

METIN AND METACTOMYOSIN*

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Calcium chloride, if injected into the muscle fiber in minimal quantity, causes an immediate local contraction. Since this initial observation of Heilbrunn and Wiercinski,¹ there has been increasing evidence that Ca^{++} is directly involved in the contraction of actomyosin.² This is rather surprising, for the Ca ion, in general, is not known to have an affinity for hydrophilous proteins. Such an affinity could, however, be expected in colloids having numerous negative groups like SO_3 , PO_3 , or COOH . Since macromolecules carrying such groups may show metachromasia,³ it appeared worthwhile to seek for a metachromatic macromolecule in muscle.

Fresh, washed or glycerinated muscle, on staining with toluidine blue, turns purple and is thus metachromatic. Microscopically, the fibers appear to be diffusely metachromatic. After disintegrating muscle in a Waring blender, the myofibrils, separated by low-speed centrifugation, stain metachromatically, whereas the free granules and supernatant are orthochromatic. Thus, the metachromasia seems to be part of the contractile component of muscle.

If freshly minced muscle of a rabbit is repeatedly washed in cold water or extracted in 50 per cent glycerol, it retains the metachromatic property. The metachromatic substance is therefore not easily washed out of muscle. If, however, a watery suspension of the washed muscle is heated to 65°C , then the juice, separated from the particles, shows, on addition of toluidine blue, a metachromatic color while the insoluble residue colors orthochromatically. Hence, denaturation by heating releases a metachromatic substance. On adding the dye in sufficient quantity to the filtrate, a metachromatic precipitate forms; the supernatant remains blue indicating that the metachromatic material is precipitated quantitatively. The precipitate can be separated by centrifugation and the dye dissociated from the precipitated substance (see below). The dye can thus be used for the simple isolation of the metachromatic material.

This material is a protein. It gives a strong biuret reaction, and is precipitated by trichloroacetic acid and ammonium sulfate (40 per cent of saturation). It does not precipitate on boiling but does so on addition of acetic acid to the hot solution.

It can also be precipitated, even at a high dilution, isoelectrically around a pH of 5.5–5.7 (addition of NaCl shifts it to a lower pH). The isoelectric precipitation can also be utilized for isolation purposes. Molisch's test was negative.

That the protein is fibrous is indicated by its high viscosity and its double refraction of flow. Addition of 0.1 M NaCl greatly decreases the viscosity. Its aqueous solution can be lyophilized.

The protein, which redissolves at higher ionic concentrations, is precipitated by 0.01 M CaCl_2 or MgCl_2 . At certain concentrations of NaCl or KCl, Ca has a stronger precipitating action than Mg, a difference which deserves further study. That the dye and the Ca or Mg precipitate one and the same protein, quantitatively, is shown by the fact that the supernatant of the ionic precipitation gives no precipi-

tation with toluidine blue, and vice versa. When a high concentration of monovalent or divalent ions is added to the metachromatic precipitate, it redissolves and becomes orthochromatic.

That this protein may form part of the actomyosin system is suggested by the following. Actomyosin (myosin B) in suspension stains metachromatically. If the actomyosin is heated to 65°C, it denatures and precipitates, whereupon the metachromatic protein goes into solution, giving a metachromatic precipitate on addition of toluidine blue. The metachromatically stained actomyosin still superprecipitates with ATP but at the same time becomes orthochromatic. Actin (G or F, prepared without special precautions for the elimination of tropomyosin) was also found to be metachromatic. Myosin, on the other hand, did not give the metachromatic reaction.

On the basis of its metachromatic property the protein described will be called "metin," while its tertiary complex with actomyosin may be termed "metactomyosin."

Metin has several properties in common with tropomyosin⁴ and has also properties common with Amberson's delta protein.⁵ Further studies are needed for its characterization.

Extraction Method.—Metin can be prepared from the muscles of a freshly killed rabbit. The skinned and eviscerated rabbit is briefly cooled on ice, its muscles excised and minced on a precooled meat mincer. The mince, say 500 gm, is suspended in 1500 ml of 0°C distilled water, gently shaken for several hr at 0°C, and then filtered through cheesecloth. The extracted muscle is pressed out by hand, suspended once more in 1500 ml of ice-cold water and, after shaking for several hours, is again separated using cheesecloth as before. The muscle is then suspended in 1000 ml of an aqueous 0.05 M NaCl solution, and rapidly heated to 65°C while stirring vigorously. The suspension is then cooled on ice, again passed through a cheesecloth, and then centrifuged. It usually gives a limpid supernate. If no NaCl is added, the filtrate is turbid. If the muscle is washed insufficiently and the bivalent ions are not eliminated, the metin extracts poorly or not at all.

Metin can be precipitated from the extract in various ways.

Precipitation by toluidine blue: The pH is adjusted to 7, and a 1 per cent watery solution of toluidine blue is added⁶ till the supernate of a small sample is intensely blue and gives no precipitate on addition of more dye. This point is usually reached with the addition of about 0.1 per cent dye. The precipitate is separated by centrifugation, washed with about 200 ml of water, and centrifuged again. Then the precipitate is dissolved in about 100 ml of a 20 per cent acetone containing 0.2–0.4 per cent acetic acid. A homogenizer can be used with advantage. The dilute acetone-acetic acid solution dissolves and dissociates the precipitate into protein and the free blue dye. The latter can be eliminated in various ways. It can be eliminated by adding powdered charcoal (Norit-A) under strong mixing. Since the Norit does not bind the dye quantitatively, it is advantageous to add it only until the fluid is discolored about halfway, then filter through a thin sheet of celite, and repeat the charcoal treatment until practically all the dye is removed. The same can be achieved by dialyzing the solution against the same solvent with liberal amounts of charcoal suspended in the outer fluid.

The metin can be precipitated from the discolored solution by adjusting the pH

to 5-5.7 and centrifuging out the isoelectrically precipitated protein, which readily redissolves on neutralization.

Dr. Eloise E. Clark, to whom our thanks are due, examined such a preparation in the ultracentrifuge and obtained a peak with a Gaussian distribution ($S_{20,w} = 2.85 \times 10^{-13}$; 4.7 mg/ml, 0.1 M NaCl). On prolonged spinning, a minute quantity of a slightly faster-sedimenting fraction ($S_{20,w} = 3.6 \times 10^{-13}$) was observed.

The greatest amount of the dye can also be eliminated by precipitating the protein solution from the acetone-acetic acid by the addition of 4 volumes of acetone which brings down the metin in the form of a voluminous precipitate. The solvent is decanted and the voluminous precipitate can be made to collapse by pressing it out with a spatula. The precipitated metin can now be slowly dissolved in neutral or slightly alkaline water. Again, homogenization may be useful. By adding butanol, remnants of the dye may be shaken out of the watery solution.

Our preparation, at this stage, seems to contain traces of nucleotides present as impurity, giving a weak absorption around 270 μ M and a weak reaction for phosphate. (A sample studied contained 4.8 mg of protein and 1 μ g of P per ml.)

Similar to other metachromatic substances, solutions of metin first color metachromatically on the addition of small quantities of dye and then precipitate only after the dye concentration approaches equimolarity with its binding sites. Approximately one dye molecule is bound by ten amino acids of the metin.

Isoelectric precipitation: Metin can also be precipitated from the primary muscle extract isoelectrically. The NaCl present shifts the isoelectric point to a lower pH. Complete precipitation is obtained in presence of 0.05 M NaCl at pH 4.5-5. The precipitate is separated by centrifugation and redissolved by neutralizing the suspension. The protein thus prepared is precipitated quantitatively by toluidine blue, indicating that it is identical with the protein obtained by the dye methods.

Precipitation by bivalent ions: From a salt-free aqueous solution, the metin can also be precipitated quantitatively by 0.01 M CaCl_2 or MgCl_2 . This precipitation is prevented by 0.05 M NaCl. If the extraction of muscle was made in the absence of NaCl, the Ca precipitate co-precipitates the substance which has caused the turbidity of the extract. It can be separated from the latter by centrifugation after redissolution. If the solution is still turbid, it can be clarified by adding 0.05 M NaCl and 0.01 MgCl_2 , and centrifuging, the salts being subsequently eliminated by isoelectric precipitation.

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¹ Heilbrunn, L. V., and F. J. Wiercinski, *J. Cellular Comp. Physiol.*, **29**, 15, (1947).

² Weber, A., and S. Wincur, *J. Biol. Chem.*, **236**, 3198, (1961).

³ By "metachromasia" is meant the property of macromolecules to be stained by certain dyes with a color which is different from the orthochromatic color of the dye itself. Toluidine blue, for instance, at high dilution (10^{-6} M), at which it is present as a monomer in water, has an absorption at 623 $m\mu$, which corresponds to its blue orthochromatic color. At a higher concentration (10^{-5}) it forms dimers which have a purplish tint and a somewhat weaker absorption at 586. If, for example, polyethylsulphonate of MW 12,900 gm (kindly supplied by Upjohn and Company, Kalamazoo, Michigan) is added, the absorption becomes considerably lower, wider, and shifts strongly to a shorter wavelength (max 526), as also is indicated by the metachromatic purple color. There is no acceptable general theory for this change. Since dimerization shifts the absorption to a somewhat shorter wavelength, it seemed reasonable to suppose that the greater metachromatic shift is due to a further stacking of the dye molecules on the negative groups of the colloid. This theory was revived by F. Bradley, and M. K. Wolf in these PROCEEDINGS, **45**, 844 (1953). How-

ever, it is easy to show that this theory is not generally acceptable. If, for instance, a dilute toluidine blue solution is added gradually to a relatively concentrated solution of a few per cent of polyethylsulfonate, the first trace of dye added, detectable by the naked eye, is already metachromatic. It seems unreasonable to suppose that this minute quantity of dye would form stacks and not distribute itself among the great excess of SO_3 groups. Similarly, if a solution of polyethylsulfonate is gradually added to a solution of toluidine blue, the maximal metachromatic shift is obtained when there is one SO_3 group for every dye molecule. Thus, there being no generally acceptable theory for metachromasia, one may sum up the situation by saying that while the weak interaction between two dye molecules in a dimer causes a small shift and a small decrease in absorbancy, the stronger interaction between the basic dye and strongly negative SO_3 , PO_3 , or COOH groups causes a greater shift with decrease and broadening of the absorption. Though most proteins contain COOH groups they are not usually metachromatic, the number of COOH groups being low and their electrostatic action being, possibly, also compensated by the NH_2 groups present.

⁴ Bailey, K., *Biochem. J.*, **43**, 271, (1948).

⁵ White, J. I., H. B. Bensusan, S. Himmelfarb, B. E. Blankenhorn, and W. R. Amberson, *Am. J. Physiol.*, **188**, 212, (1957).

⁶ Other metachromatic dyes, like acridine orange, can equally be used.