width of about 30 kilometers, with a ring cross section of perhaps 3 to 4 kilometers in the horizontal. I am not aware that a cloud of such form and size has been observed at any level within the atmosphere before. Interesting questions about the source of the requisite water vapor are posed by its unprecedented altitude (*6*).

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6. I thank Leon Salanave for alerting me to the cloud when it became visible in the Tucson sky and for further technical assistance, and I. E. Daniels and C. E. Peterson for permission to reproduce their photographs. The cooperation of the numerous Arizonans submitting reports is gratefully acknowledged. Supported by the Office of Naval Research under contract NR 082–164.

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Potentiation by Adrenaline of a Proteolytic Activity Associated with Purified Myosin

Abstract. *Proteolytic activity accompanies myosin through three reprecipitations. The fact that this activity can be potentiated by very small doses of* l*-adrenaline supports the view that adrenaline receptors are protein in nature and that adrenaline-like compounds exert their action through modification of the activity of enzymes.*

Correlation of certain disparate experimental observations led us to examine muscle myofibrillar elements for a catheptic protease activated by adrenaline. Elevated levels of adrenaline and increased catheptic activity have been observed in dystrophic mouse muscle (*1*, *2*). Catheptic activity has been noted in isolated myofibrils (*3*), in crude myosin (*4*), in actin preparations (*5*), and in a KCl extractable fraction of muscle (*6*). In physiological experiments, adrenaline has increased the amino acid levels in plasma of eviscerated rats (*7*) and under other circumstances has increased the output of urinary nonprotein nitrogen (*8*).

Preliminary experimentation revealed proteolytic activity in crude preparations of rabbit myosin that could be stimulated by adrenaline. We purified the myosin in these preparations by repeated reprecipitation, according to procedures described by Mommaerts (*9*), and studied the effect of *l*-adrenaline and *d*-adrenaline on this activity after the third cycle. Thus, crude myosin was separated from whole muscle homogenate by extraction with cold Weber's solution and was subjected to three subsequent cyclings involving selective solubilization and precipitation via time- and temperature-controlled manipulation of ionic strength (*9*). Each myosin fraction manifested proteolytic activity at *p*H 3.5, 5.0, and 6.0; however, we are reporting only on the work carried out at *p*H 3.5, thus delimiting the protease examined as a cathepsin. The enzyme assay employed 0.4*M* acetate buffer, *p*H 3.5, and hemoglobin as substrate. The concentration of adrenaline varied from 10 to 10^{-8} μg/ml. Increase in free tyrosine during incubation at 35°C served to define proteolytic activity. Incubation times varied from 5 minutes to 2 hours. Tyrosine was measured by fluorimetry after it had been conjugated with nitrosonaphthol and had formed the yellow fluorophore (*10*). This method permits separation of tyrosine from other compounds including adrenaline which fluoresce in the ultraviolet and allows data to be obtained on the relationship between concentration and fluorescence intensity at tyrosine concentrations too dilute for assay by traditional ultraviolet or visible absorption methods.

Release of tyrosine linear with time occurred with the crude myosin fraction. In five of seven experiments with 2 to 10 μg of adrenaline per milliliter enhancement was observed as a change in slope of this line, the increase lying between 40 and 200 percent. On the other hand, 5 μg/ml of adrenochrome did not cause potentiation. In two of seven experiments the killing of the rabbit was not rapid. In extracts prepared from these animals the endogenous cathepsin was initially high and adrenaline did not increase the activity. This phenomenon was occasionally observed in the purified fractions as well.

In Figs. 1 and 2 we have charted the results of some of our studies. Tubes were incubated for 60 minutes and analyzed in duplicate; trichloroacetic acid was employed to stop the reaction. All incubations were run at

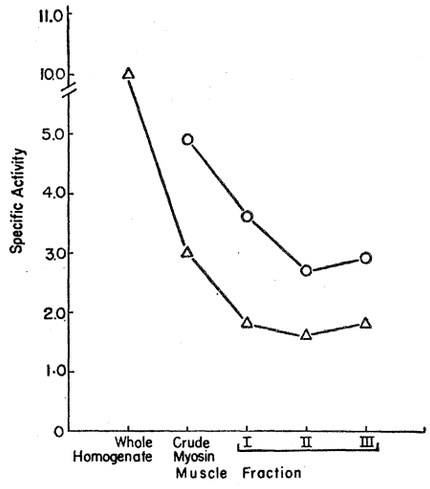

Fig. 1. Change in specific catheptic activity (micrograms of tyrosine released per milligram of protein in a 60-minute incubation) with separation and progressive purification of the muscle myosin fraction. Incubations were performed at two concentrations of enzyme protein. Triangles: 1.1 mg of protein per milliliter; circles: 0.59 mg of protein per milliliter.

one of two constant enzyme concentrations, 1:5 dilutions of two stocks containing 1.1 and 0.59 mg of protein per milliliter.

The work of Nardone (*4*) suggested the association of a proteolytic activity with myosin. Our preparations had an activity and solubility minimum in preliminary studies at about *p*H 5.0 which is close to the isoelectric point of myosin. Our methods, though they produce physical crystallinity, fall short of producing more than 90 percent homogeneity of the proteins by electrophoretic analysis (*9*). Thus, whether the enzyme activity described represents a function intrinsic to myosin or that of a contaminant molecule cannot be settled now.

What may be the meaning of the initial loss in specific activity we ob-

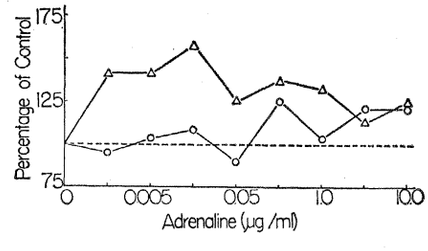

Fig. 2. Effect of *l*-adrenaline (triangles) and *d*-adrenaline (circles) on the catheptic activity of myosin fraction reprecipitated three times, expressed as percentage of control.

served during purification (Fig. 1)? Proteolytic activity has been associated with muscle fractions other than myosin; thus, we may be dealing in the initial purifications with a separation of several proteases of differing specific activity, that of myosin being the weaker. Preliminary evidence for this separation of activities has already been gathered.

Dilution of the enzyme increased the specific activity at all stages of purification, an increase which suggests (Fig. 1) the persistent presence of a precursor, inhibitor, or an enzyme-enzyme masking interaction that reduces the activity of the preparation.

We have examined the effects of diverse small molecules on the activity of the third reprecipitation fraction. Thus, $10^{-3}M$ cysteine, magnesium ion, and ferrous ammonium ions all potentiate its activity. The positive effect of cysteine allows us to distinguish the activity we observe from the muscle cathepsin extractable with KCl which Snoke and Neurath examined (*6*). Cupric ions do not inhibit our enzyme at concentrations reported to inhibit the catheptic activity associated with actin and described by Drabikowski (*5*).

The most notable effect is the potentiation obtained from *l*-adrenaline at physiological and lower than physiological dose levels (Fig. 2). Although equal to *l*-adrenaline in its potentiating effect at concentrations above 1 μg/ml, *d*-adrenaline is inactive in the lower dose range. This relative insensitivity of the *d*-form parallels the reduced effect that the *d*-form of adenaline exerts on physiological systems in vivo (*11*). The muscle catheptic enzyme under investigation, inasmuch as it is as sensitive to adrenaline as the natural receptor system, may itself be an adrenaline receptor in vivo, a phenomenon possibly related to the powerful effect exerted by adrenaline on the mechanisms of muscle contraction.

The unusual multiple activity peaks observed for the dose-response curve of adrenaline deserve comment. They are reproducible and depend for their specific relation to dose on the length of time employed for the prior triple reprecipitation (*12*).

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Tarichatoxin: Isolation and Purification

Abstract. *The potent neurotoxin occurring in the embryos of the California newt,* Taricha torosa, *has been obtained in crystalline form. With a lethal subcutaneous dose of approximately 0.14 micrograms for a 20-gram mouse, it is, along with saxitoxin and tetrodotoxin, one of the most toxic nonprotein substances known.*

In connection with transplantation experiments, Twitty and Johnson (*1*) in 1934 discovered that the eggs and embryos of the California newt *Taricha torosa* (formerly *Triturus torosus*) carried a toxic substance which, upon injection into other species of newts and salamanders, caused paralysis. This substance caused death when injected into other amphibians and mammals. The purification of this neurotoxin was undertaken in 1940 by Horsburgh, Tatum, and Hall (*2*), and in 1942 by van Wagtendonk, Fuhrman, Tatum, and Field (*3*) who purified it so that it had a toxicity of approximately 70 mouse units (*2*) per milligram—that is, 1/70 mg would kill a 20-gram mouse in 10 minutes when injected subcutaneously. Chemical work established that this preparation was dialyzable, soluble in water, soluble in methanol and ethanol, insoluble in other organic solvents, and unstable in strong acid and base. Its mode of action has been studied by Fuhrman *et al.* (*4*).

We have renewed the chemical investigation of this toxin, which we call tarichatoxin, and have isolated a crystalline material with an activity of approximately 7000 mouse units per milligram. By comparison, the previous preparation (*2*) was only about 1 percent toxin. Thus, tarichatoxin ranks with saxitoxin (*5*) from shellfish and tetrodotoxin (*6*) from the Japanese Fugu as one of the most toxic nonprotein substances known.

Purification resulted from the following: *Taricha torosa* eggs, 95 liters, collected (*7*) during the spawning season in the vicinity of Stanford University, were ground with 47 liters of water, and 1.2 kg of sodium chloride in 1-gallon batches in a Waring blender. This homogenate was treated with 40 percent of its volume of acetone; the coagulated gelatinous solid which floated to the top was filtered through a mat of glass wool and washed with methanol. The extracted solids, which contained only a few percent of the original toxic activity, were discarded. The filtrate and washings were concentrated to 2 to 3 liters and then dialyzed against three successive 10-liter portions of distilled water. The dialysates were evaporated and extracted with methanol to give 113 g of material with an activity of 10 to 20 mouse units per milligram. This crude toxin was stirred with 200 ml of methanol; 18 g of almost inactive material, which did not dissolve, was removed by centrifugation. The soluble portion was deposited by evaporation on an equal weight of silicic acid and chromatographed in three batches on acid-washed silicic acid (Mallinckrodt) which had been exhaustively washed with distilled water and dried at 120°C for 24 hours. The material was chromatographed with chloroform successively enriched with methanol. The most active fractions, after evaporation, gave material of activity about 1000 mouse units per milligram. During preparative electrophoresis the toxin moved toward the anode with 0.5 percent acetic acid as solvent and electrolyte. After solvent evaporation, this gave 537 mg of material with activity of approximately 3000 mouse units per milligram.

Purification was also followed by thin-layer chromatography on Merck silica gel G with 4 percent acetic acid in absolute ethanol as solvent. The spots could be developed with ceric sulfate reagent or by spraying with 10 percent potassium hydroxide in methanol followed by heating at 120°C for 15 minutes; this procedure gave yellow