THE LOCATION OF ADENINE NUCLEOTIDE
IN THE STRIATED MUSCLE OF THE TOAD

D. K. HILL
From the Postgraduate Medical School of London, Hammersmith Hospital, London, England

ABSTRACT
The location of tritiated adenine nucleotide was studied by autoradiography in toad muscle which had been fixed in an acetic acid/ethanol mixture, with lead acetate present to act as the nucleotide precipitant. The muscles were embedded in Araldite. The autoradiographs were examined in both the light microscope and the electron microscope. In confirmation of earlier work, it was found that at least 50 per cent of the adenine nucleotide is concentrated in the I band, near the A-I boundary, and evidence is now available to suggest that this fraction is located in the interfibrillar spaces rather than in the substance of the fibrils. Electron micrographs of unstained sections failed to show any structural features at the site in question, though the triads were made visible by the lead. However, when sections were stained with an alkaline solution containing lead, or with uranyl acetate, "vesicles" were revealed at the appropriate site and it is thought that these may be elements of a transverse network (reticulum) of tubules containing the adenine nucleotide. The location of phosphocreatine could not be investigated because this substance was lost from the muscle during the preparative procedure.

INTRODUCTION
In earlier work (8) the location of adenine nucleotide in striated muscle was studied by autoradiography. The muscle, containing tritium-labelled adenine, was fixed with osmium tetroxide, and a lanthanum salt was added to precipitate the nucleotide. About 70 per cent of the adenine nucleotide was found to be aggregated in narrow transverse "bands," a pair to each sacromere, lying within the isotropic (I) region, close to the boundary with the anisotropic (A) band.

There were three main reasons for making the further study described here. First, an improved method of fixation was required. Autoradiographs of transverse sections of a muscle prepared with the osmium tetroxide/lanthanum fixative had shown a marked non-uniformity of distribution of radioactivity; this was attributable to the low permeability of the fibres to the lanthanum, which resulted in a transverse diffusion of the adenine nucleotide towards the site of precipitation in the parts of the fibres which were nearest to the surface of the muscle (where the lanthanum concentration would build up faster than in the deeper parts of the fibres). It has now been found that this transverse diffusion can be prevented by using a non-aqueous fixative, in which the adenine nucleotide is not soluble, for the first stage of the preparation. A mixture of acetic acid and ethanol was chosen for the purpose, with the addition of lead acetate. Lead would not be expected to combine with, and "bind," adenine nucleotide under such conditions, but the idea was that it would penetrate the muscle and be ready to combine and act as the nucleotide precipitant.
during the second stage when the muscle was transferred to an aqueous solution at a higher pH.

The muscle structure was found to be not uniformly well preserved by this fixative, but in selected parts the preservation was reasonably good, and intact myofilaments could be demonstrated.

The second requirement was for a more detailed investigation of the exact location of the adenine nucleotide. The earlier results had shown only that the location was in the form of bands running across the fibre, but it was not possible to decide whether the nucleotide was aggregated in the interfibrillar spaces or, alternatively, within the body of the fibrils. The earlier work had made use of only the light microscope for the autoradiographic analysis. The light microscope method is again used for the present experiments, but in addition the electron microscope has now been employed for the transverse analysis of autoradiographic preparations. The better resolution which is thereby obtained is not actually the only advantage in using the electron microscope for this purpose: equally important has been the improvement in the visualization of the interfibrillar spaces which are clearly seen in electron micrographs, but are practically invisible under the light microscope.

The transverse analysis has now given the first definite indication that, in resting muscle, the adenine nucleotide is aggregated in the interfibrillar spaces rather than in the substance of the fibrils.

In addition to the autoradiographs, it was desirable also to obtain electron micrographs of a muscle which had been fixed in the presence of a heavy-metal nucleotide precipitant. These might show the presence of an electron-opaque deposit, or structural component, at a site corresponding with that indicated by the autoradiography. Osmium tetroxide/lanthanum–fixed material showed no sign of the existence of any such deposit. It became clear that any unnecessary electron-opaque stain, such as osmium tetroxide, had to be avoided if there was to be any chance of observing the relevant features. The acetic acid/ethanol/lead fixative was tried. It resulted in a remarkably “empty” background, when Araldite was used as the embedding medium, against which low-contrast structures lying in the interfibrillar spaces could be readily observed. However, it proved that sections had to be additionally stained with lead or uranium before certain features, thought to be associated with the adenine nucleotide, could be shown to be present in the appropriate site near the A-I boundary.

Phosphocreatine (PC) was also the subject of similar earlier work (10), and it had been hoped to continue the investigation along the lines described here. This, however, proved impossible because tritiated creatine has been found to be almost entirely lost from a muscle subjected to fixation by the present method.

In the past the frog was used as the experimental animal (8, 10). For the present experiments it was decided to use the toad instead, because it is much easier to obtain very small specimens from the dealers. Small size of the animal has two advantages: first, there is considerable saving of radioactive material when this is administered by injecting the whole animal; secondly, the time required for fixation and embedding may be greatly reduced by making use of a muscle as small as a sartorius of a 5- to 10-gram toad. The muscle may weigh as little as 8 mg. It will be seen that the autoradiographic analysis of distribution of adenine nucleotide in the toad muscle has proved to be similar to that found earlier with the frog.

The term adenine nucleotide has generally been used in preference to adenosine triphosphate (ATP) because all forms of adenine nucleotide were presumably labelled with tritium, including that which is a component of the nucleic acids. But it has been shown (7) that the greater part of the isotope which becomes bound in the living muscle is attached to a component with the chromatographic characteristics of ATP. The conclusions, therefore, should be regarded primarily as relating to ATP.

MATERIALS AND METHODS

Radioactive Labelling of the Muscles

Very small (5 to 10 gm) toads (Bufo bufo) were used. The adenine nucleotide, or creatine phosphate, was labelled by injection of tritiated adenine or creatine into the dorsal lymph sac (7, 8, 10). The H2-adenine and H3-creatine, prepared in the laboratory (9, 10), had activities of 1290 mc/mmole (9.6 mc/mg), and 360 mc/mmole (2.75 mc/mg), respectively. The amounts injected and the results of the radioactive assays are given in Table I. Radioactive measurements were made, after extracting the tritiated material from the pieces of muscle in 0.1 N HCl, by
TABLE I

Summary of Information Concerning the Radioactive Labelling of the Muscles, and Details of the Fixation Procedure

<table>
<thead>
<tr>
<th>Serial number of experiment</th>
<th>47</th>
<th>48</th>
<th>49</th>
<th>50</th>
<th>51</th>
<th>52</th>
<th>53</th>
<th>54</th>
<th>65</th>
</tr>
</thead>
<tbody>
<tr>
<td>Refers to notes below</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>A</td>
<td>C</td>
<td>A</td>
<td>C</td>
<td>A</td>
<td>C</td>
<td>A</td>
<td>C</td>
<td>A</td>
</tr>
<tr>
<td>2 (μc/g)</td>
<td>830</td>
<td>990</td>
<td>862</td>
<td>835</td>
<td>810</td>
<td>805</td>
<td>1190</td>
<td>995</td>
<td>630</td>
</tr>
<tr>
<td>3 (days)</td>
<td>2.8</td>
<td>2.8</td>
<td>4.8</td>
<td>4.8</td>
<td>3.2</td>
<td>3.2</td>
<td>2.9</td>
<td>2.9</td>
<td>2.9</td>
</tr>
<tr>
<td>(°C)</td>
<td>15</td>
<td>15</td>
<td>10</td>
<td>10</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>20</td>
</tr>
<tr>
<td>4 (μc/g)</td>
<td>147</td>
<td>322</td>
<td>60</td>
<td>124</td>
<td>82</td>
<td>313</td>
<td>136</td>
<td>171</td>
<td>70</td>
</tr>
<tr>
<td>(0.18)</td>
<td>(0.35)</td>
<td>(0.07)</td>
<td>(0.15)</td>
<td>(0.10)</td>
<td>(0.39)</td>
<td>(0.11)</td>
<td>(0.17)</td>
<td>(0.11)</td>
<td></td>
</tr>
<tr>
<td>5 a</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 (per cent)</td>
<td>d</td>
<td>44</td>
<td>113</td>
<td>65</td>
<td>106</td>
<td>81</td>
<td>93</td>
<td>58</td>
<td>95</td>
</tr>
<tr>
<td>7 e</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8 (per cent)</td>
<td>85</td>
<td>1.5</td>
<td>75</td>
<td>4.2</td>
<td>103</td>
<td>7.4</td>
<td>77</td>
<td>8.5</td>
<td>66</td>
</tr>
<tr>
<td>9 (μc/g)</td>
<td>125</td>
<td>--</td>
<td>51</td>
<td>--</td>
<td>89</td>
<td>--</td>
<td>97</td>
<td>--</td>
<td>44</td>
</tr>
</tbody>
</table>

3. Time (days) between injection and dissection of the muscles; temperature (°C) at which the toad was kept after injection.
4. Amount of isotope in control muscle (μc/g). The figure in brackets gives the same quantity in terms of the amount of isotope injected per gram body weight (i.e. μc/g muscle per μc/g body weight).
5. Fixative: a. 10 mM lead acetate, 25 per cent acetic acid, 75 per cent ethanol (v/v); 30 minutes at 0°C, 30 minutes at 20°C.
   b. 100 mM lead acetate, 25 per cent acetic acid, 75 per cent ethanol (v/v); 30 minutes at 0°C, 30 minutes at 20°C.
   c. 25 per cent acetic acid, 75 per cent ethanol (v/v); 60 minutes at 0°C.
6. Amount of isotope present in the muscle after exposure to the fixative, expressed as per cent of the control value. A piece of the fixed muscle was cut off for each assay, and its weight was estimated from a measurement of its length. d. No assay was made; autoradiographs of a piece of the muscle which was embedded immediately after fixation showed that practically 100 per cent of the isotope must have been retained.
7. After fixation the muscles were immersed in:
   e. 10 mM lead acetate, 100 mM acetate buffer pH 4; 60 to 120 minutes at 20°C.
   f. 10 mM lead acetate, 90 per cent ethanol, 10 per cent water (v/v); 30 min at 20°C; followed by 10 mM lead acetate, 100 mM acetate buffer pH 4; 75 minutes at 20°C.
   g. 100 mM lead acetate, 100 mM acetate buffer pH 4; 120 minutes at 20°C.
8. The amount of isotope present in the muscle after exposure to aqueous lead acetate (pH 4), expressed as the per cent of the amount remaining in the muscle after fixation.
9. Amount of isotope present in the embedded muscle, estimated from the above data. (It was shown that the tritiated adenine remaining after exposure to aqueous lead acetate, pH 4, was wholly retained during dehydration and embedding.)

Preparation, Fixation, and Embedding Procedures

Sartorius muscles (8 to 20 mg) were used. After dissection they were fixed to glass holders and soaked at 0°C for 2 hours in oxygenated calcium-free solution (composition: NaCl, 96 mM; KCl, 5.0 mM; ethylenediaminetetraacetic acid, neutralized to pH 7.0 with NaOH, 1.0 mM). Deprivation of calcium diminishes the response to any form of stimulation, including that due to the fixative (10). Immediately before fixation, the muscles were stretched to about 110 per cent of the normal extended length in the body.

The muscles were fixed in acetic acid/ethanol/lead acetate, or acetic acid/lead acetate (Table I); this was followed either directly, or after an intermediate stage in water/ethanol/lead acetate, by treatment with an aqueous solution of lead acetate at pH 4.

D. K. Hill

Adenine Nucleotide in Striated Toad Muscle
This pH was chosen as being high enough to ensure combination of the adenine nucleotide and phosphocreatine with lead ions, but it is sufficiently far below the ionization range of the protein carboxyl groups to prevent staining of the protein.

In later experiments for electron microscopy alone, with non-radioactive muscles, a number of modifications of the above method of fixation were tried, in order to improve the preservation of the muscle. It was found that an increase in the proportion of acetic acid gave a definite improvement, and tended to reduce the loss of material from the centre of the A band which is referred to later. In addition, the substitution of methanol for ethanol, and the addition of acrolein (14, 20) were tested, but these changes appeared to make little difference to the state of preservation. The compositions of the solutions were: 100 mM lead acetate, 40 per cent acetic acid, 55 per cent methanol, 5 per cent acrolein (stabilized with quinol, sp gr 0.865); followed by 100 mM lead acetate, 90 mM acetate buffer, (pH 4), and 10 per cent acrolein.

The muscles were washed to remove excess lead salt by immersion for about 15 hours in a solution of 5 per cent acetic acid and 95 per cent ethanol, at 20°C. There was no need for a further stage in pure ethanol since acetic acid is miscible with Araldite, and indeed the acid appeared to accelerate the rate of substitution. Small pieces of the muscles were put in liquid Araldite without activator (15 parts resin CY212, 15 parts hardener HY964, 2 parts plasticizer (dibutyl phthalate), v/v) at 50°C for about 15 hours, followed by a final curing at 96°C 20°C. There was no need for a further stage in pure methanol, 5 per cent acetic acid, 95 per cent ethanol, at 20°C since acetic acid is miscible with Araldite, but with unsatisfactory results. In the first place, tissues fixed in organic fixatives are embedded in methacyrlate only with great difficulty, owing to bubble formation (20); second, in such portions as were satisfactorily embedded the muscle structure proved to be much less well preserved than it was with Araldite; this showed particularly as a loss of material at the centre of the A band. Again, considerable difficulty was experienced in cutting sections thinner than about 60 μm.

**Autoradiography**

**LIGHT MICROSCOPY:** Autoradiographs for light microscopy were made with sections 0.75 μ thick, using Kodak AR. 10 stripping film (8). After processing and drying, the muscle was stained, through the film, by immersion of the supporting slide for 1 to 5 minutes in a solution of basic fuchsin in a mixture of acetone and water (0.5 per cent basic fuchsin, 50 per cent acetone). The stain was removed from the gelatin of the film by immersion for 30 minutes in 10 mM sodium acetate buffer, pH 4, with continuous agitation on a mechanical shaker. The specimen was finally washed and dried, and mounted under a coverglass. The autoradiograph was photographed with yellow-green light through an oil-immersion apochromatic objective lens (na 1.32) on Kodak P. 200 plates. Analysis of the autoradiographic distribution was performed by the method described previously (10).

Autoradiographs were also required for a low-power examination of the over-all distribution of the radioactive material in a cross-section of the muscle. In order to make the silver grains conspicuous at low power, the autoradiograph was over-developed by immersion for 30 minutes in Kodak D. 19b developer. Specimens prepared in this way were not stained with basic fuchsin.

**ELECTRON MICROSCOPY:** Autoradiographs for examination by electron microscopy were made with...
sections 100 to 150 μm thick, using Ilford L4 fine-grain liquid emulsion and following the procedure of Caro and van Tubergen (4). These authors' first method of applying the emulsion to the section was employed. The coated grids were stored in a light-tight box over silica gel, at 20°C. After an exposure time of 10 to 40 days the autoradiographs were developed for 5 minutes at 20°C in Kodak Microdol-X and, after rinsing, were immersed in an acid, hardening, fixing bath for 5 minutes. In order to obtain clearer electron micrographs, several attempts were made to remove the gelatin of the emulsion, after processing, by digestion with trypsin (0.01 per cent, in 20 mM phosphate buffer pH 7.3) at temperatures varying from 20 to 37°C. These were unsuccessful, since practically all the silver grains were lost with the gelatin.

RESULTS

Radioactive Assays: Retention of Adenine and Creatine

CONTROL MUSCLES: The results listed in Table I (row 4) show that both adenine and creatine were taken up and bound in amounts which are comparable with the values recorded in the earlier experiments (8–10).

RETENTION OF ADENINE DURING PREPARATION: The adenine nucleotide appears to have been wholly retained during the first stage in the acetic acid/ethanol fixative. At the second stage, in aqueous solution at pH 4, the results are more variable, but it is clear that at least two-thirds to three-quarters of the adenine nucleotide was retained in the muscle.

RETENTION OF CREATINE DURING PREPARATION: Creatine phosphate was probably not fully retained during the first stage, and about one-half was lost in experiments 48 and 54. The proportional rate of loss was much greater during the exposure to aqueous solution during the second stage: 91 to 99 per cent of the amount left after the first stage was lost during the second. The reason why this occurred will be discussed later.

ACTIVITY OF THE EMBEDDED MUSCLE: The muscles labelled with tritiated adenine had activities of 44 to 125 μc/g (Table I, row 9). This was sufficient to give ample grain yields for autoradiography by light microscopy, with conveniently short exposure times of 5 to 10 days.

The activity was rather low for autoradiography by electron microscopy because, as will be seen, the analysis could be performed only in areas where the emulsion layer was extremely thin, and this reduced the efficiency. Even after exposure times of 30 to 40 days the grain yield in such areas was too low to give as much significance to the analysis as could be desired.

Preservation of the Muscle: Observations by Phase Contrast, Polarized Light, and Electron Microscopy

Acetic acid/alcohol mixtures are regularly used by histologists and cytologists as fixatives for various applications. Caspersson and Thorell (5) used such a fixative for observations of the distribution of adenine nucleotide in insect muscle fibres. Occasional references are found to their employment for preserving nucleic acids for electron microscopy (e.g., reference 20). There is, however, no known example of any successful preservation of the finer structures of muscle by this type of fixative. It is therefore necessary to enquire into the state of preservation of the muscles used for the present experiments. This was done, first, with the aid of the light microscope, using the strength of the birefringence of the A bands as a general indication as to the integrity of the protein structure. When muscle is embedded in Araldite its “form” birefringence becomes extremely weak, because the refractive index of the Araldite, 1.50, is very nearly equal to that of dry protein, 1.54, (1). This means that the “intrinsic” birefringence caused by elements of molecular size will be dominant, and the integrity of these elements can be assessed by the strength of the birefringence, or at least a comparison can be made of different parts. An example is shown in Fig. 1. It was clear that the birefringence is not uniform, and in parts it practically vanishes. This suggested that the preservation of the protein structure was not uniformly good. Selection of the appropriate regions for comparison by electron microscopy would have been difficult if it had not been found, using light microscope sections viewed by phase contrast, that staining with permanganate produced a distinctly different pattern as between the regions of strong and weak birefringence (Fig. 2); and since permanganate is also an electron stain the corresponding regions could be identified in electron micrographs. Permanganate has the additional advantage that it stains the protein of the myofilaments.

It was found that the filamentary structure
appeared to be intact (Fig. 7) only in those parts where there was a wide band in I, showing comparatively dense staining by permanganate. In regions of lower birefringence there was no over-all density increment in the I band, and the filamentary structure was then not seen. In such regions it was always noticed that there was a narrow band, in the centre of the A band, where the density was less than elsewhere; it is possible that this indicates a loss of protein. (In parts where the preservation is good a similar pale band may be seen, but it is much less conspicuous, for example see Fig. 7.)

Sections were always cut near the surface of the muscle because it was found that the deeper parts showed a smaller proportion of well preserved structure.

There was considerable variation among different experiments; some muscles showed better preservation than others. Fortunately, one of the muscles which was labelled with tritiated adenine (experiment 53) was unusually well preserved; within 100 μ of the surface about one-half of the field showed good preservation.

When a new block face was prepared for electron microscopy a section was always first examined by phase contrast after staining it with permanganate. Unless this showed moderate or good preservation (as indicated by the width of the dense bands) over almost the whole section, the block was rejected. It was practically impossible to limit the electron microscopic observations strictly to the good regions when sections were stained by lead or by uranyl acetate, because the filaments cannot be
seen, but there was no suggestion of any essential variation, as between the different parts, in the features under investigation.

Section of good parts for autoradiography by light microscopy was not so difficult, because it was found that the stain pattern produced by basic fuchsin was exactly similar, in the distribution of wide and narrow bands, to that seen after using permanganate on another section cut at the same time. This meant that regions of an autoradiograph after the stage of acid fixation in order to take samples for radioactive assay. It was found that in these muscles the sarcomere length in the embedded preparation was in every case considerably less than it was in some experiments (not otherwise referred to here) in which the stage in aqueous solution at pH 4 had been omitted. The sarcomere lengths averaged 2.3 and 2.7 μ, respectively, for the alternative methods of preparation. The latter figure is about what would be expected for the

which showed wide bands (Fig. 4) could be taken to be well preserved; the analysis was confined to these.

Transverse sections were not used except for studying the distribution of radioactivity in the muscle as a whole, because the above criteria for assessing the state of preservation of the individual fibres were not applicable.

**Sarcomere Length: the Length of the A Band**

In all the experiments listed in Table I, the muscle had to be cut away from the glass holder extension at which the muscles were set before fixation (the extended length in the body is about 2.5 μ (2), and the muscles were elongated by 10 per cent before fixation). It follows that exposure to the aqueous solution must have resulted in a longitudinal shrinkage of about 15 per cent. Though it cannot necessarily be assumed that this shrinkage occurred equally in the A and I bands, no data are available, either from birefringence measurements (the birefringence in Araldite-embedded material is too weak for accurate measurements to be made) or from observation of the lengths of the A filaments in electron micro-

![Figure 2](image-url)

**Figure 2** Sections stained with permanganate. Light microscope; phase contrast. The stain lies in the I bands (1); this is confirmed by examining thicker sections, similarly stained, under the polarizing microscope.

a, region showing strong birefringence in the A band; the whole of the I band is stained. × 5000.

b, region showing weak birefringence; the stain is taken up only in the centre of the I band. × 5000.
graphs (the ends of the filaments could not be seen with sufficient clarity, see for example Fig. 7), to enable a shrinkage factor to be applied separately to the two parts of the sarcomere. The factor of 15 per cent must therefore be used for both. In the living muscle the A band has a constant length of 1.5 μ (12); it follows that the corresponding length after preparation should be taken as 1.3 μ.

A muscle which has been fixed in acetic acid/ethanol is very soft, and there is evidence that damage may occur if tension is not released during the subsequent stage in aqueous solution. A number of experiments were made in which the muscles, initially set to give a sarcomere length of about 2.7 μ, were left attached to the glass holder throughout the later stages of preparation. As a result of the tendency to shrinkage, a high tension developed while the muscles were in aqueous solution at pH 4. Parts of these muscles were later found to have sarcomeres as much as 3.1 to 3.2 μ long, and it was observed, by phase contrast examination, that in these regions some fibres showed distinct gaps at the middle of the A band, suggesting that the fibrils had ruptured at this point. It seems likely that under high tension the weaker parts may give and thus become abnormally extended and perhaps torn. For this reason, when using the present method of fixation it has seemed better to release tension after the primary non-aqueous stage, even though considerable shrinkage then occurs, rather than risk damage by forcible restraint.

Figure 3 Autoradiograph of a transverse section, 0.75 μ thick, cut at 35° to the long axis of the muscle. Exposure, 30 days. The autoradiograph was over-developed to show the silver grains at low magnification. The outlines of the individual fibres are discernible only in parts, but it is clear that the radioactivity is uniformly distributed within each fibre. Some of the deeper fibres (D) possess less activity than do those nearer the surface of the muscle. X 250. 
Autoradiography

Light Microscopy

Transverse sections: The distribution of radioactivity in a transverse section of a muscle is shown in Fig. 3. The activity within any one fibre is seen to be evenly distributed. After fixation with an osmium tetroxide/lanthanum mixture (8) there was a very marked non-uniformity within the individual fibre. One reason for employing a non-aqueous fixative in the present work was to prevent the appearance of such an artefact; it is seen to have been effective in this respect.

The activity is not uniformly distributed over the muscle as a whole, and there appears to be a band of deeper fibres which possess a comparatively low concentration of the isotope. A similar deficiency in the deeper parts of the muscle was observed previously, for both adenine nucleotide (see reference 8, plate 1) and phosphocreatine (reference 10, plate 1).

The longitudinal distribution of the adenine nucleotide: Part of an autoradiograph of a longitudinal section of a muscle is shown in Fig. 4. The distribution of the silver grains, in relation to the boundaries of the A and I bands, analyzed in the way described previously (7, 10), is shown in Fig. 5. There is evidently a preferred site at the position of the arrow C; this is inside the I band, near the A-I boundary. The position of this "centre" is the same as that found previously (8) after fixation by the osmium tetroxide/lanthanum mixture.

There is some doubt concerning the form of the...
autoradiographic distribution which should be produced by a transverse "line source" in the muscle. In practice it appears (10) to be narrower than, in theory (11), it should be. This uncertainty means that it is not possible to calculate accurately what proportion of the whole activity in the muscle is concentrated at the preferred site. On this occasion an estimate is made by assuming that there are two distributions in the muscle, one consisting of a highly concentrated line source at C, the other being a uniform distribution spread evenly throughout the whole of the muscle. The concentration of the latter is taken as the average, 29, of the counts between arrows D and E in Fig. 5; the number of grains attributable to this distribution is 754 out of a total of 1564. The difference, 810, represents the effect of the supposed aggregate at C; this is 52 per cent of the whole. In other words, this fraction may be taken as being concentrated in the I band near the A-I boundary. It should be pointed out, however, that other assumptions could be made which would give higher or lower values for the fraction in question. The earlier calculation (8) was based on an assumption which, if it had been applied in the present instance, would have given a considerably higher value for the proportion of activity concentrated at C.

**Electron Microscopy**

**The Transverse Distribution of the Adenine Nucleotide:** Autoradiography by light microscopy gives no solution to the problem of finding the transverse distribution of the adenine nucleotide in muscle. It shows only the position of the centres of concentration on the long axis of the muscle, but provides no evidence as to whether these lie within the fibrils or, alternatively, in the interfibrillar spaces.

The mean width of the fibril as seen by electron microscopy in longitudinal sections was measured and found to be 0.490 μ (149 fibrils). It is assumed that the fibrils have circular form in transverse section, so the mean width observed in longitudinal section has to be multiplied by $4/\pi$ to obtain the mean diameter, 0.625 μ. The resolving power with light microscope autoradiography should be just sufficient (11), with fibrils of this size, to allow a solution of the problem. The method fails to do so, however, not primarily on account of a lack of resolving power, but rather because the interfibrillar spaces cannot be seen clearly under the light microscope, either in transverse section, or in longitudinal sections which are cut sufficiently thinly to avoid serious depth effects. The electron microscope overcomes this difficulty because the interfibrillar spaces show up, over part of their length, in electron micrographs of the processed autoradiographs (Fig. 6). The electron-opaque element is presumably silver, from the emulsion, which has stained certain components of the muscle at some stage during the photographic processing.

The method using electron microscopy has a second advantage, in that the resolution with autoradiography is appreciably better than it is with light microscopy (3).

The interfibrillar material seen by electron microscopy shows very low contrast with the background. A sufficiently clear picture was obtained only when the layer of emulsion was very thin, because the residual gelatin tended to reduce the contrast and clarity of the underlying structures. The efficiency of the autoradiographic process, in terms of the total yield of silver grains, unfortunately proved, for this reason, to be very low in areas which were suitable for observing the interfibrillar spaces.
The pattern seen in Fig. 6 a resembles that shown after staining sections with uranyl acetate (Fig. 13). The resemblance became more marked after the gelatin had been removed by trypsin digestion (this removed the silver grains as well). The uranyl-stained complex undoubtedly lies in the I band, so there seems little doubt that the interfibrillar 'lines' seen in the autoradiographs also have their location in that part of the sarcomere.

It was decided to restrict the transverse analysis of the autoradiographs to include only those grains which lay over the I bands, where the interfibrillar spaces are seen. It has been shown that the main centre of aggregation of the adenine nucleotide lies in the I band; since this fraction of the nucleotide is of primary interest, it seemed preferable to limit the analysis accordingly. In any case, the positions of the interfibrillar spaces in the A bands are not generally definable with much accuracy by interpolation.

The position of a grain was defined as the centre of the cluster of smaller particles of which it is seen to be composed. This position was then measured, in the transverse sense, with reference to the interfibrillar spaces between which the grain lay. The measurement was made to an accuracy of one-tenth part of the interval between these spaces. The values so obtained were put into three groups, A, B, and C, representing equal areas of field. Group A included those grains which lay adjacent to the interfibrillar spaces; B, those over the intermediate area; and C, those which were located over the centre one-third of the fibril.

A large number of micrographs was examined, but almost all of them had to be discarded because the muscle structure was not seen with sufficient clarity. This was due mainly to the presence of the gelatin residue from the emulsion, but even when this was not the limiting factor the pattern of interfibrillar lines was not sufficiently regular (Fig. 6) to allow many grains to be satisfactorily referred to the boundaries of the fibril. A total of only 158 grains could be found for which locations could be recorded with adequate precision. (The procedure could be criticized on the grounds that, by rejecting those fields in which the interfibrillar lines showed insufficient regularity, a significant bias was being introduced into the analysis; the distribution of the isotope in a regular area might, for some reason, be different from that in a non-regular area. Unfortunately, this defect in the method of analysis is unavoidable.)

The results were as follows:

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of grains</th>
<th>sd</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>102 (64 per cent)</td>
<td>10</td>
</tr>
<tr>
<td>B</td>
<td>34 (22 per cent)</td>
<td>6</td>
</tr>
<tr>
<td>C</td>
<td>22 (14 per cent)</td>
<td>5</td>
</tr>
</tbody>
</table>

(The standard deviation is simply taken as the square root of the number of grains, since errors inherent in the analysis itself are thought to be relatively unimportant in the present instance.)

The conclusion is that the number of grains located near the interfibrillar spaces is significantly higher than the number found near the centres of the fibrils.

A more detailed quantitative treatment does not seem justified with the present data.

For reasons mentioned above, this analysis refers only to radioactive material which lies in the I band of the muscle. This includes the fraction which, according to the autoradiographic analysis done with the light microscope, is concentrated near the A-I boundary. Now there are grounds for suggesting that this fraction may be concen-
trated in the interfibrillar spaces rather than in
the substance of the fibrils.

It is the purpose of the following electron micro-
scope studies to see whether any structure is
visible, or can be made visible by suitable staining,
at the site in question.

**Electron Microscopy**

**Unstained sections:** Unstained sections
which are thinner than about 80 m€ are almost
entirely featureless. No protein structures are
visible, and lead deposits are generally not present
in sufficient quantity to enable any pattern to be
recognized. When such a section is stained with
permanganate the A and I bands become visible,
and the protein filaments may be seen (Fig. 7) in
those parts which are well preserved.

The sections had to be cut at a thickness of at
least 100 m€ in order to show a definite pattern
of lead deposits. The structures which are then
seen to be stained with lead are thought to be the
three-element complexes of the sarcoplasmic re-
ticulum known as the “triads” (16, 17).

The contrast may vary considerably in different
parts of a section. The parts of the muscle which
are well preserved (as judged by the criteria

![Electron micrograph of a section, 60 mμ thick, stained with 5 per cent potassium permanganate for 8 minutes. This part of the muscle showed strong birefringence, and by light microscopy wide permanganate-stained bands in I were seen (Fig. 3 a); the greater density in I is also seen, here, by electron microscopy. (The difference in density of the two I bands is an artefact due to uneven brightness of the electron beam.) The contrast is very low, but the filamentary structure of the A band (A) can just be
seen to be intact. More prolonged staining with permanganate causes damage of the filamentary structure. × 32,000.](image)
already mentioned) appear always to show the triads the least clearly. In such parts oval-shaped vesicles showing negative contrast were often seen (Fig. 8) in the interfibrillar spaces between the triads and the A-I boundary. These hollow vesicles often show signs of being divided transversely into two compartments.

The triads are seen with greater contrast and clarity in parts of the muscle where the preservation of the finer protein structure is thought to be not so good. The three-element nature of the triad is sometimes clearly visible (Fig. 9).

**Sections stained with lead:** The electron microscopic examination of stained sections has been confined to those parts of the muscle which show the best preservation, as judged by the criteria mentioned previously. The pattern seen after staining with an alkaline solution containing lead is shown in Figs. 10, 11, and 12. The sections were too thin for the triads to be seen, and the pattern consists basically of pairs of vesicles, or groups of vesicles, in the interfibrillar spaces; these vesicles are lined up in distinct rows which run transversely across the muscle fibre. There can be little doubt that these vesicles lie in the I band, because when sections are stained by uranyl acetate (see below) the triads are shown up as well as the vesicles, and the location is then easily recognized.

The object was to see whether any structures can be demonstrated by electron microscopy at the location where the adenine nucleotide is

---

**Figure 8**  Electron micrograph of an unstained section, 150 mµ thick, showing a part of the muscle which is well preserved. The centres of the A and I bands are indicated. The triads are seen as pairs of dark structures in I. There is no sign of any positive-contrast structures in the interfibrillar spaces near the A-I boundaries, but hollow, oval-shaped vesicles may be seen there (arrow); these sometimes appear divided. × 22,000.
thought to be concentrated. The question is: do the vesicles occupy the appropriate site? According to the autoradiography (Fig. 5) the centre of concentration lies about one-quarter of the way from the A-I boundary to the Z line. But the centres of the groups of vesicles are nearly half way between the A-I boundary and the Z line. The difference in location, though it amounts to only about 0.13 μ, is probably significant (though there is some uncertainty (see reference 10, pp. 42-43) as to an acceptable estimate of the resolving power with autoradiography), and, if this is so, it must be concluded that the vesicles, if regarded as a single system, are not in the proper position. However, it is possible that no discrepancy really exists, because the vesicles which are displayed may not in fact be components of a single intercommunicating system; there may be two or more independent systems, separated longitudinally from one another. It is conceivable that the adenine nucleotide is associated not with the complex of vesicles as a whole, but only with that part of it which lies nearest to the A-I boundary. It seems fairly clear (Figs. 11 and 12) that parts of the complex often do lie in the appropriate position, even though this is not true of the complex considered as a whole.

There is some evidence (10) that phosphocreatine is located closer to the Z line than is the adenine nucleotide. Perhaps the phosphocreatine occupies another part of the vesicular complex.

**SECTIONS STAINED WITH URANYL ACETATE:**

The stain pattern given by uranyl acetate (Figs. 13 and 14) differs, in one important respect, from that seen after treatment with lead: the triads are stained densely by uranium, whereas they are not visible with lead. The pairs of vesicle complexes in line with the triads are again apparent, but the stain is now seen not to be sharply concentrated in their walls and appears to have spread to a zone outside their periphery.

**DISCUSSION**

It is well known that, apart from the nucleic acid and other minor fractions, adenine nucleotide is very readily extractable from broken-up muscle cells by neutral aqueous solutions. It therefore is largely not normally bound to the fibrous proteins. Yet a proportion of the nucleotide, probably not less than one-half of the total, has been shown not to be uniformly distributed throughout the muscle fibre but is concentrated in a part of the I band. The only conclusion which appears consistent with the facts is that the fraction of the nucleotide which shows this longitudinal localization lies within some part of the system of tubules of the sarcoplasmic reticulum. This reticulum is situated in the interfibrillar spaces and does not penetrate between the myofilaments of the fibrils.

According to this argument it is not surprising to find, as has been found from the present experiments, that a proportion of the nucleotide of resting muscle lies in the interfibrillar spaces rather than in the substance of the fibrils.

There is evidence, from earlier work (8), that the fraction of the nucleotide which is localized in the longitudinal sense has, at the same time, freedom to diffuse transversely across the fibre, so it appears that the reticulum which contains the relevant fraction of nucleotide has transverse continuity and permits diffusion in that sense.

In amphibian muscle the only part of the reticulum which displays a recognizable constancy in its form and location is the triad complex at the Z line (16); at any rate, it can be said that no regular arrangement of tubules has been described at the region which is of present interest, near the A-I boundary. Veratti (17), in much earlier studies with the light microscope, found that muscles of a number of species do possess transverse reticula at the junction of the A and I bands, but he was unable to demonstrate any such formation in amphibian muscle.

**FIGURE 9** Electron micrograph of an unstained section, 150 μ thick, showing a part of the muscle where the preservation of the finer protein structure is probably not so good. There is a lower density (A) at the centre of the A band: this is a regular feature of the less well preserved regions. The triads are more clearly visible than they are (Fig. 8) when the preservation is good. The three-element nature of the triad is sometimes apparent (arrow); this feature is more easily seen when there has been a "glancing tangential" cut through the triad complex, and its transverse width in section does not, then, indicate the true interfibrillar spacing. This field was selected because it happened to show an abnormally large number of triads cut in this manner. × 23,500.
Figure 10  Low-power electron micrograph of a 100 mμ section, stained with alkaline lead solution. The centres of an A and of an I band are marked (A, I). The pattern consists of rows of vesicles lined up transversely in the I band. X 12,500.

Figure 11  Similar to Fig. 10. Electron micrograph of a 100 mμ section, stained with alkaline lead solution. The extent of the A band (1.3 μ) is shown (A). The vesicular structures are often of multiple form (arrows). X 21,000.
FIGURE 12 Similar to Figs. 10 and 11. Electron micrograph of a 100 μm section, stained with alkaline lead solution. The extent of the A band (1.2 μ) is shown (A). The vesicular structures are often of multiple form (arrows). X 52,000.

FIGURE 13 Low-power electron micrograph of an 80 μm section, stained with uranyl acetate. The centre of an A and of an I band are marked. The triads stained densely. The vesicles in line with the triads are seen, but the stain has spread to a zone outside their periphery. The vesicles are sometimes of multiple form (arrows). X 15,500.
The Identity of the Material Seen After Staining the Sections

The pK value for ionization of the first hydrogen of the primary phosphoryl group of ATP is about 2, and that for the phosphoryl group of PC is less than 2 (6). The pK of protein carboxyl groups is about 5. The aqueous solution used at the second stage of the procedure was buffered at pH 4 in order to promote combination of lead with ATP and PC, without staining the protein. Nucleic acids and other phosphate compounds might have reacted too, but there was no question of the lead acting as a general ‘stain’ at this pH. If any electron-opaque deposit had been found at the site indicated by autoradiography, there would have been good grounds for supposing it to be the lead salt of adenine nucleotide. No such deposit was found. The most likely explanation is that the lead was lost during a later stage of the preparation, probably in the 5 per cent acetic acid/95 per cent ethanol solution which was used to remove excess lead acetate. Though the adenine nucleotide itself is extremely insoluble in this mixture, and would therefore be retained, the lead may have been dissociated from it by the acid and could then have diffused away. (If this is the correct explanation it follows that the fixation procedure actually adopted was unnecessarily complicated, since the same distribution of adenine nucleotide would presumably have been found after fixation simply in an acetic acid/ethanol mixture, and without transference, secondarily, to an aqueous solution.)

It was necessary to resort to staining by an alkaline solution of lead, or by uranyl acetate, before any electron-opaque material was revealed at the sites in question. Unfortunately, under alkaline conditions (pH 12, in this case) lead can no longer be expected to react specifically with phosphate compounds, but combination with protein (through its carboxyl and sulfydryl groups) and perhaps with glycogen (19) could have taken place. The nature of the chemical reactions which occur when the uranyl ion is employed has been discussed by Huxley and Zubay (13); though there is reason to think that phosphate groups may be almost exclusively involved in certain types of material, no such specificity can be claimed in the present case.

The conclusion must therefore be restricted to pointing out the significance in the fact that structures may be demonstrated in a position which, on other grounds, is thought to be that where much of the adenine nucleotide is concentrated in resting muscle. There are insufficient grounds for arguing that the lead deposit which is seen is the salt of adenine nucleotide.

Composition of the “Triad Substance”

The only parts of the muscle which show up clearly in electron micrographs of unstained sections are the triads. It is clear that a substance must be present here which has exceptionally strong affinity for the lead. This could be inorganic phosphate, which forms an extremely insoluble compound with lead. The amount of free inorganic phosphate in resting muscle is only about 1 mmole/kg (18), but a breakdown of some PC (normally about 25 mmole/kg) or ATP (about 5 mmole/kg) could yield much higher values.

Some parts of the muscle did not show good preservation of the protein structure. It seems likely that breakdown of PC or ATP, with formation of inorganic phosphate, may well have accompanied the process of degradation. It was seen that a relatively large amount of lead was deposited in the triads in the less well preserved parts of the muscle. This observation provides evidence in favour of the suggestion that the triad deposit is inorganic lead phosphate.

The Loss of Creatine Phosphate

The intention had been to obtain further information on the location of phosphocreatine, but this proved impossible because it was not retained in the muscle. In earlier work (10), it was pointed out that the lanthanum salt of phosphocreatine is not very insoluble and that the amount lost from a muscle must be dependent on the time of exposure. Similar considerations apply in the

Figure 14  Electron micrograph of an 80 m\(\mu\) section, stained with uranyl acetate. The centres of an A and of an I band are marked. The extent of the A band (1.3 \(\mu\)) is shown. The triads are densely stained. The vesicles in the interfibrillar spaces are sometimes of multiple form (arrows). \(\times 31,500\).
present case. Phosphocreatine is not very insoluble either in an acetic acid/ethanol/lead acetate mixture or in a solution of lead acetate at pH 4. It would seem that the time factor must be of critical importance and that the procedure adopted is, for that reason, not suitable for fixing phosphocreatine.

CONCLUSION

The question must be asked: is the location of adenine nucleotide in vivo the same as that which has been found in the fixed muscle? What is the likelihood that the process of fixation has caused binding at an "artificial" site? No definite answer can be given. But it can at least be said that the possibility of the result's being an artefact now seems considerably reduced, in view of the present finding that the longitudinal distribution of nucleotide is much the same after acetic acid/ethanol fixation as it was (8) after aqueous fixation at neutral pH. However, some confirmation by a method which is applicable to the living muscle is obviously desirable. There seems no clear way to achieving this purpose with vertebrate muscle, where the short sarcomeres preclude any useful application of ultraviolet optical methods such as those which have been used (5) to study the distribution of adenine nucleotide in the muscle fibres of insects.

This work was supported by a grant from Muscular Dystrophy Associations of America, Inc. The author is indebted to Dr. A. G. E. Pearse for kindly allowing him the use of the electron microscope, and to Mr. Ralph Nunn for much valuable assistance with the electron microscopy.

Received for publication, May 28, 1963.

BIBLIOGRAPHY