Attention has recently been focused on the appearance of myofilaments in freeze-fractured and freeze-etched replicas (1, 2). From these studies it is apparent that cross fracturing of unfixed myofibrils always reveals thick (myosin) filaments as projections in both etched and unetched preparations. Furthermore, in an oblique cross fracture, there is a very striking change in appearance on crossing the M line: the broken-off ends of the myosin filaments extend above the fracture face to a much greater extent on one side (the M+ zone) than on the other (the M- zone) (1, 2, 3). It has been suggested that this difference, termed the M+/M- phenomenon (1), is dependent on the molecular polarity of the thick filaments (2). Using a complementary replica technique, we have investigated some of the factors involved in determining the freeze-fractured appearance of these filaments.

**MATERIALS**

Samples of flight muscle were taken from the thorax of the Puriri moth (*Aenetus virescens* Walk.). There were two methods of treatment: (a) storage for an extended period at -20°C in a solution containing 50% glycerol, 100 mM KCl, and 20 mM phosphate buffer, or (b) fixation for 1 hr at room temperature in 2.5% phosphate-buffered glutaraldehyde (4), followed by storage in glycerol. For preparation of complementary (“double”) replicas, samples of glycerinated muscle in bundles of up to three fibers were placed in a Chalcroft-Bullivant double-fracture holder (5) and frozen in Freon 12 in the manner previously described (5, 6). The specimen was snapped under liquid nitrogen and carried over to the evaporator in a type II Bullivant and Ames freeze-fracturing device (6-8). Pt/C shadowing and carbon backing were done with the specimen temperature below -140°C, and hence etching did not take place. Complementary pairs of replicas were cleaned and mounted as previously described (5).

**RESULTS**

Oblique fractures of unfixed glycerinated muscle (Figs. 1, 3 a, 3 b) reveal two different appearances of the thick filaments: short projections (the M- zone) and long projections (the M+ zone). Z levels are seen as bands of closely packed minor projections within a broader featureless zone, the I band (Fig. 1). The differentiation of thick-filament profiles occurs midway between successive Z levels, that is, across the M line (Fig. 1). It follows that there is a similar (but reversed) differentiation of M+/M- filament profiles across the I bands (Fig. 1). As sarcomeres tend to occur in register in individual fibers of flight muscle (9, 10), this description fits both individual myofibrils and groups of myofibrils.

In unfixed muscle at higher magnification complementary pairs of replicas (Figs. 3 a, 3 b) reveal an exact matching of projection to projection. In almost all cases a long projection in the M+ zone of one replica corresponds to a short projection in the M- zone of the other replica. There are some cases of nonconformity where short projections occurring in the M+ zone are matched by long projections in the corresponding M- zone. Quite frequently, a small groove adjoins one of the short projections in the M- zone (Figs. 3 a, 3 b). Only in a very few cases is there a depression or groove not accompanied by a projection (Fig. 3 b).

Oblique fractures of fixed muscle (Figs. 2, 4 a, 4 b) do not show the M+/M- phenomenon. The broken-off, thick filament projections appear the same all the way across the A band, and are about equal in size to the short M- Zone projections of unfixed muscle. A complementary pair of replicas (Figs. 4 a, 4 b) shows that after fixation there is almost equal breaking, but with some random fluctuation. As in the unfixed muscle, there is an exact matching of projection to projection.

The results described are not special features of the particular muscle or preparation schedule employed. We have looked at other insect flight muscle and at various vertebrate striated muscles. In all cases unfixed glycerinated muscle showed the M+/M- phenomenon, while glutaraldehyde-fixed glycerinated muscle did not.
DISCUSSION

The lack of depressions in the M – region which could correspond to projections in the M + region was noted in an earlier publication (2), and it was suggested that a degree of plastic extension or deformation might have occurred in the thick filaments, in which case depressions need not be expected. The present finding of the precise matching of projection for projection in the complementary replicas confirms this suggestion. The short grooves adjoining projections, particularly on the M – face (Figs. 3 a, 3 b), are due to the pulling of filaments out of the frozen matrix. Similar grooves were occasionally seen in Lethocerus flight muscle (1). They would be expected on one face of an oblique fracture. Their presence does not argue against plastic deformation having occurred, for matching projections are still almost always found on both complementary replicas even where there is a groove.

The thick filament is composed of myosin molecules, each with a globular head portion plus a short rodlike region comprising heavy meromyosin (HMM) and a long rodlike tail portion of light meromyosin (LMM). These molecules are arranged in such a way that the heads are always directed away from the midpoint or M region of the complete filament (11).

Since there are matching projections, it is evident that the deformation zone must extend some distance on either side of the fracture plane. By plastic deformation is implied an irreversible

Figure 1. Oblique fracture of unfixed Charagia flight muscle, showing four myofibrils, each demonstrating the M+/M– phenomenon. A, A band; I, I band; M, M line region; Z, Z line region. In all micrographs an encircled arrow indicates the direction of platinum shadowing. Scale line = 1 µ. X 17,400.

Figure 2. Oblique fracture of glutaraldehyde-fixed Charagia flight muscle. The M+/M– phenomenon is not seen. The dense band above the Z line (Z) is an undigested tracheole attached to the underside of the replica. A, A band; I, I band; M, M line region. Scale line = 1 µ. X 21,600.
Figures 3a and 3b. Complementary pair of replicas showing oblique fracture of a single myofibril of unfixed flight muscle. The complementary replicas were always photographed in the electron microscope with one grid inverted with respect to the other. Thus the micrographs do not appear as mirror images of each other; and the complementarity is easy to see directly. Long projections in the M+ region of one replica are exactly matched by short projections in the M− region of the other replica. A matching reference point is indicated by an asterisk. The three arrowheads indicate filaments which have fractured contrary to the general trend, with longer projections in the M− zone. In fig. 3a, in the M− zone, a number of short projections accompanied by a small groove can be seen. Two of these are circled. In Fig. 3b, in the M− zone, a groove without a projection is arrowed. The dense horizontal band in Fig. 3b is a fold in the replica. M, M line region. Scale line = 0.5 μ. × 70,000.
change in the organization of the myosin filament, which may involve (a) shearing of intermolecular bonds between myosin tails allowing them to slip past one another, (b) extension of myosin tails involving breakage of intramolecular bonds, and (c) slippage between the filaments and matrix. Slippage cannot be the only factor, for the filaments are obviously locally extended. In addition, glutaralde-

FIGURES 4 a and 4 b Complementary pair of replicas showing oblique fracture of a single myofibril of fixed flight muscle (same myofibril as seen in bottom left of Fig. 2). There is exact matching of filament for filament but the M+/M− phenomenon is not evident. There is some random fluctuation in the size of the filaments, and occasionally some show a subunit structure. A matching row of projections is indicated by arrowheads. An asterisk is placed midway between two matching features. Scale line = 0.5 μ. X 79,000.
hyde is a known cross-linker of proteins (4), and its
dramatic abolition of the M + /M − phenomenon
argues in favor of the breakage of inter- or intramolecular bonds being the basis of filament fracturing.

In unfixed material (Figs. 3 a, 3 b) it appears
that the balance of forces between intramolecular
and intermolecular bonds is such as to result in an
asymmetrical fracture of the extended filaments,
with a high probability of it occurring closer to the
M − surface. The nonconformist long projections
in the M − zone may be a statistical phenomenon.
However, it has been noted that they usually occur
in regions where cross bridges are readily seen (1),
and it may be that the fracturing properties of a
filament are locally modified by strong bonding
between HMM and the actin filaments.

After glutaraldehyde fixation (Figs. 4 a, 4 b) the
fracturing of thick filaments is approximately
symmetrical. As both complementary filaments of
a pair protrude above the fracture face, it is clear
that plastic deformation still occurs. The crosslinking action of the aldehyde has modified the
properties of the myosin filaments so that their
native polarity is no longer the predominant factor
in determining how they fracture.

In summary we have been able to demonstrate
that: (1) Plastic deformation does occur when thick
(myosin) filaments (either unfixed or fixed) are
freeze-fractured. (2) An asymmetrical break occurs
in unfixed material. (3) Glutaraldehyde fixation
modifies the fracturing properties of thick fila-
ments, resulting in a symmetrical break.

Our demonstration that these filaments deform
during fracture, taken along with other work show-
ing deformation of collagen and polystyrene latex
(12), may have some bearing on the understanding
of freeze fracturing of membranes. Plastic deforma-
tion of the intramembranous particles, which are
thought to be protein, would be one explanation
for the lack of small-scale complementarity be-
tween the two faces produced when a membrane is
fractured (5, 6, 13, 14).

Received for publication 24 May 1972, and in revised form 3
July 1972.

REFERENCES

1. RAYNS, D. G. 1972. J. Ultrastruct. Res. 40:103.
2. BERTAUD, W. S., D. G. RAYNS, and F. O. SIMP-
SON. 1968. Nature (Lond.). 220:381.
3. BERTAUD, W. S., D. G. RAYNS, and F. O. SIMP-
SON. 1970. J. Cell Sci. 6:537.
4. SABATINI, D. D., K. BENSCH, and R. J. BARNETT.
1963. J. Cell Biol. 17:19.
5. CHALCROFT, J. P., and S. BULLIVANT. 1970. J.
Cell Biol. 47:49.
6. BULLIVANT, S. 1969. Micron. 1:46.
7. BULLIVANT, S., and A. AMES. 1966. J. Cell Biol.
29:435.
8. BULLIVANT, S., R. S. WEINSTEIN, and K. SOMEDA.
1968. J. Cell Biol. 39(2, Pt. 2):19 a.
9. REEDY, M. K. 1967. Am. Zool. 7:465.
10. ASHURST, D. E. 1967. J. Cell Sci. 24:35.
11. HUXLEY, H. E. 1963. J. Mol. Biol. 7:281.
12. CLARK, A. W., and D. BRANTON. 1968. Z. Zell-
forsh. Mikrosk. Anat. 91:586.
13. BRANTON, D. Annu. Rev. Plant Physiol. 1969. 20:
209.
14. MEYER, H. W., and H. WINKELMANN. 1969.
Protoplasma. 68:253.