THE PURIFICATION OF 5'-ADENYLIC ACID DEAMINASE FROM FROZEN RABBIT SKELETAL MUSCLE

Thesis for the Degree of M. S.

MICHIGAN STATE UNIVERSITY

Carl L. Winely

1965

ABSTRACT

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by Carl L. Winely

In 1957 Ya Pin Lee reported crystalization of 5'-adenylic acid deaminase from rabbit skeletal muscle. Though the specific activity of Lee's purified enzyme (17.24 uM AMP converted per minute per mg of protein) was superior to earlier isolations (Schmidt, 1928; Kalckar, 1947; Nikiforuk & Colowick, 1956) the final recovery was only 5 per cent. Consequently, the goal of this study was to isolate deaminase of similar purity in greater yield.

Since skeletal muscle myosin is complexed with deaminase (Ferdman and Nechiporenko, 1946; Hermann and Josepovits, 1949), a myosin extraction was made from frozen rabbit muscle. The complexed deaminase was obtained by low salt precipitation after actomyosin was removed. Myosin was denatured by heating at 54°C for 4 minutes, and nucleic acid was precipitated by protamine sulfate.

The deaminase activity of all fractions was determined by the method of Kalckar (1947). The protein concentrations were calculated from measured absorbances at 260 mu and 280 mu by the method of Warburg and Christian (1941).

The isolated enzyme converted 17.5 uM AMP/min/mg of protein. The recovery was 32 per cent and the amount of deami-

nase obtained per gram of rabbit tissue was 34 times greater than reported by Lee (1957).

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Biochemistry

1965

ACKNOWLEDGEMENTS

The author is grateful to Dr. C. H. Suelter and Dr. E. W. Byrnes for guidance and assistance throughout the course of this work.

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INTRODUCTION

Muscle 5'adenylic acid deaminase (AMP deaminase) is complexed with myosin (Ferdman, 1946, and Hermann, 1949). Though the physiological function of the enzyme has not been elucidated as yet, other indirect evidence also suggests that the enzyme might be related to muscular contraction. In muscular atrophy, the amount of deaminase which is complexed with myosin declines, and the quantity of deaminase found in aqueous extracts increases (Mechiporenko and Ferdman, 1953). Muscular dystrophy in mice was also shown to influence the amount of deaminase present. In comparing the muscle extracts of the affected animals with those of the controls, Pennington (1961) found that the affected possessed only one third of the mean activity of the normal animals.

To propose a specific function for deaminase in contraction is difficult, but it seems that an influence upon myosin via myokinase might be possible. The results of Cain, Infante, and Davies (1962) show that when work is done by rectus abdominis muscles, ATP is used by ATPase during single contractions:

(1) $ATP \xrightarrow[\text{Myosin}]{\text{ATPase}} ADP + \text{inorganic phosphate} + \text{work}$

This ATP is rapidly reconstituted by the action of creatine phosphoryltransferase and/or myokinase, while the contraction is taking place.

(2) $PCP + ADP \xrightarrow{\text{creatine phosphoryltransferase}} ATP + \text{creatine}$

(3) $2\ ADP \xrightleftharpoons{\text{myokinase}} ATP + AMP$

Myokinase activity is important because by cyclic regeneration of ATP from ADP, free energy is in effect utilized from the breakdown of both the labile phosphates of the ATP. That is, ADP, Pi, and energy are the products of reaction (1); two moles of ADP are then converted to one mole of ATP and AMP by reaction (3); the second labile phosphate of an original mole of ATP is then utilized by reaction (1). The myokinase reaction is also important in that it maintains low levels of ADP, which is an inhibitor of myosin ATPase. Since a value of 1.2 has been reported by Bowen (1956) for the equilibrium constant of the myokinase reaction, high concentrations of AMP would tend to maintain a high steady state concentration of ADP. However, if 5'-adenylic acid deaminase irreversibly deaminates the AMP, the myokinase reaction would then favor formation of ATP.

Another possible role of 5'-adenylic deaminase is the control of phosphorylase b. Glycogen, in the presence of phosphate, is converted to glucose-1-phosphate by polysaccharide phosphorylase. This enzyme has been found in two forms in muscle; phosphorylase a and phosphorylase b (Green and Cori, 1943). AMP is a necessary cofactor for phosphorylase b with a concentration of 3×10^{-5} M required for half-maximal activity (Fisher and Krebs, 1958). Though phosphorylase a is active in the absence of AMP, small concentrations (2×10^{-6} M) do exert a stimulatory effect (Cori, Cori, and Green, 1943). Contrary to this, IMP will not activate phosphorylase a or phosphorylase b; therefore, 5'-adenylic acid deaminase might

function in the control of phosphorylase by regulating the amount of available AMP.

It is apparent from the above that 5'-adenylic deaminase may be extremely important in muscular contraction, glycogen breakdown, or some other process which is dependent upon a given level of AMP. Consequently, a complete characterization of the in vivo processes of the enzyme, including the catalytic mechanism of action, is desirable. In order to perform this characterization, the enzyme must be available in highly purified form. A purification procedure for seemingly "crystalline deaminase" from rabbit muscle does exist (Lee, 1957), but with only a 5 per cent recovery; therefore, a procedure which results in a greater yield of the enzyme is necessary. For this reason, an attempt was made by the author to develop such a process.

HISTORICAL

Muscle adenylic acid (5'-adenosine monophosphate) was at first thought to be identical with yeast adenylic acid (3'-adenosine monophosphate). However, Embden and Schmidt (1929) proved that the two acids not only differ in chemical structure, but also in their behavior in muscle extracts. Though yeast adenylic acid was not deaminated by muscle extracts, muscle adenylic acid readily formed inosinic acid both biologically and also by treatment with nitrous acid. At about this time, Parnas and Mozolowski (1927) found that the low content of ammonia present in fresh muscle rapidly increased in injured muscle. Parnas (1929) showed that within two minutes after traumatic injury, the relative concentrations of purines present in muscle changed from an initial 82% adenine and 18% hypoxanthine to 77% hypoxanthine and 23% adenine. By isolation of the nucleotides, he showed that in both rigor and fatigue, muscles form inosinic acid at the expense of adenylic acid.

The enzyme responsible for this effect was shown to be AMP deaminase and was first described in 1928 by Schmidt. Using sodium carbonate extracts of saline-washed, minced muscle, he demonstrated the presence of adenosine and adenylic acid deaminase activities. Adenosine deaminase activity was removed from the preparation by adsorption with alumina gel. This procedure thus demonstrated that two different enzymes

function in the deamination of adenylic acid and adenosine. Schmidt was also able to isolate inosinic acid and ammonia as the products of adenylic acid deamination. The reaction may therefore be expressed as:

$$AMP + H_2O \longrightarrow IMP + NH_3$$

After this first isolation, AMP deaminase was sought in other tissues. Though skeletal muscle deaminase was found to be most active, the enzyme was also found in heart, smooth muscle, brain, peripheral nerve, liver, kidney, spleen, and lung (Nechiporenko, 1949; Eidel'man, 1935; Kutscher, 1948; P. Satta, 1954). In addition to showing tissue differences, the relative levels of deaminase also vary within species. A study on deaminase in skeletal muscle from various species gave the following relative levels: guinea pig 200, man 165, rabbit 123, cat 104, chicken 100, rat 70, and ox 42 (Kutscher, 1948).

The AMP deaminase found in muscle is concentrated in the myosin fraction. The only myosin reported to date which lacks deaminase activity but retains adenosinetriphosphatase activity is that of dog heart (Nechiporenko, 1953). However, after dog heart myosin is mixed with rabbit AMP deaminase prepared from an aqueous extract, the product obtained by dilution with cold water retains deaminase activity. This activity survives many reprecipitations which indicates that the original lack of deaminase in the heart myosin was due to deficiency in the native state rather than the absence of a binding site on the myosin molecule.

After Schmidt's original work, no further important purification of rabbit muscle 5'-adenylic acid deaminase occurred until approximately 20 years later. At this time Kalckar (1947) succeeded in isolating 5'-adenylic deaminase from rabbit muscle by two methods. In the first preparation, skeletal muscle was ground and extracted with three volumes of chilled water and left in an ice chest overnight. The lactate, which formed from glycogen, acidified the mixture to about pH 6, which brought about flocculation and sedimentation of the deaminase. The precipitate was extracted with 1 M ammonium acetate, pH 8, centrifuged, and subjected to ammonium sulfate fractionation. The fraction which precipitated between 0.3 and 0.5 saturation showed the highest deaminase activity.

A second procedure yielded deaminase of higher specific activity, but less total activity. A solution of myosin prepared according to Bailey (1942) was dialyzed against 0.02 M ammonium acetate, pH 8, for 5 to 6 hours. The myosin precipitate was centrifuged off and the deaminase which remained in the supernatant fluid was precipitated at pH 6 by adding succinate buffer (0.2 volume of 0.3 M pH 5.9). The precipitate was redissolved in a small volume of 0.1 M ammonium acetate, pH 8, and then subjected to ammonium sulfate fractionation. The fraction between 0.3 and 0.5 saturation was again the most active. The activity of this preparation corresponded to the deamination of 7.4×10^{-3} uM of adenylic acid per minute per mg of protein.

The assay procedure which Kalckar used deserves mention for it is more convenient than the detection of NH_3 (Schmidt,

1928). Kalckar (1947) found that the deamination of adenine to hypoxanthine caused a marked change in the absorption spectrum. At 265 mu the optical extinction decreased to approximately 40 per cent; whereas, at 240 mu the extinction increased. Since the deamination of AMP had the same changes in absorption, Kalckar was able to estimate the activity of AMP deaminase by the decrease in optical density at 265 mu of an AMP solution.

In 1949, Hermann and Josepovits observed that actin-free myosin from rabbit muscle, crystallized according to Szent-Györgyi (1943), had a high deaminase activity after three recrystallizations. They were able to show that the deaminase activity of crystalline myosin was as great or greater than that of Schmidt or Kalckar.

The protein portion of Schmidt's deaminase was then proven to contain myosin by the following evidence: (1) salt fractionation and solubility tests; (2) an increase in viscosity upon the addition of actin; and (3) a reduction in viscosity by the addition of adenosinetriphosphate. Similar observations were made with the deaminase prepared according to Kalckar.

Hermann and Josepovits (1949) concluded that adenylic deaminase was bound to the myosin fraction as strongly as adenosinetriphosphatase and that the two activities were of the same magnitude. Since they could devise no means of separating the two activities, these workers concluded that both were due to myosin and no other protein was present.

This view was generally accepted until Engelhardt et. al. (1952) succeeded in separating the two enzymes by heat fractionation. The separation was confirmed by Lyubimova and

Matlina (1954) and was later well characterized by Locker (1956).

Locker (1956) found that when the myosin solutions were coagulated at 53°, pH 6.2, 8-18 per cent of the protein remained in solution. This material was then divided by dialysis against water into two parts: (a) a myosin-like fraction, P, comprising 4-13 per cent of the myosin and containing a well defined major component P_1 as shown by electrophoresis; and (b) a soluble fraction, D, containing three electrophoretically separated components (D_1, D_2, and D_3) in the approximate proportions 4:5:1. The AMP deaminase activity of P was 3-4 times greater than that of myosin while that of D was low.

At the same time that Locker described the heat fractionation of myosin, Nikiforuk and Colowick (1956) prepared deaminase of high activity. In this procedure, the crude extraction was carried out by the method of Schmidt (1928). The enzyme was then adsorbed onto alumina C_γ and eluted with 1 M Na_2HPO_4. Saturated $(NH_4)_2SO_4$ at pH 7.6 was added. The fraction which precipitated between 0.27 and 0.45 saturation was dissolved in 0.1 M Na_2HPO_4. The enzyme was further purified by the paper chromatography salting out procedure of Mitchell (1949). The specific activity of the purified fraction was 1.13 uM AMP converted per min per mg of protein; therefore, the enzyme was about 135 times as active as that of Kalckar (7.4×10^{-3} uM converted/min/mg). However, the total recovery of Nikiforuk's and Colowick's procedure was only 2.1 per cent.

After the heat treatment by Locker (1956), several puri-

fication steps were well characterized:

(1) Precipitation of myosin-deaminase solutions by dilution with water

(2) Heat fractionation

(3) Ammonium sulfate fractionation

Ya-Pin Lee (1957) employed these techniques and reported the isolation of "crystalline" deaminase. However, since the overall yield obtained was only 5 per cent, this problem was undertaken to obtain enzyme of high activity in greater yield. For comparative purposes, the procedure of Lee is listed in detail below:

Original Extraction:

Fresh rabbit muscle was homogenized for 1 minute in a Waring blender with 3.5 volumes of a solution containing 0.3 M KCl, 0.09 M KH_2PO_4, and 0.06 M K_2HPO_4 at pH 6.5. After extracting the deaminase by stirring at 3° C for 1 hour, the residue was removed by centrifugation at 1500 x g for 30 minutes. The residue was then reextracted by stirring an additional hour in two volumes of the same buffer. After again centrifuging, the combined supernatant liquid was passed through two layers of cheesecloth to remove the lipid layer.

Low Salt Fractionation:

The combined extract was diluted with 9 volumes of chilled water with stirring over 15 minutes at 3° and then stirred an additional 10 minutes. The suspension was centrifuged (Sharples) or allowed to stand at 3° overnight and the supernatant then aspirated and the precipitate obtained by centrifugation. The

precipitate was dissolved in 0.5 M KCl and the protein concentration adjusted to 15 mg/ml.

Heat Fractionation:

The solution was brought to 0.02 M $MgCl_2$ by the addition of 1 M $MgCl_2$ and the pH was adjusted to 6.8 with 1 M K_2HPO_4. 1000 ml portions of this solution were heated in a 2 liter stainless steel beaker at $50^\circ \pm 1^\circ$ for 2 minutes. The solution was quickly cooled to 3° and the denatured protein separated by centrifugation. The precipitate was suspended in 0.5 M KCl and heated to 45°. The coagulated elastic protein was quickly filtered through one layer of cheesecloth. The supernatant fluid and the filtrate were combined.

Ethanol Fractionation:

The pH of the solution was adjusted to 6.5 with 0.5 N acetic acid and chilled to -2° in a -10° bath. 95 per cent ethanol was added to a concentration of 7 per cent (v/v). The suspension was centrifuged and the supernatant was filtered through a thin layer of Celite on a Buchner funnel. The precipitate was again centrifuged. The filtrate and supernatant liquid were combined and brought to 23 per cent ethanol (v/v) maintaining a temperature of -5° through out the addition. The temperature was then lowered to -10° C and the precipitate was obtained by centrifugation at -10° C. The precipitate was dissolved in 0.5 M KCl to a protein concentration of 5 mg/ml.

Ammonium Sulfate Fractionation:

Most of the deaminase activity was obtained between 1.26 and 2.26 M ammonium sulfate by the addition of solid ammonium sulfate at pH 6.5 at 3°. The precipitate, collected by centrifugation, was dissolved in 0.5 M KCl to give a protein concentration of about 5 mg/ml.

Low Salt Fractionation:

The above fraction was adjusted to pH 6.5 and dialyzed against 10 volumes of 0.02 M KCl solution with stirring at 3° for 8 hours. The precipitate, was dissolved in 0.5 M KCl to give a protein concentration of 5 mg/ml.

Calcium Phosphate Gel Fractionation:

Two ml of calcium phosphate gel (20 mg of dry weight per ml), prepared by the method of Keilin and Hartree, was added to each 10 ml of the low salt fraction. After adjusting the pH to 6.5 with 0.5 N acetic acid, the preparation was stirred for 30 minutes at 3° before centrifugation. If all of the enzyme was not absorbed, successive small amounts of Ca phosphate gel (0.3 ml of gel suspension per 10 ml of initial solution) were added and collected by centrifugation. Each of the gel fractions was washed individually with 0.3 M KCl solution and eluted twice with 0.08 M K_2HPO_4 pH 8.5 (half volume of the gel suspension which was added), at 3° for 2 hours in each elution. The combined gel residues were eluted with 0.1 M K_2HPO_4, pH 8.5, solution overnight and the eluate was saved.

Crystalline Deaminase:

The eluates of high specific activity (more than 4000 units per mg) were collected and the pH was adjusted to 8.0. The solution was chilled to -3° in a -10° bath and 0.15 volume of 95 per cent ethanol was added slowly with mild stirring. After the temperature dropped to -8°, the precipitate was collected. A small amount of 0.5 M KCl was added to make a saturated solution at room temperature. This clear viscous solution was cooled very slowly with mild stirring. The crystals appeared during the drop in temperature.

TABLE I

Results of Lee (1957)

3 kilos of rabbit skeletal muscle.

Fraction No.		Total protein (mg)	Total* units	Units* per mg protein	Purification	Yield
1	Muscle extract	340,000	6,320	.0186	1	(100)
2	Low salt ppt.	168,000	5,670	.0338	1.8	90
3	Heat-treated fraction	49,500	4,180	.0845	4.5	66
4	Ethanol fraction	3,960	2,100	.531	29	34
5	Ammonium sulfate fraction	1,320	1,605	1.217	65.5	25.5
6	Low salt fraction	850	1,580	1.859	100	24
7	Ca phosphate gel eluate	120	914	7.605	410	14.5
8	Crystals (1st crop only)	18	312	17.24	920	5

* The units used by Lee (1957) were converted to uM ATP converted per min.

After Lee (1957) reported the purification of deaminase, Locker (1959) prepared deaminase from myosin by a more simple and rapid method. The activity of his preparation (23°) was equivalent to 2.1 uM AMP converted/min/mg protein. This is slightly greater than the activity of Lee's enzyme after the second low salt fractionation.

Locker's method consisted of two heat fractionations at 53°, pH 6.2. As mentioned previously, after the first heat fractionation a myosin-like precipitate, P, was obtained when the soluble fraction was dialyzed against water. A second heat coagulation was then carried out on the dissolved P fraction in the presence of 0.02 M tripolyphosphate, giving a strong displacement of activity into the soluble fraction obtained by dialysis against water (D_2 fraction). It was this D_2 fraction which had an activity of 230,000 ul NH_3/mg protein/h (2.1 uM AMP/mg protein). The corresponding P_2 fraction had a fifth of this activity.

Currie and Webster (1962) attempted to dissociate the actomyosin-deaminase without using a heat treatment. They found that precipitation of rat muscle actomyosin solutions at low ionic strength in the presence of inorganic phosphate resulted in a high yield of deaminase in the filtrate and a 15-fold increase in specific activity. Comparative studies made on various protein preparations from both rat and rabbit muscle showed that this phenomenon does not occur with myosin or actomyosin from rabbit.

The purification steps involved in their procedure were:

a muscle extraction which formed an actomyosin-deaminase complex; precipitation of the complex by dilution with cold water; dissociation of deaminase from actomyosin by phosphate (u = 0.05); RNase treatment and ammonium sulfate fractionation; and separation on DEAE-cellulose.

The purified rat enzyme showed an absorption maximum at 278 mu and a 280/260 ratio of 1.86. The mean value for the Michaelis-Menten constant in succinate buffer pH 6.4 at 30^{o} was $1.41 \pm 0.15 \times 10^{-3}$ M. The specific activity was 15.81 uM AMP/min/mg with a recovery value of approximately 25 per cent. The properties of the enzyme agreed favorably with those of Lee's deaminase prepared from rabbit muscle. With Lee's enzyme the K_M was 1.41×10^{-3} M and the specific activity was 17.24 uM AMP/min/mg. The recovery and absorption ratio obtained by Webster and Currie are superior to those of Lee for his recovery was only 5 per cent and the 280/260 was 1.2.

METHODS AND MATERIALS

The substrate (5'-adenylic acid) was purchased from either Sigma Biochemical Corporation or California Corporation for Biochemical Research as the sodium salt or as the crystalline acid. The sodium salt was used directly and the acid was neutralized by the addition of KOH before use.

Protamine sulfate was purchased from California Corporation for Biochemical Research.

Distilled water used for dilutions was deionized by a Crystalab Deionizer.

Protein was measured by the 280/260 mu absorption method of Warburg and Christian (1941).

The activity of 5'-adenylic acid deaminase was assayed by the method of Kalckar (1947) as modified by Lee (1957). The reaction mixture contained 4.5×10^{-5} M 5'-adenylic acid and 0.1 M succinate buffer, pH 6.4. The reaction, carried out at 30°, was started by addition of diluted enzyme solution. With exception of the final protein solution which was diluted with distilled water, all dilutions were made with 0.5 M KCl. The reaction was measured at 265 mu with a Beckman DU spectrophotometer equipped with a Gilford attachment. Using the initial optical density change observed, the activity was calculated by the equation:

$$\frac{\Delta O.D._{265\ mu}}{(min)\ (mg.\ protein)\ (8.86)} = \text{uM AMP converted/min/mg protein}$$

The factor 8.86 used in the previous equation was derived from the basic equation for absorbance:

$$O.D. = ECl \qquad (1)$$

O.D. = optical density or absorbance

E = molar extinction coefficient

l = length of light path

Since cuvettes of 1 cm width were used, equation (1) reduces to:

$$O.D. = EC \qquad (2)$$

The total absorbance for a solution containing both AMP and IMP is:

$$O.D. = E_{AMP}C_{AMP} + E_{IMP}C_{IMP} \qquad (3)$$

If the absorbance is measured at time t_1 and t_2 the change in optical density is:

$$\Delta O.D. = E_{AMP}C_{AMP_{t_1}} + E_{IMP}C_{IMP_{t_1}} - E_{AMP}C_{AMP_{t_2}} - E_{IMP}C_{IMP_{t_2}} \qquad (4)$$

Combining similar terms:

$$\Delta O.D. = E_{AMP}(C_{AMP_{t_1}} - C_{AMP_{t_2}}) + E_{IMP}(C_{IMP_{t_1}} - C_{IMP_{t_2}}) \qquad (5)$$

This may be expressed as:

$$\Delta O.D. = E_{AMP}\Delta C_{AMP} + E_{IMP}\Delta C_{IMP} \qquad (6)$$

Since the increase in concentration of IMP equals the decrease in concentration of AMP, then

$$\Delta C_{AMP} = -\Delta C_{IMP} \qquad (7)$$

Substitution of equation (7) in equation (6) results in:

$$\Delta O.D. = \Delta C_{AMP}(E_{AMP} - E_{IMP}) \qquad (8)$$

The experimentally determined extinction coefficients at 265 mu in succinate buffer are $E_{AMP} = 14.10 \times 10^3$ and $E_{IMP} = 5.24 \times 10^3$. Consequently, the equation for calculation

of uM of AMP is:

$$\frac{\Delta O.D.}{8.86} = \text{uM AMP}$$

PURIFICATION PROCEDURE

Original Fractionation:

Rabbits were suffocated with ether, bled, and the back and leg muscles immediately excised, chilled in ice, and then frozen. The frozen muscle was extracted by cutting the tissue into small pieces and homogenizing in a Waring blender for 1 minute with 3.0 volumes of a buffer containing 0.3 M KCl, 0.09 M KH_2PO_4, and 0.06 M K_2HPO_4 at pH 6.5 and 3° C. After addition of 4 volumes of buffer, the mixture was stirred for 1 hour. The extract was then centrifuged at 14,000 x G to separate the muscle residue. Lipid material was removed by pouring the liquid through two layers of cheesecloth.

Freezing:

The extract was frozen by one of two methods: (1) placing the solution in a -20°C freezer overnight or (2) freezing in a dry ice acetone bath. Identical deaminase activity was obtained with the two methods. After thawing slowly in a 20°C water bath, the solution was centrifuged at 14,000 x G for 20 minutes and the precipitate discarded.

Calcium Chloride Fractionation:

The cloudy supernatant was made 10^{-2} M in $CaCl_2$ by the slow addition of 1 M $CaCl_2$ over a 20 minute period. The solution was then stirred for an additional 20 minutes. The

preparation was centrifuged for 20 minutes at 14,000 x G and the precipitate discarded.

0.3 M K^+ Precipitation:

Assuming the extract was 0.5 M. in potassium ion concentration, it was diluted with cold, deionized, distilled water to a concentration of 0.3 M. This addition was carried out over a 20 minute period with efficient stirring. After 20 minutes additional stirring, the solution was centrifuged at 14,000 x G for 20 minutes. The insoluble material was discarded.

Myosin Precipitation:

The potassium ion concentration was lowered to 0.05 M by the slow addition of cold (3°C), deionized, distilled water. The solution was stirred throughout the water addition and then stirred for an additional 30 minutes. The preparation was maintained at 3°C overnight. With sufficient time lapse, the insoluble protein precipitated and settled to the bottom of the container. The clear, water layer was aspirated and the lower cloudy layer was centrifuged at 14,000 x G for 20 minutes to separate insoluble material.

Heat Fractionation:

The protein concentration was adjusted to 10 mg/ml with 0.5 M KCl, and the pH adjusted to 6.4. The solution was placed in a 70°C water bath and with constant stirring was heated at 55° ± 1 for 4 minutes. After quickly cooling the solution in

an ice bath, it was centrifuged at 40,000 x G for 20 minutes. The clear supernatent was obtained and 0.5 M KCl was added to the precipitate. Following 30 minutes of stirring, this solution was centrifuged at 40,000 x G. If the specific activity of the supernatent was equivalent to the previous supernatent, the two fractions were combined.

Protamine Sulfate Fractionation:

One ml of a 2% protamine sulfate solution for each 10 ml of enzyme solution was slowly added with stirring at 3°C. After 15 minutes additional stirring, the solution was centrifuged at 40,000 x G for 15 minutes. The deaminase was in the liquid fraction.

Dialysis:

Dialysis tubing was prepared by boiling it in a 10^{-2} M EDTA solution, and then boiling in distilled water. The tubing was washed with cold, deionized, distilled water and the enzyme was added. Following dialysis against cold, deionized, distilled water (3 hours), the solution was centrifuged at 40,000 x G for 15 minutes. At this point, the enzyme was water soluble.

RESULTS

Table II

200 gm Rabbit Skeletal Muscle

Fraction	280/260	Total Protein mg.	Specific Activity	Total Activity	Purification	Yield
Muscle Extract	0.77	11,800	0.195	2,300	1.0	100
Freeze	0.80	8,870	0.270	2,400	1.4	104
$CaCl_2$ Ppt.	0.80	8,670	0.30	2,600	1.5	113
0.3 M K^+ Ppt.	0.71	3,680	0.61	2,240	3.1	97.5
Myosin Ppt.	1.1	900	2.10	1,880	11	81.5
Heat Treatment	0.73	215	6.18	1,330	31	58.0
Protamine Sulfate	1.14	106	11.00	1,170	56	50.7
Dialysis	1.14	41	17.50	718	90	31.9

DISCUSSION

Using frozen muscle rather than fresh muscle resulted in a 5 fold increase in total deaminase activity and a 10 fold increase in specific activity in comparison with the method of Lee (1957). Freezing the muscle, of course, disrupts the tissue to some extent, the effect being to increase the amount of deaminase activity obtainable from the muscle fibrils while denaturing some of the contaminating proteins. In order to obtain maximum activity it was found that 7 ml of buffer per gm of tissue was required. This volume of buffer lowered the viscosity of the extract and shortened the extraction time. The decreased viscosity resulted in a higher concentration of deaminase in the aqueous phase through more efficient stirring. The shortened extraction time was desirable for the concentration of actomyosin and other contaminating proteins was lowered. Actually, this method yields only 50% of the total protein obtained by Lee (1957) which accounts for the specific activity increase mentioned previously.

Freezing the extract after removal of the tissue residue caused the clear solution to become cloudy. A white precipitate was then obtained by centrifugation. Upon assaying the supernatant, the specific activity showed an increase of approximately 40%. The total yield was calculated to be 104%. This value was very reproducible though sometimes the recovery was greater than this. Repeated freezing and thawing at this

point had no effect.

The protein removed by freezing was not conclusively identified. However, indirect evidence was obtained which suggested that the material was actomyosin. Actomyosin was considered as a possibility mainly because Seagran (1956) found that actomyosin prepared from frozen fish muscle was insoluble if frozen in salt solution. If the precipitate was actomyosin, it seemed that freezing a preparation containing a greater concentration of actomyosin should cause increased precipitation. Such a preparation was obtained by stirring the extract for two hours rather than one. After freezing, two-thirds of the protein precipitated. The specific activity of the deaminase rose by a factor of 6 and the total recovery was 111%. Thus, it appeared that the insoluble protein was actomyosin. Also of interest was that the per cent recovery was greater that normally observed (111% and 104% respectively).

The removal of contaminating protein by freezing explains the increase in specific activity but does not explain the observed increase in total deaminase. Assuming that the insoluble protein was actomyosin, the normal actomyosin-deaminase complex must have been disrupted or the recovery would have been less than 100%. This would have been particularly true in the preparation carried out with a longer extraction time since two-thirds of the protein precipitated. Assuming, therefore, that the initial actomyosin-deaminase complex was disrupted by freezing, a logical explanation for the increased recovery would be that the deaminase reactive site became more

accessible after the actomyosin was precipitated. In other words, perhaps bound actomyosin sterically hindered the deaminase activity. Thus, if the above did occur, the substrate would have been more readily bound and the subsequent activation would explain the observed recovery increase.

Another possibility which must be considered is that soluble actomyosin does not bind deaminase. In this event, increased yield would probably be a consequence of some occurrence unrelated to actomyosin precipitation. For instance, if freezing changed the structure of deaminase slightly so that the enzyme became more active, then the recovery value would increase.

Of course, no direct evidence is available to explain the actual effect of freezing. However, of the two possibilities suggested above, the former seems most likely as it is related to actomyosin concentration. If the mechanism was unrelated to actomyosin, it seems unlikely that the extract with the greater actomyosin concentration would have had a greater activation than was normally observed.

Another technique which further increased the recovery of deaminase above the original value was the addition of 10^{-2} M $CaCl_2$. Upon slow addition of 1 M $CaCl_2$, the solution became cloudy. After reaching 10^{-2} M, a precipitate was obtained upon centrifugation. Evidence suggesting that the insoluble protein was actomyosin is the work of Weber and Winicur (1961) and Maruyama and Watanabe (1962) concerning the role of Ca^{+2} in the super-precipitation of actomyosin.

Indirect evidence was again obtained by doing a preparation by a two hour extraction time which increased the concentration of actomyosin. It was believed that with more actomyosin, greater precipitation should be obtained. As with freezing, the addition of $CaCl_2$ to this preparation resulted not only in increased precipitation but also increased recovery. The recovery was 126% whereas the normal value was 113%.

One explanation for the recovery increase would be the same as that proposed for the effect of freezing; namely, the release of deaminase which had been previously inhibited. Another possibility is that Ca^{+2} activates the deaminase. Though the concentration of Ca^{+2} was only 10^{-5} M in the reaction cuvette since the protein was diluted, it could have been higher at the reaction site due to adsorption of Ca^{+2} by myosin-deaminase complex. Unfortunately, evidence is not available to distinguish which, if indeed either, suggestion is valid.

Another interesting observation was made with $CaCl_2$. If the concentration of $CaCl_2$ was increased to 1.2×10^{-1} M, no deaminase activity remained in the supernatant. As the 280/260 ratio was only 0.64, this seemed a desirable means of separating deaminase from nucleic acid. Unfortunately, attempts to dissolve the precipitate were unsuccessful.

An actomyosin precipitation was suggested by the work of Protzehl and Weber (1952). It was reported that at pH 6.6, actomyosin was insoluble at 0.3 ionic strength and below. Though it was feared that a large amount of deaminase might

also be precipitated, the extract was slowly diluted to 0.3. As seen on Table II, approximately a two fold increase in specific activity was obtained. Correspondingly, about 67% of the protein was removed. Some deaminase was lost be precipitation (16%), but not nearly as much as one might expect. If, as expected, most of the precipitating protein was actomyosin, it would seem that deaminase binds preferentially to myosin. Indeed, the first three steps in this procedure suggest the following possibilities: (1) deaminase binds preferentially to myosin, (2) the deaminase-actomyosin complex is easily dissociated, or (3) deaminase and actin bind upon the same site on the myosin molecule.

The myosin precipitation used in this procedure was essentially the same as that of Lee (1957). However, the procedure was actually suggested by two lines of evidence: (1) deaminase exists as a complex with myosin, (2) and myosin precipitates at low salt concentrations. Though no direct evidence exists for the deaminase, myosin complex, Ferdman and Nechiporenko (1946) and Hermann and Josepovits (1949) showed that myosin had deaminase activity. Hermann and Josepovits were unable to separate the two activities and concluded that it was myosin which possessed deaminase activity and no other protein was present. Although this observation has been shown to be false, it has importance for it shows that the so called "crystalline myosin" was contaminated with deaminase. This "crystalline myosin" was prepared by the method of Szent-Györgyi (1943). In this procedure myosin is repeatedly pre-

cipitated by dilution with cold water to 0.04 ionic strength. After precipitation, the myosin is redissolved by the addition of solid KCl to u = 0.5. By using this method, a considerable increase in activity (3.5 fold) was observed. However, about 16% of the deaminase was not recovered. Part of this loss was undoubtedly due to incomplete recovery of the precipitated myosin for some of the particles do not settle completely and are aspirated with the surface liquid. However, indirect evidence was obtained during this work which suggests that purified deaminase does not have the same solubility properties as myosin; therefore, perhaps a small amount of myosin free deaminase was lost in the aqueous fraction.

At this point, it was observed that the activity was equivalent to that attained by Lee after his ethanol extraction. For this reason, an ammonium sulfate fractionation similar to Lee's was attempted. The results were disappointing both with ammonium sulfate and later with an ethanol fractionation. A heat treatment had not been tried because of the large loss of activity expected. However, when further purification attempts failed, heat denaturation of myosin was carried out. As seen in Table II, the results were rewarding in that a 3 fold increase in specific activity was obtained. On the other hand, 23% of the deaminase was lost. A portion of this was, of course, denatured as was the myosin. However, by stirring the coagulated myosin in 0.5 M KCl, deaminase equal to or greater than 10% of the total activity was obtained. It would seem, therefore, that a major faction of the unrecovered

deaminase was not denatured, but was bound to the coagulated myosin. Unfortunately, the results from the heat treatment were not always reproducible. The effectiveness of the coagulation seemed to be greatly dependent upon the protein concentration and also upon the time required to reach 54°C. The best results were obtained with small samples (5 ml) because the desired temperature was obtained rapidly.

According to Locker (1959), the recovery of deaminase from heat coagulated myosin was dependent upon both salt and phosphate concentration. He found that the greatest yield was obtained at a KCl concentration of 1 M. He also found that pyrophosphate added before heating, would increase the yield of water insoluble deaminase. On the other hand, the addition of tripolyphosphate increased the concentration of water soluble deaminase. No attempt has been made as yet to test the effectiveness of these methods upon the heat treatment carried out by the author.

Also of interest in the work of Locker (1959) was the detection of RNA. He found that the concentration of nucleotide and nucleic acid in myosin was 0.4%. This agreed closely with the results of Mihalyi, Laki, and Knoller (1957), who reported 0.5 - 0.8 per cent RNA. Locker further found that the RNA present in myosin was concentrated in the fractions surviving the heat treatment. Consequently, he proposed that the RNA exerts a stabilizing effect upon the portion of the protein to which it is attached. A similar observation was made by the author. A value of 0.73 was found for the 280/260

mu ratio. Since the ratio previous to heating was 1.10, apparently the nucleotide concentration relative to the protein concentration had increased. No attempt was made to determine this nucleotide or nucleic acid concentration.

Because of the evidence mentioned above, it was assumed that the preparation was contaminated with nucleic acid. Consequently, the extract was diluted with cold water to an ionic strength of 0.04. It was hoped that the deaminase would precipitate while leaving the nucleotides or nucleic acid in solution. Unfortunately, the absorption ratio (280/260) of the redissolved precipitate did not show an increase. The next attempt was the addition of 1 M $MnSO_4$ to a concentration of 0.05 M (Ochoa et al., 1951). This method was more successful for the specific activity increased, but the absorption ratio rose only slightly. Finally, the use of protamine sulfate (E. Racker, 1947) did prove fruitful. The 280/260 mu ratio increased to 1.14 and the specific activity increased by a factor of 1.7.

The enzyme solution was then dialyzed vs water in order to precipitate the deaminase so that it could be concentrated. After dialysis, the solution was centrifuged and the precipitate was dissolved with difficulty in 0.5 M KCl. Upon measuring the activity, the solution was found to be inactive. When the dialysate was tested, the specific activity was found to be equal to that of Lee's crystalline deaminase. Though it was rather surprising that the enzyme was water soluble, Locker (1959) had similar results with heat treatments. Upon

heating a myosin solution at 53°C, pH 6.2, Locker obtained a water soluble and a water insoluble fraction. All of the deaminase activity was in the insoluble fraction. The insoluble fraction was dissolved in 1.0 M KCl and reheated at 53°. After this second coagulation, two fractions were again formed, but the water soluble fraction had high deaminase activity. Thus water soluble deaminase has been prepared by two different methods. It is possible that this solubility is related to the RNA content. In one case, removal of nucleic acid by protamine sulfate makes the enzyme water soluble. In the other, Locker reported an increase in his water soluble fraction by the addition of low concentrations (0.01-0.02 M) of tripolyphosphate. Possibly the tripolyphosphate replaces the RNA on the enzyme and thereby allows the deaminase to become water soluble.

The absorption ratio observed after treatment with protamine sulfate (1.14) was not as high as expected. This was attributed to excess protamine in solution. Removal of this contaminating protein by Sephadex and DEAE cellulose was not successful. A separation of the two by ultracentrifugation was not attempted, but should be possible because of the difference in molecular weights of the two proteins.

One disadvantage to the purification procedure reported here is the final protein concentration. The average value obtained was 1 mg/ml. Attempts to concentrate the protein by either water adsorption through dialysis tubing with Sephadex G-200 or by lyophilization were usually unsuccessful.

On one occasion lyophilization yielded deaminase with a 280/260 mu ratio of 1.3 and a specific activity equal to twice the "crystalline activity" of Lee's deaminase. Unfortunately, this could not be repeated. Ultracentrifugation as a means of concentrating the protein was not attempted.

SUMMARY

1. A purification procedure for the isolation of 5'-adenylic acid deaminase from frozen rabbit muscle was developed.

2. Specific activity of the enzyme was equivalent to Lee's "crystalline" deaminase.

3. The recovery was 31.9 per cent.

4. The amount of deaminase, of crystalline activity, obtained per gm of muscle was 34 times as great as previously reported (Table I and II).

5. The purified enzyme was water soluble.

6. Homogeneity could not be shown due to protamine sulfate contamination.

BIBLIOGRAPHY

Bailey, K., Biochem. J. 36, 121 (1942).

Bowen, W. J. and Kerwin, T. D., Arch. Biochem. Biophys. 64, 278 (1956).

Cain, D. F., Infante, A. A., Davies, R. E., Nature 196, 214 (1962).

Cori, C. F., Cori, G. T., and Green, A. A., J. Biol. Chem. 151, 39 (1943).

Currie, R. D., and Webster, H. L., Biochim. Biophys. Acta, 64, 30 (1962).

Embden, G., and Schmidt, G., Z. Physiol. Chem. 181, 130 (1929).

Engle hardt, V. A., Lyubimova, M. N., Venkstern, T. V., Timofeeva, M. Y., and Babskaya, Y. B., Doklady Akad. Nauk, S. S. S. R., 85, 397 (1952).

Ferdman, D. L., and Nechiporenko, Z. Y., Ukrain. Biochem. J., 18, 105 (1946).

Fisher, E. H. and Krebs, E. G., J. Biol. Chem. 231, 65 (1958).

Green, A. A. and Cori, G. T., J. Biol. Chem. 151, 21 (1943).

Hermann, V. S., and Josepovits, G., Nature, 164, 845 (1949).

Kalckar, H. M., J. Biol. Chem., 167, 445 (1947).

Kalckar, H. M., J. Biol. Chem., 167, 461, (1947).

Korkes, S., Del Campillo, A., Gunsalus, I. C., and Ochoa, S., J. Biol. Chem., 193, 721 (1951).

Kutscher, W. and Sarreother, W., Klin. Wochschr. 26, 698 (1948).

Lee, Y.-P., J. Biol. Chem., 227, 987 (1957).

Locker, R. H., Biochim. Biophys. Acta, 20, 514 (1956).

Locker, R. H., Biochim. Biophys. Acta., 32, 189 (1959).

Lyubimova, M. N., and Natlina, E. S., Doklady Akad. Nauk. S. S. S. R., 94, 927 (1954).

Maruyama, K. and Watanabe, S., J. Biol. Chem., 237, 3437 (1962).

Mihalyi, E., Laki, K., and Knoller, M. J., Arch. Biochem. Biophys., 68, 130 (1957).

Mitchell, H. K., Gordan, M., and Haskins, F. A., J. Biol. Chem., 180, 1071 (1949).

Nechiporenko, Z. Yu, Ukrain. Biochim. Zhur. 25, 62 (1953).

Nechiporenko, Z. Y., and Ferdman, D. L., Doklady Akad. Nauk. S. S. S. R. 92, 803 (1953).

Nechiporenko, Z. Y., and Pogrebins'ka, E. N., Ukrain. Biochim. Zhur. 21, 150 (1949).

Nikiforuk, G. and Colowick, S., J. Biol. Chem., 219, 119 (1956).

Parnas, J. K., Biochem. Z. 206, 16 (1929).

Parnas, J. K. and Mozolowski, W., Biochem. Z. 134, 399 (1927).

Pennington, R. J., Nature, 192, 884 (1961).

Racker, R., J. Biol. Chem., 167, 843 (1947).

Schmidt, G., Z. Physiol. Chem., 179, 243 (1928).

Seagran, H. L., Food Research 21, 506 (1956).

Szent-Gyorgyi, A., Studies from the Inst. of Med. Chem., Univ. Szeged, 3, 76 (1943).

Warburg, O. and Christian, W. Biochem. Z., 310 (1941) 384.

Weber, A. and Winicur, S., J. Biol. Chem., 236, 3198 (1961).

Weber, H. H., and Portzehl. H., Advances in Protein Chem. 1, 161 (1952).