Virchows Arch. path. Anat. 342, 244—251 (1967)

# The Presence and Localization of Masked Lipids in Mouse Muscle: A Histochemical Study

VIVIANE MAGGI and R. ALLEN

Tissue and Organ Culture Unit, Imperial Cancer Research Fund, Lincoln's Inn Fields, London W.C. 2

Received January 28, 1967

*Vorkommen und Lokalisation von maskierten Lipiden in der Muskulatur der Maus: eine histochemische Untersuchung*

*Zusammenfassung.* In Herz- und Skeletmuskulatur der Maus fanden sich zwei Lipide. Diese Lipidfraktionen lassen sich durch die histologische Routinetechnik nicht darstellen. Die eine Lipidfraktion aber wird demaskiert und anfärbbar, wenn Muskelschnitte einer Temperatureinwirkung von 18—60° für eine bestimmte Zeit ausgesetzt werden. Es handelt sich bei dieser Fraktion um ein bandförmig angeordnetes Phospholipid, dessen Herkunft ungeklärt ist. Die andere Lipidfraktion wird durch Einwirkung von Natrium-Ionen in den Sarkolemm-Formationen demaskiert. Bei dieser Lipidfraktion scheint es sich nicht um ein Phospholipid zu handeln. — Die Funktionen beider Lipidfraktionen werden erörtert.

*Summary.* A lipid fraction has been found in the heart and voluntary muscle of the mouse. This fraction is not stained by routine histological methods, but is unmasked and becomes stainable after the sections of muscle have been exposed to temperatures varying between 18°C and 60°C. The fraction appears to be a phospholipid and to be localized in bands, the nature of which it has been impossible to ascertain.

Sodium ions unmask a second lipid fraction in the muscle sarcolemma; this latter fraction does not seem to be a phospholipid.

The function of these lipid fractions is briefly discussed.

Lipids exist in the tissues in at least two forms. One can be demonstrated by the extraction procedures and by histochemical stains, whilst the other is tightly bound to a protein support (MAGGI and BRANDER, 1965) and cannot be extracted by the conventional methods nor can it be stained by the routine histochemical techniques. This paper describes the results of further histochemical investigations on the nature, distribution and possible role of this masked lipid fraction in the heart and voluntary muscles of the mouse.

## Material and Methods

C 57 adult male mice were killed by dislocation of the neck and pieces of heart, tongue and quadriceps muscle were removed rapidly and frozen in Arcton 12, precooled in liquid nitrogen. Frozen sections were cut at a thickness of 8—12 μ in a Bright's cryostat.

The procedure of PERRY and CORSI and of PERRY and ZYDOWO was used for the isolation of myofibrils from the quadriceps of the thigh. The final preparations of myofibrils were smeared on slides. These and the frozen sections were used unfixed or fixed for ten minutes in formol-calcium (BAKER) and subjected to the following treatments prior to staining:

A. The effects of temperature on the availability of lipids to staining were tested by leaving the preparations for 18 hours at −30°C or at room temperature (ca. +20°C) or at +60°C.

B. The effects of some salts on lipid staining were tested by immersing the slides at room temperature for 18 hours in 0.15 M sodium chloride at pH 7.0, or 0.3 M potassium chloride at pH 7.8, or 0.3 M calcium chloride at pH 6.8.

C. The combined effects of temperature and electrolytes on lipid staining were tested by immersing the preparations in 0.15 M sodium chloride, or 0.3 M potassium chloride, or 0.3 M calcium chloride at +60°C for 18 hours.

D. The material was stained without previous treatment.

The material treated as described above was stained for lipids as follows: 1. A saturated solution of 3.4-benzpyrene in aqueous caffeine (BERG, 1951). The preparations were mounted in buffered glycerol at pH 7.5 and fluorescence was examined with a Zeiss microscope equipped with a HBO 200 mercury burner. Autofluorescence was assessed on unstained material; 2. A saturated solution of Sudan Black B in acetone; 3. The acid haematein method for phospholipids (BAKER, 1946). The specificity of the method was controlled by the bromination procedure of CHAYEN et al. (1964). When a positive reaction was obtained with any of the methods given above, the lipid nature of the positive components was tested by staining sections from the same block after extraction with chloroform:methanol:concentrated hydrochloric acid 200:10:1 v/v.

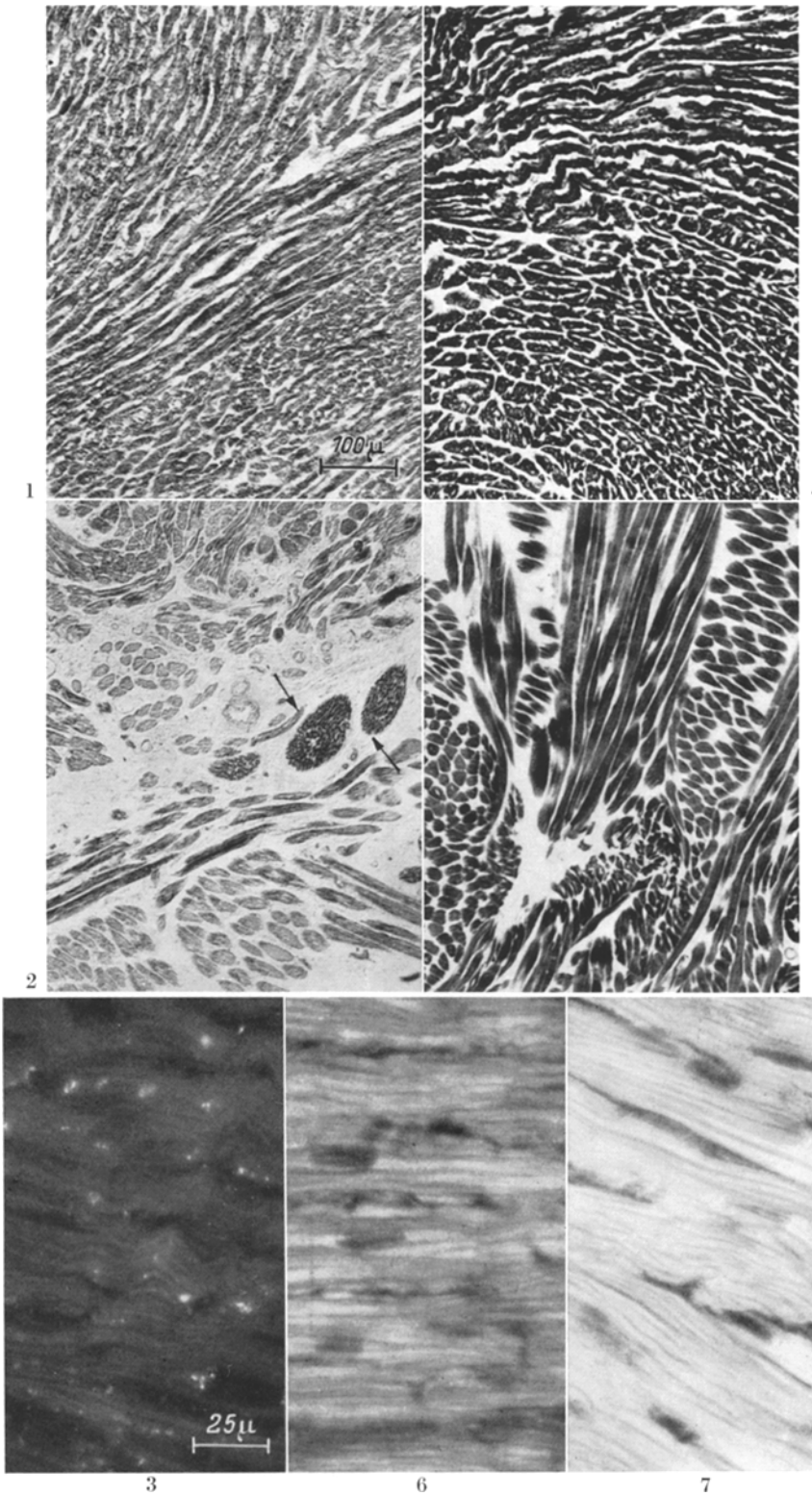
## Results

The intensity of the response to both 3.4-benzpyrene and acid haematein of untreated material is shown in Figs. 1—3 and the Table. Isolated myofibrils and thigh muscle and tongue sections were unstained by both stains, whereas heart sections occasionally showed some stained areas. The material kept at −30°C for 18 hours did not differ from the untreated material. When the slides were left at room temperature for 18 hours there was an increase of both fluorescence and acid haematein staining in the sections (Figs. 4—6) though not in the isolated myofibrils. The reactions were even more marked in sections kept at +60°C for 18 hours (Fig. 7). Isolated myofibrils treated similarly showed the same reaction (Fig. 8). The staining in the muscles was localized to bands of dimensions varying between 0.8 and 1.2 μ, depending on the treatment used (Figs. 8—12).

Pretreatment of the sections with brominated water (CHAYEN et al., 1964) prevented acid haematein staining. Pretreatment of the sections with acidified methanol: chloroform resulted in a lack of response to both acid haematein and 3.4-benzpyrene. Sections stained immediately after cutting, or after having been kept at −30°C, or at room temperature (+20°C ca.) for 18 hours, did not stain with Sudan Black B, but there was a faint staining in sections kept at +60°C for 18 hours.

Table. *Effect of temperature and of ions on unmasking of lipids in muscle sections*

| Temperature °C | Electrolytes | Time | Stain | |
|---|---|---|---|---|
| | | | Acid Haematein | 3.4-benzpyrene |
| −30° C | — | 10—30 min. | ± | ± |
| −30° C | — | 18 hours | ± | ± |
| +18—20° C | — | 18 hours | ++ | ++ |
| +60° C | — | 18 hours | +++ | +++ |
| +18—20° C | 0.15 M NaCl | 18 hours | — | +++ |
| +60° C | 0.15 M NaCl | 18 hours | — | +++ |
| +18—20° C | 0.3 M KCl | 18 hours | — | ++ |
| +60° C | 0.3 M KCl | 18 hours | — | +++ |
| +18—20° C | 0.3 M $CaCl_2$ | 18 hours | + | ++ |
| +60° C | 0.3 M $CaCl_2$ | 18 hours | ± | +++ |




Figs. 1—7 (for legends see p. 247)

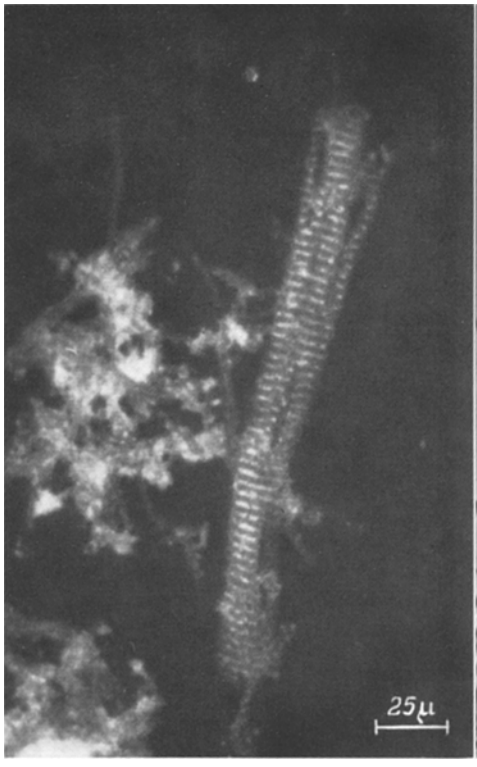



Fig. 8

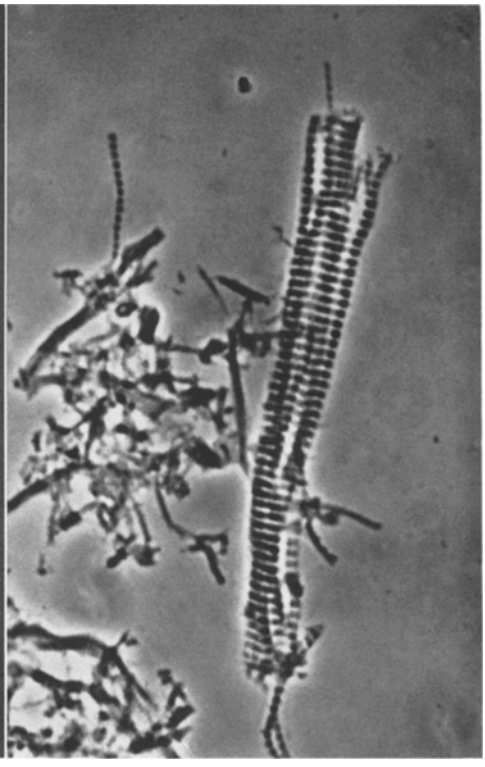


Fig. 9

Figs. 1—12. All photographs of fluorescent material were taken with the same exposure time and developed and printed under the same conditions, to give a semi-quantitative measure of the stain intensity. Exciter filters BG 38/2.5 and BG 3/4 (Schott and gen.) and barrier filters 50 and 44 were used with a Zeiss photomicroscope equipped with a HB 200 mercury lamp

Fig. 1. Frozen section of heart stained with acid haematein immediately after cutting. The image is due to the brown background; there is no blue-black stain due to phospholipids. × 100

Fig. 2. Frozen section of tongue stained with acid haematein immediately after cutting. No black-blue stain due to phospholipids in the muscle. Nerve fibres (arrows) are positive. × 100

Fig. 3. Frozen section of heart stained with 3.4-benzpyrene immediately after cutting. The intensity of the fluorescence corresponds to that present in unstained sections. × 400

Fig. 4. Frozen section of heart stained with acid haematein after 18 hours at room temperature (ca. 20°C). The fibres are stained blue-black. × 100

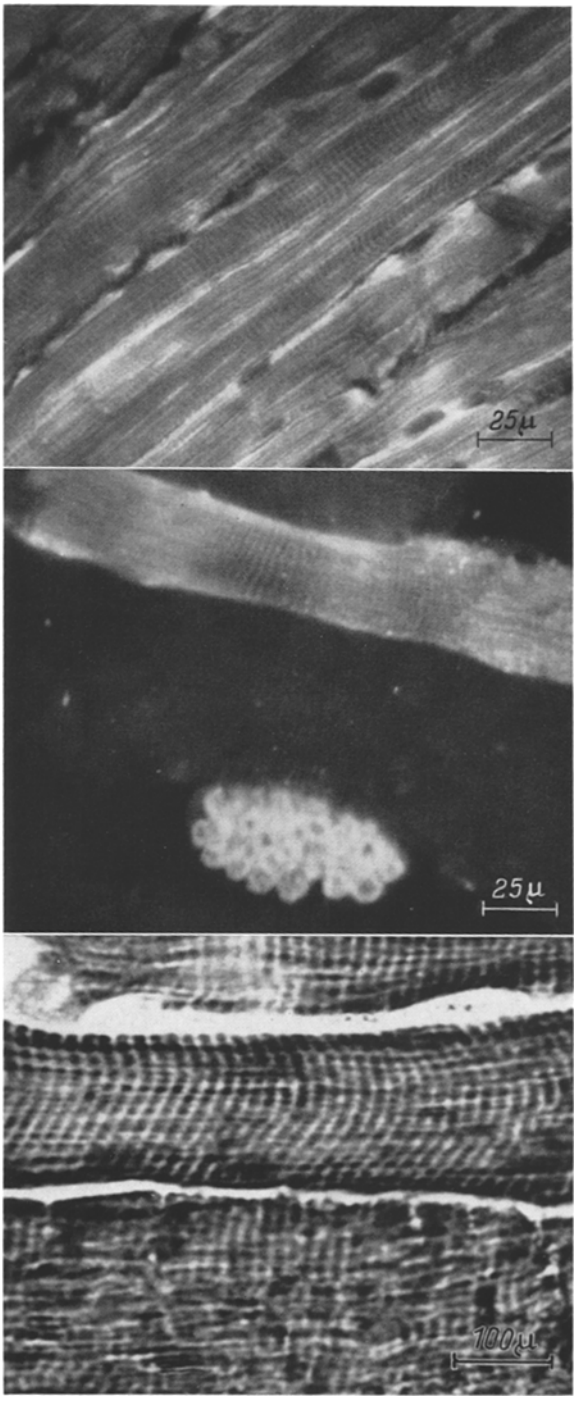
Fig. 5. Frozen section of tongue stained with acid haematein after 18 hours at room temperature. The fibres are intensely positive for phospholipids. × 100

Fig. 6. Frozen section of heart stained with 3.4-benzpyrene after 18 hours at room temperature. The tissue fluoresces very intensely. × 400

Fig. 7. Frozen section from the same experiment as in Fig. 6 but stained with 3.4-benzpyrene after 18 hours at 60°C. The increase in fluorescence with respect to Fig. 6 is remarkable. × 400

Fig. 8. Isolated myofibrils from quadriceps muscle stained with 3.4-benzpyrene after 18 hours at 60°C. There is a clearly visible fluorescent band pattern. × 400

Fig. 9. Same preparation as in Fig. 8 but photographed with phase contrast objective. The lighter bands appear to correspond to the stained bands in Fig. 8. × 400




10

11

12

Fig. 10. Frozen section of tongue stained with, 3.4-benzpyrene after 18 hours at room temperature. There is a distinct fluorescent band pattern. × 400

Figs. 11 and 12 (for legends see p. 249)

The intensity of fluorescence in sections kept in 0.15 M sodium chloride at room temperature and stained with 3.4-benzpyrene was of the same order as that of the sections kept dry at +60°C (Table). The sections kept in 0.15 M sodium chloride at +60°C for 18 hours showed no further increase in fluorescence. A considerable amount of fluorescence could be observed also in the sarcolemma in the tongue and quadriceps.

These phenomena were more marked in heart than in tongue, and more in tongue than in quadriceps. No difference could be observed between the sections kept in potassium or calcium chloride at room temperature and those kept dry at room temperature, or between sections kept in potassium or calcium chloride at +60°C and those kept dry at +60°C. Sections kept in either sodium or calcium chloride at room temperature did not stain with Sudan Black B; when they were kept in the same solutions, but at +60°C, there was a faint reaction. With acid haematein no staining took place when sodium or potassium chloride were used, at either room temperature or at +60°C. However, when sections were kept in calcium chloride either at room temperature or at +60°C the intensity of the response to stain was comparable to that of the controls.

When sections treated as described above were extracted with acidified methanol: chloroform they did not stain with any of the methods employed.

## Discussion

These data show that muscle cells contain a lipid fraction which cannot be stained by the usual methods, without pretreatment. After a prolonged exposure to temperatures between +18°C and +20°C this fraction can be stained by both 3.4-benzpyrene, a general lipid stain, and by acid haematein, a dye specific for phospholipids (Baker, 1946). An increase of temperature to +60°C increases the intensity of the staining.

The localization of the stain as a band pattern raises the problem of the nature of these bands. Since the frozen sections used in the experiments described here were of necessity rather thick, their phase contrast image yielded a distorted band pattern, which made identification in these optical conditions unreliable. Attempts to identify the bands by their size have shown that this varies considerably according to the staining technique used. When acid haematein was used, the length of the stained bands was between 0.8 μ and 1.0 μ, and the length of the sarcomere about 1.7 μ. When 3.4-benzpyrene was used, the stained bands measured about 1.2 μ and the sarcomere 2.5 μ to 3.0 μ. This was the same size as that of the sarcomere measured in isolated myofibrils kept at +60°C for 18 hours and examined by phase contrast, although during examination with fluorescence microscopy the sarcomere in the isolated myofibrils appeared longer. It is well-known that, due to the optical conditions during observation of fluorescent specimens, these appear larger than when observed in phase contrast or in bright field (Nairn, 1964).

Fig. 11. Frozen section of quadriceps muscle stained with 3.4-benzpyrene after 18 hours at room temperature. No control is presented since they were totally negative. The band pattern is very distinct. The myelin sheath of a nerve (bottom) fluoresces brightly. × 400

Fig. 12. Frozen section of quadriceps muscle stained with acid haematein after 18 hours at room temperature, showing a distinct band pattern. Controls were negative. × 1300

Thus, from the data presented in this paper it may be concluded that the muscle fibres of the mouse contain a lipid fraction which is unmasked by heat and is probably a phospholipid, since it is stained by acid haematein as well as with 3.4-benzpyrene. This fraction appears to be present in bands which alternate with unstained bands. The identification of these bands has not been possible. If they were A bands, the hypothesis could be advanced that the lipid fraction described in this paper may represent the lipid moiety of calcium-activated adenosintriphosphatase (myosin), as suggested by FORTE et al. (1966). In this respect, the failure of ionic solutions at both room temperature (+20°C. ca..) and +60°C to unmask this fraction could be due to the fact that these ions partly extract myosin (calcium-activated adenosintriphosphatase) from muscle fibres (HUXLEY, 1960). If, as it appears more likely from the observations on isolated myofibrils (see Figs. 8 and 9), this fraction is present in the I bands, this does not exclude its possible participation in the formation of actomyosin and hence its role in muscular contraction. The intensity of the stain obtained after the application of unmasking procedures suggests that, whatever the localization of this lipid fraction, it represents quantitatively an important part of the structure of the bands.

A second masked lipid fraction is present in the sarcolemma of the muscle fibres. This fraction is unmasked by sodium ions but not by potassium or calcium ions, and is unlikely to be a phospholipid since it is not stained by acid haematein. It is possible that this fraction may be similar to that described by FEYRTER (1950) in various tissues. A support to this hypothesis comes from the data of DOERR (1952) who found a lipoprotein component to be present in human hearts at sites corresponding to those described in this paper. According to the German authors this fraction is stained by the aqueous thionin-tartaric acid method (FEYRTER, 1950; DOERR, 1952). The fact that a material of this sort is present in a membrane structure — the sarcolemma — raises the problem of the composition of the membranes in the muscle cells. That the unmasking is effected by sodium but not by potassium or calcium, suggests that an action on the polarity of the lipid components of the membrane may be involved.

The authors wish to express their gratitude to Professor JEAN HANSON for her kind advice in discussing this manuscript and for having suggested some of the experiments described here, and to Dr. L. M. FRANKS for helpful discussion.

## References

BAKER, J. R.: The histochemical recognition of lipine. Quart. J. micr. Sci. **87**, 441—470 (1946).

BERG, N. O.: A histological study of masked lipids. Stainability, distribution and functional variations. Acta path. microbiol. scand., Suppl. **40**, 1—192 (1951).

CHAYEN, J., L. BITENSKY, and C. LONG: The acid haematein method and the detection of masked lipids. Abstr. 2nd. Int. Congress of Histo-and Cytochemistry, p. 148. Berlin-Göttingen-Heidelberg: Springer 1964.

DOERR, W.: Die „basophile (mukoide) Degeneration" des Herzmuskels. Z. Kreisl.-Forsch. **41**, 42—49 (1952).

FEYRTER, F.: Über die Ursachen der besonderen Erscheinungen bei der sogenannten Einschlußfärbung (in einem wäßrigen Weinsteinsäure-Thionin-Gemisch). Z. mikr.-anat. Forsch. **55**, 543—553 (1950).

FORTE, J. G., G. M. FORTE, and R. F. BILS: Phospholipid involvement in gastric microsomal ATPase. Exp. Cell Res. **42**, 662—672 (1966).

HUXLEY, H. E.: Muscle cells. In: The cell, vol. IV, p. 365—481. New York and London: Acad. Press 1960.

MAGGI, V., et W. BRANDER: Contribution à l'étude de la nature des lipides liés de la paroi artérielle. I. Histochimie. Acta histochem. (Jena) **20**, 166—173 (1965).

NAIRN, R. C.: Fluorescence microscopy and photomicrography. In: Fluorescent protein tracing, 2nd. Ed. (ed. R. C. NAIRN), p. 73. Edinburgh and London: E. & S. Livingstone Ltd. 1964.

VIVIANE MAGGI, M. D. Ph. D.
Imperial Cancer Research Fund
Lincoln's Inn Fields
London, W.C. 2, Great Britain