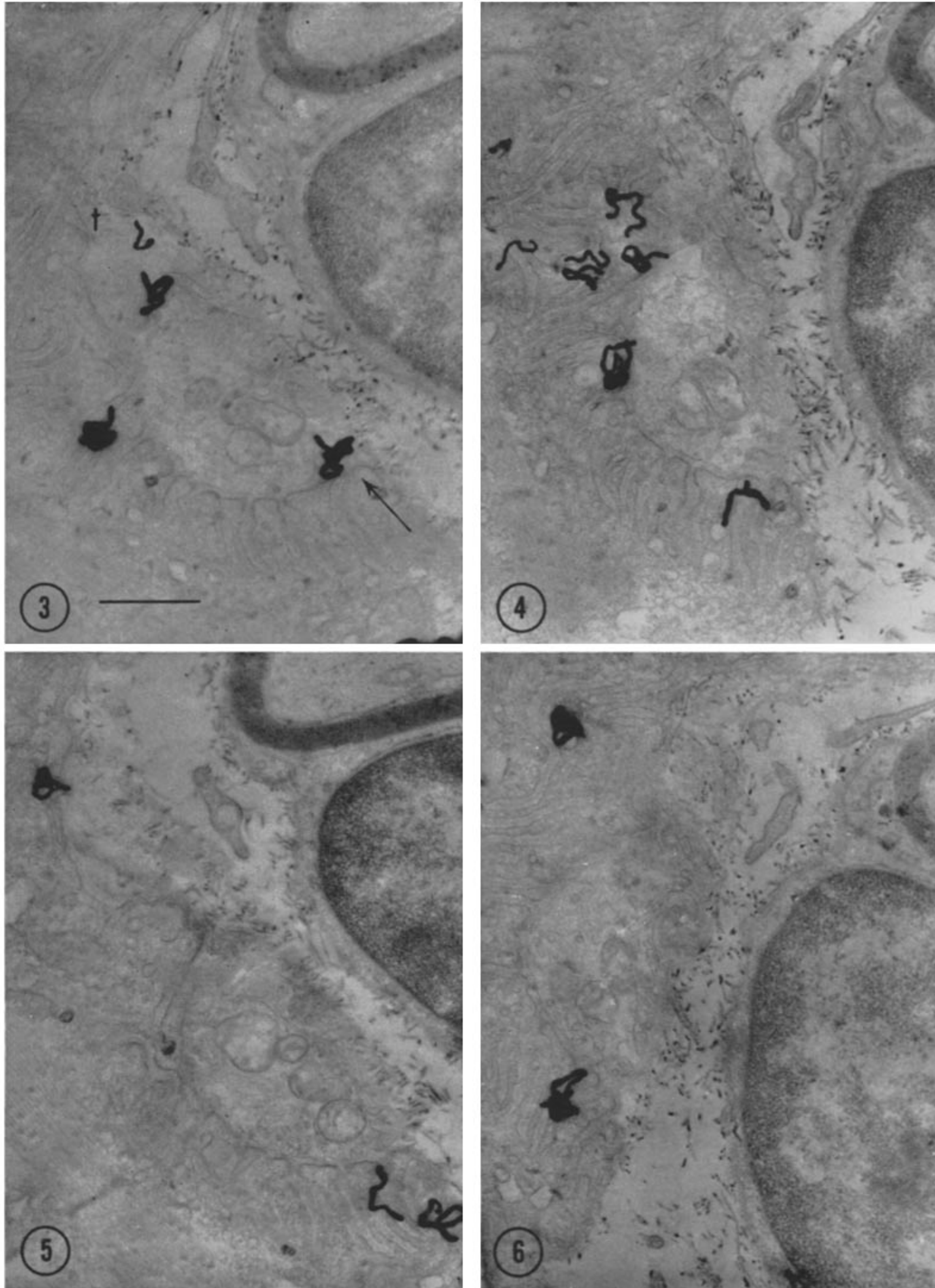


FIGURE 1 Radioautogram of a section through an end plate, treated with DFP-^{3}H. Nerve end bulb (*n*); junctional fold (*jf*). Silver section, coated with a monolayer of centrifuged Kodak NTE emulsion, developed with Gold latensification, Elon-ascorbic acid. Arrows point to developed grains. The radioautogram emphasizes one of the problems in using this high resolution procedure for quantitation—the small developed grains are hard to identify positively. × 34,000.

scribed in numerous publications (Andersson-Cedergren, 1959; reviewed by Couteaux, 1958; Zacks, 1964, 53–67). The nerve fiber that has divested itself of its myelin sheath terminates as an end bulb in a depression or trough formed by the invagination of the muscle plasma membrane. At the interface between the nerve and muscle, the muscle plasma membrane is thrown into numerous folds, called junctional folds, which form a zone approximately 0.5– 1 μ wide adjacent to the axonal end bulb. The gap between the axon and the muscle plasma membranes is called the primary cleft and is approximately 600 A wide. The space between the membranes of the junctional folds is known as the secondary cleft and varies in width from 500 A at the mouth to about 800 A at the base. The term subneural apparatus is often used to refer to the junctional folds plus clefts. Overlying the nerve-muscle contact area is a cap consisting of connective tissue and Schwann cell elements, designated by Couteaux as the teloglial cap.

In order to express the data as concentrations of radioactivity in the tissue, the areas occupied by the different end plate components in micrographs of equal magnification were traced on paper, cut out, weighed, and converted to square microns. Since the sections were of uniform thickness, the total volume of a given tissue component and the concentration of radioactivity in these components could be obtained. Since there was a distinct muscle background (determined in an identical fashion), the concentrations were expressed as a multiple of muscle background.

FIGURE 2 Part of an end plate in a survey radioautogram. Note location of most of the developed grains over the junctional fold zone. Schwann nucleus and myelinated nerve fiber at upper right. Nerve end bulb (*n*); junctional folds (*jf*); teloglial cap (*t*). Gold section, coated with Ilford L4 emulsion, developed with Microdol X, 27 wk of exposure. × 17,500.



FIGURES 3–6 Radioautograms obtained from sections at different levels of the same end plate as seen in Fig. 2. Gold sections, Ilford L4 emulsion, Microdol X developed. 15-27 wk' exposure. Fig. 3 has one grain in the end bulb (arrow) and two on the teloglial cap (*t*); all the rest of the grains are on the junctional folds. × 15,000.

TABLE I

Distribution of AChase within Motor End Plate

Component	Radioactivity per unit volume as multiple of muscle background	No. of molecules of AChase per μ^3	No. of molecules of AChase per end plate
Muscle	1.0	—	—
Total end plate	23.0	1.0×10^4	1.5×10^7
Teloglial cap	10.0	4.0×10^3	1.5×10^6
Junctional folds	45.0	2.0×10^4	1.3×10^7
Nerve end bulb	4.5	—	—

These values are 10% higher than obtained from the actual grain count, to correct for a loss due to fixation by glutaraldehyde.

In column three the number of molecules of AChase per total end plate was obtained by multiplying the number of molecules per μ^3 of end plate by an estimated volume of the end plate.

The number of molecules in the teloglial cap and junctional folds per end plate were obtained from the percentage of total grains found overlying these components (see text).

No values are given for AChase in the end bulb because of the uncertainties involved (see text).

Absolute quantitation of AChase molecules was also obtained. These were calculated, knowing the number of decays which could be expected in a given time per mole of DFP (from the specific activity of the DFP), and knowing the number of such decays which are needed to give, on the average, one developed grain. The sensitivity studies of Bachmann and Salpeter, (1967), show that under the experimental conditions employed here, and with a monolayer of Ilford L4 emulsion developed with Microdol X for 3 min at 24°C, one developed grain can be expected per 10 radioactive decays in the specimen.

EVALUATION OF PROCEDURES: The reliability or probable error of the measurements derived from quantitative radioautography as here employed depends on the uncertainties introduced by the various steps of specimen preparation. It is impossible to evaluate them all with equal certainty. One must distinguish the uncertainties due to cytochemistry (i.e. extraction, diffusion, saturation, and specificity) and those due to electron microscope radioautography. The problem of enzyme inactivation and extraction by fixation and subsequent treatment of the tissue appears minimal in the present preparation. By grain counting of light microscope radioautograms of sternomastoid end plates similarly reacted, it has been established that glutaraldehyde fixation prior to labeling lowers the uptake of DFP by esterases at the end plate by less than 10%. (Rogers et al. 1966) End-plate AChase has been shown to be generally resistant to both extraction (Christoff et al., 1966) and diffusion, even after the drastic treatment of being soaked in distilled water (Couteaux, 1955). Once the enzyme is phosphorylated, there is good reason to believe that OsO_4 postfixation and alcohol dehydration will not reactivate it. For a review of the irreversible nature of AChase phosphorylation, see O'Brien (1960, 99–106).

The problem of specificity is somewhat more complicated. It is well known that DFP is not specific for acetylcholinesterase but will phosphorylate other esterases (see review by O'Brien, 1960) and, slowly, nonenzyme sites (Ashbolt and Rydon, 1957). Finally, the lipophilicity of DFP appears to cause some difficulty in removing all the nonspecific adsorption of DFP to tissue components even with prolonged washes in nonradioactive DFP and buffer as was employed here. The label over the bulk of the muscle seen in the present material appears to be primarily of this latter type since it was essentially unaltered when the tissue was treated with radioactive DFP, with or without pretreatment with nonradioactive DFP.[1]

A detailed account of the validity and specificity of the labeling procedures employed will be published by Rogers et al. (1967, in preparation). For the purposes of the present study, it is important to establish that the treatment used here had carried to saturation all the reactions at the end plate (i.e., phosphorylation of all reactive sites with nonradioactive DFP; reactivation by 2-PAM, and subsequent phosphorylation of these reactivated sites with DFP-^{3}H). This was demonstrated by showing that increasing the concentration of the reagents or prolonging the incubation periods did not increase the amount of incorporation as judged by light microscope track radioautography. Furthermore, since the period between phosphorylation with nonradioactive DFP and reactivation with 2-PAM was short, the possibility that reactivation was prevented by "ageing" of the DP-AChase complex (Berends et al., 1959) was negligible. (Further problems of specificity are considered in a forthcoming review by Barnard and Rogers, 1967.)

In the present study, the claim for specificity of labeling rests on the selective reactivation of phos-

[1] Recent studies of washing procedures have shown that the muscle background can be greatly reduced by incorporating a wash in organic solvents (e.g. alcohol-chloroform) after the unlabeled DFP and buffer washes (Rogers, personal communication).

phorylated acetylcholinesterase by pyridine-2-aldoxime methiodide (2-PAM) (Wilson and Ginsburg, 1955; Wilson, Ginsburg and Quan, 1958), since only these reactivated sites were available for phosphorylation with radioactive DFP. Recent work shows that about 33% of DFP-phosphorylated sites at the end plate are reactivated by 2-PAM, and that about the same percentage is protected from DFP by eserine and 284C51 (Burroughs Wellcome), two reversible inhibitors of AChase (Rogers, et al., 1966). This provides additional indirect evidence for the specificity of 2-PAM reactivation. It should, however, be borne in mind that all the quantitation here presented refers directly only to the phosphorylation of 2-PAM-reactivated enzyme sites, and only indirectly to molecules of AChase.

The uncertainties of the electron microscope radioautographic procedures arise from several independent factors and have already been discussed in great detail elsewhere (Bachmann and Salpeter, 1965, 1967). In this study, electron microscope radioautography is used as a cytochemical tool to complement information available from electron microscope histochemistry. The radioautographic technique lacks the resolution and high sensitivity (due to amplification) of the histochemical procedures. It can, however, be used quantitatively. The accuracy of this quantitation has recently been discussed by Bachmann and Salpeter (1967). In calibrations of emulsion sensitivity under conditions close to that of radioautography, they show that such calibrations can deviate by 15–30%. These deviations are probably due to errors in judging emulsion and section thickness and also to others of an unknown nature.

Combining all the errors due to cytochemistry and radioautography, there seems to be no reason why the over-all accuracy of the present results should not be within a factor of two for the absolute quantitation, and considerably better than that for the relative results.

RESULTS

Labeling at End Plates

An arbitrary decision was made to consider any developed grain within 2 μ of the axonal plasma membrane as being associated with the end plate. In this way, a zone of muscle at least 1 μ thick is included—a distance about 5 times larger than the expected resolution. Of 168 grains found associated with the end plate, 80–85% lay over the junctional folds, about 5% over the terminal nerve end bulb, about 8–10% over the teloglial cap, and about 2–4% in muscle. The relative areas occupied by these three end plate components were 43, 27, and 30%, respectively.

The distance from the midpoint of each developed grain to the midpoint of the primary cleft was measured. The distribution of grains (excepting those in the teloglial cap) is presented in Fig. 7. The histogram demonstrates that the radioactivity is primarily distributed over the junctional fold region. The nature of the labeling over the terminal nerve end bulb will be discussed. The density of developed grains as a multiple of muscle background is presented in Table I.

Table I also gives absolute values for the concentration of AChase present in the tissue. In the present preparation (DFP 2.4 c/mmole) after 1 wk' exposure, one developed grain represented on the average 1 x 10^5 molecules of AChase. To calculate actual AChase molecules, these assumptions were made: (*a*) One molecule of DFP phosphorylates one active center of the enzyme. This is known from much biochemical evidence reviewed by Koelle (1963). (See also Dixon and Webb, 1964). (A molecule of AChase is considered synonymous with a protein carrying only one active center.) (*b*) The schedule used, i.e. DFP; 2-PAM; DFP-^{3}H, labels only AChase molecules and labels all of them under the conditions here employed (see Discussion). (*c*) There was no significant latent image fading during the exposure period. Bachmann and Salpeter (1967) have shown that this assumption is justified within the error of the present study. The value given in Table I for AChase molecules at the junctional fold area is most likely too low since it was calculated only from the developed grains directly overlying this tissue. Yet, since radioactive decays radiate away from the source, a certain number will cause developed grains over the adjacent tissue, the spread being determined by the resolution of the preparation. No equivalent spread of radiation into the junctional fold region is available to compensate for this loss, since both muscle and nerve end bulb have a lower activity per area. It can be estimated that the value here given should be about 10%, or at most 20%, higher.

Labeling Other Than at End Plates

MUSCLE BACKGROUND: Aside from the labeling at end plates, radioactivity was observed at various other sites. Unfortunately the nonspecific background was relatively high. Labeling density over the bulk of the muscle was about 5% of that

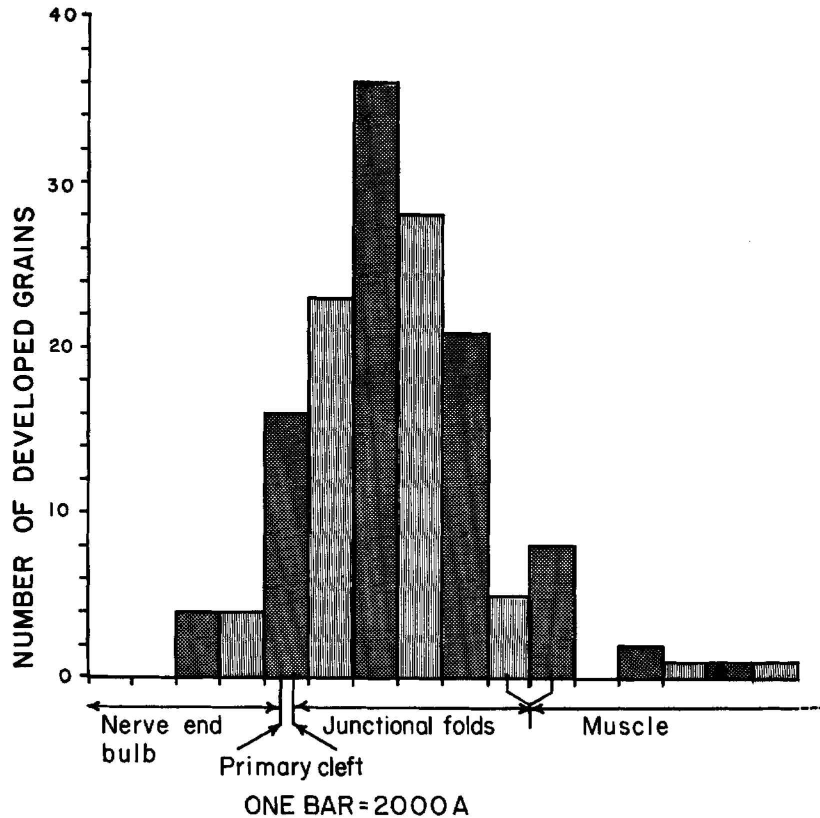


FIGURE 7 Histogram based on 168 grains found within 2 μ of primary cleft (grain overlying teloglial cap not shown).

over the end plate. These muscle grains are equivalent to what would be produced by 400 molecules of AChase per μ^3 of tissue (4×10^{15} molecules per ml), and varied by as much as 50% from specimen to specimen. Since the amount of this general label depended on the washing procedure and was unaltered whether the tissue was treated with nonradioactive DFP, followed by radioactive DFP or whether it was just treated with radioactive DFP (Rogers, personal communication), the muscle label presumably represents a little enzyme phosphorylation but mainly nonspecific adsorption. Therefore, it would be difficult to positively identify levels of AChase of less than about 500 molecules of AChase per μ^3 above this background. Because of these limitations, no quantitation of the labeling in nerve or even erythrocytes was attempted. Random scanning of these structures revealed negligible labeling compared to muscle background. Cohen and Warringa (1953) have calculated that there are about 520 active AChase groups per ox erythrocyte—a figure well below easy detection by the present procedure. A systematic study of labeling in nervous tissue may prove feasible with improved washing procedures to eliminate nonspecific background.

MAST CELLS: One structure was found to be very heavily labeled in the present material, considerably above the label in the end plate. This was the mast cell, located between the muscle fibers in the connective tissue. (The label was over the granules.) Lagunoff and Benditt (1963) have reported a mast cell enzyme which is inhibited by DFP. According to Darzynkiewicz and Barnard (1967), the mast cells do not contain any AChase. Yet they have other esterases in such vast quantities that the present procedure of preincubation with nonradioactive DFP, although adequate to saturate all the esterases at end plates, does not fully saturate the muscle mast cell esterases, still leaving a lot available for the subsequent incubation

in DFP-^{3}H. In the electron microscope the labeled mast cells can easily be identified by their characteristic granules and thus be distinguished from end plates. Their general location between muscle fibers, randomly distributed, can however, introduce a problem of identification when un-unstained material is viewed with the light microscope. This provides a possible explanation for the heavily labeled structures reported as end plates in the publication by Barnard and Ostrowski (1964), results which are inconsistent with those reported here.

DISCUSSION

VALIDITY OF THE ELECTRON MICROSCOPE RADIOAUTOGRAPHIC QUANTITATION: Quantitation of end plate AChase, by electron microscope radioautography was compared with that obtained by different means. Rogers et al. (1966) have reported that there are 2–3 $\times$ 10^7 molecules of AChase per mouse sternomastoid end plate. In that determination, radioactive (^{32}P or ^{3}H) DFP was used, with analyses by either quantitative light microscope track radioautography or liquid scintillation counting. If, therefore, the results of enzyme density per unit volume of end plate reported here were multiplied by the total volume of an end plate, the two results should be comparable. Unfortunately the total volume of an end plate was not easy to estimate. From light microscope views of squashed specimen lightly stained by the Koelle reaction (Koelle and Friedenwald, 1949; Pearse, 1961, 891) the end plates have dimensions approximately 50 $\times$ 30 μ (Rogers, personal communication). Cross-sections seen with the electron microscope show that the end plates consist of numerous terminal twigs of nerve end bulbs plus subneural apparatus. The average depth of the end plates at these terminals is about 3 μ. It is quite apparent, however, that the terminals constitute only a fraction of the end plate, yet these are the labeled components of the end plate and comprise the regions used in the present study for the density determinations. Assuming that the shape of the end plate is close to that of a spheroid and that only approximately 50% of the end plate consists of terminal twigs, the relevant volume was estimated to be between 1000 and 2000 μ^3. By multiplying this volume by the density of AChase, the total number of AChase molecules per end plate is found to be 1–2 $\times$ 10^7 molecules. Although there is considerable uncertainty in the estimation of the relevant end plate volume, this value coincides sufficiently with that obtained by light microscope track radioautography and scintillation counting to give a degree of confidence in the validity of the procedures employed.

LABEL IN SUBNEURAL APPARATUS: The resolution of the electron microscope technique is not sufficient to determine whether the radioactivity over the junctional folds is associated purely with the subneural apparatus or also with the axonal membrane, nor whether it is associated just with the membranes or with the primary and secondary clefts. Bachmann and Salpeter (1965) estimate that with silver sections and monolayers of Kodak NTE the resolution is not better than approximately 800 A. Thus data from Group II were also inadequate to settle this question. Several electron microscope histochemical studies on the localization of AChase at motor end plates, each using somewhat different procedures, have failed to produce agreement on these points (Birks and Brown, 1960; Lehrer and Ornstein, 1959; Barrnett, 1961; Zacks and Blumberg, 1961; Miledi, 1964). Since, however, the post junctional membrane is believed to contain the receptors for acetylcholine, it was tempting to assume that the AChase was functionally associated with the surface of the postjunctional muscle membrane. Using the radioautograms already analyzed, I measured the total length of postjunctional membrane with a "map measurer." The junctional folds were found to produce a sixfold increase in length in the boundary around the nerve. Assuming that each membrane profile represents a membrane segment extending in depth to the full thickness of the section, a value was obtained for the surface area of postjunctional membrane. All the grains overlying the junctional fold region and those found within 3000 A of this zone were counted, and this total was divided by the membrane area. The 3000 A on either side of the junctional fold was added in order to include the scattered radiation from the edge of the hot junctional fold zone. From theoretical considerations (Bachmann and Salpeter, 1965) one can expect that 75% of the radiation originating at an edge will cross the emulsion within 3000 A of that edge in specimen of thickness and emulsion of Group I. Since the hot junctional fold is wide (about 1 μ) relative to the resolution, the additional grains represented only about 6% of the total.

The density per unit area of the muscle postjunctional membrane was calculated to be about 4400 molecules of AChase per μ^2. (This was 14 times higher than background, i.e. grains per μ^2 of muscle plasma membrane, calculated the same way.) Some electron microscope histochemical studies have suggested that the prejunctional axonal membrane also contains AChase (Barrnett, 1961; Miledi, 1964). If an equal enzyme density on both pre- and postjunctional membranes is assumed, the density per unit area would be 3600 AChase molecules per μ^2. This would be more than a factor of 10 higher than the muscle plasma membrane background.

Nachmansohn (1963, 732) has pointed out that muscle sections rich in end plates have about 3 to 5 times more AChase than muscle sections without end plates, and that the subneural junctional folds increase the area of surface membrane at the junction by a factor of the same order, i.e., 3 to 5 times. However, it does not follow from this (unlike Nachmansohn's claim) that the higher concentration of AChase at the subneural apparatus is only apparent, and that the AChase is evenly distributed along the muscle membrane. Since the end plate comprises only a small fraction of the surface of any given muscle section, even of those rich in end plates, the overall increase in innervated muscle membrane area is probably less than 50%. The experimental data presented in the present paper show a local AChase concentration per area of post junctional surface membrane which exceeds by a factor of 14 the labeling (mainly nonspecific) at the noninnervated muscle membrane.

A recent study (Salpeter, Rogers, and Barnard, 1967, unpublished) analyzed the radioactivity at the end plates of external ocular muscle by the same technique described here. The external ocular muscle has a large fraction of tonic fibers (Hess and Pilar, 1963) and the end plates on these fibers, in contrast to those on the twitch fibers, have a postjunctional membrane with greatly reduced junctional folds (Reger, 1961). Although the total radioactivity at these ocular junctions is much less than that at the sternomastoid junctions, the density per unit area of postjunctional membrane is essentially the same in the two junctions. Further experiments on different junctions (for instance on those known to have very high concentrations of AChase relative to their size, or on those completely devoid of junctional folds as are the intrafusal muscle end plate) are necessary to determine whether AChase density on the postjunctional membrane is constant.

LABEL IN NERVE END BULB: The question of whether there is any AChase in the nerve end bulb remains unresolved (Barrnett, 1961; Miledi, 1964; Zacks and Blumberg, 1961). The electron microscope radioautographic examination shows that the developed grains over the terminal nerve bulb are about 4.5 times above muscle background in density. Yet the geometry of the end bulb, i.e., a small volume surrounded by a zone with more than 10 times higher radioactivity, introduces another source of background there as a result of radiation spread from the relatively hot junctional fold area (see histogram, Fig. 7). Furthermore, the total number of developed grains found over the nerve end bulb is low, introducing a relatively large sampling error (only 5% of the 168 grains at the end plate). Thus the uncertainties involved in the present preparation are large enough to prevent a meaningful determination of whether there is any real AChase in the end bulb and, if there is, where it is located. The shape of the histogram suggests that the developed grains over the terminal axon are primarily produced by radiation from the junctional fold zone. However, it is clearly established here that, even if there is AChase in the end bulb, the concentration per unit volume of tissue must be less than 10% of that at the junctional fold region. The possibility must be considered that the low label in the nerve end bulb reflects the inability of 2-PAM to penetrate the axonal permeability barriers (Hinterbuchner and Nachmansohn, 1960) thus leaving the end bulb AChase phosphorylated with nonradioactive DP. However, since the Schwann sheath is the primary permeability barrier (Nachmansohn, 1966) and is absent at this level of the nerve, it was assumed that permeability would not be a problem. In any case, a preliminary analysis of end plates which had been incubated only in DFP-^{3}H (without a prior DFP, 2-PAM sequence) shows a similar relative distribution of radioactivity. No analysis of nerve at a distance from the end bulb has been done.

In conclusion, this study shows the potential of electron microscope radioautography as a quantitative tool. Hopefully, refining the techniques for enhancing radioautographic sensitivity, and improving biological specimen preparation, will allow the use of the higher resolution methods for problems of enzyme cytochemistry.

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