to take into account the existence of the folded configurations without internal hydrogen bonds in other acetylaminoacid-N-methylamides.

As shown above the equilibrium ratio of different configurations of acetylaminoacid-N-methylamides depends on the nature of the solvent. This result suggests that the configuration of a polypeptide chain changes with environment. However, it should be realized that this change is essentially due to the change in *intramolecular* and *intermolecular* hydrogen bonds and not due to the change of the stable positions of the internal rotation potential in which the steric repulsion between the rotating groups plays the most important part.[7]

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, UNIVERSITY OF WISCONSIN]

Studies of the Enzyme Fumarase. II.[1] Isolation and Physical Properties of Crystalline Enzyme

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A method is described for the isolation and crystallization of fumarase from pig heart muscle which differs from methods which have previously been reported. Based on the muscle extract, a 2600-fold increase in specific activity and a recovery of 20% are obtained. This protein yields a single boundary in the electrophoresis apparatus and in the velocity ultracentrifuge. The ultraviolet absorption is characteristic of a protein, and it has not been possible to detect an enzyme–substrate complex spectrophotometrically.

Fumarase catalyzes the reversible hydration of fumarate to *l*-malate. It has been found in numerous animal and plant tissues, and in 1941, Laki and Laki[2] reported a method for the isolation and crystallization of fumarase from ox heart. The protein obtained, however, was shown in 1948[3] and 1950[4] to be impure, and in 1951,[5] Massey reported that he had crystallized fumarase from pig heart muscle extract with about a tenfold increase in specific activity over the preparation of Laki and Laki. The procedure developed in this Laboratory differs substantially from that of Massey.[6] However, side by side comparisons of the two crystalline enzyme preparations show that kinetic constants for the enzyme prepared by the two different methods are the same within experimental error as is the specific activity, and that the ultraviolet absorption spectra are superimposable. Both preparations yield a single boundary in the electrophoresis apparatus and ultracentrifuge.

Experimental

Assay.—*Fumarase activity* is determined spectrophotometrically[1] using ultraviolet light at 250 mμ, in a solution of 0.05 *M* sodium phosphate buffer at *p*H 7.3 and 25° with an *l*-malate concentration of 0.05 *M*. The number of units of activity for an aliquot of enzyme solution in 3 ml. of the buffer is arbitrarily defined as the initial rate of change in optical density per 10 sec. × 10^3 due to the formation of fumarate.

The *specific activity* is defined as the ratio of the activity for a given aliquot of enzyme solution to the optical density at 250 mμ of the same aliquot in 3 ml. of 0.05 *M* phosphate buffer of *p*H 7.3 and at 25°.

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Preparation.—The procedure given by Massey is similar to that followed originally by Laki and Laki in that the fumarase remains in the supernatant of the initial extract after the *p*H is lowered and calcium phosphate gel is used to adsorb the enzyme in the purification procedure. In the present method, fumarase is precipitated from the initial extract by lowering the *p*H and the temperature, and calcium phosphate gel is not used.

The following procedure has been used in obtaining the results summarized in Table I. Twenty fresh pig hearts are freed of fat and connective tissues and ground in a meat grinder. The meat is washed 6 times with 6–7 liters of cold (5°) distilled water per wash and strained in cheesecloth. The meat is then suspended in 6 l. of 0.01 *M* phosphate buffer of *p*H 7.3 which is warm enough (about 60°) to make

TABLE I

ISOLATION OF CRYSTALLINE FUMARASE

Step			Volume (*V*) in ml.	Activity (*A*) for 0.1 ml. enzyme solution	Total units $AV \times 10^{-6}$	Specific activity	Purity, %
Washes 1–6			45,000	30	13.5	40	
Original extract			5,000	70	5.3	130	0.04
Re-extraction			3,000	65			
*p*H 6.0, S[a]			8,000	57	4.5	190	.06
P[b]				0			
*p*H 5.3, S			8,000	10	0.8	50	
P			200	1500	3.0	440	.13
$(NH_4)_2SO_4$ fractionation							
(1)	0–35%	P	125	300	0.37	108	
	35–65%	P	50	3500	1.4	3500	1.0
	65%	S	260	110	0.29	265	
(2)	15–45%	P	20	1000	0.2	800	
	45–60%	P	35	3800	1.3	6100	1.8
	60%	S	20	196	0.04	680	
(3)	50%	P (A)	12	1000	0.12	2000	
	50%	P (B)	15	6000	1.1	50000	15
	50%	S	25	252	0.05	500	
B suspended in 15% satd. $(NH_4)_2SO_4$		S	25	956	0.24	4000	
		P	14	8500	1.1	330000	
Repeated crystallization			10	11000	1.1	336000	100

[a] Supernatant. [b] Precipitate.

the temperature of the slurry 35°. It has been found that extraction at 35° for 10 minutes with dilute phosphate buffer gives the highest specific activity and very nearly the maximum yield. Therefore, the slurry is stirred for 10 minutes at this temperature, the extract strained off, and the meat re-extracted with 4 l. of the phosphate buffer at 35°. The extract is cooled to 5–10°, and 1 *M* sodium acetate buffer (*p*H 4.6) is added dropwise until *p*H 6.0 is obtained (measured at 25°). The extract is then centrifuged at 0–5° in a laboratory-size Sharples Supercentrifuge, at 17,000 r.p.m., and the precipitate discarded. The supernatant is then adjusted to *p*H 5.3 and centrifuged again at 0–5°. The precipitate obtained is suspended in 200 ml. of 0.01 *M* phosphate buffer (*p*H 7.3) and allowed to stand for about 0.5 hr. at 25°.

An equal volume of 70% saturated ammonium sulfate solution[7] is added to the suspension which is then allowed to stand for about 1 hour at 5° with occasional stirring. The precipitate is centrifuged off in a Servall SS-1 high speed centrifuge at 16,000 *g* and discarded. The supernatant is dialyzed overnight with stirring at 5° against an ammonium sulfate solution, the concentration of which is high enough to produce a 65% saturated solution after dialysis. The precipitate which forms is spun down, dissolved in 15% ammonium sulfate, and the supernatant discarded. A second ammonium sulfate fractionation is carried out by the above dialysis technique, the fumarase precipitating between 45 and 60% saturation.

Crystallization.—The precipitate from the second fractionation is dissolved in 0.01 *M* phosphate buffer (*p*H 7.3) and dialyzed with stirring against an ammonium sulfate solution such that the resultant degree of saturation will be 50%. The dialysis is allowed to continue for 3–4 hr. while a heavy precipitate A forms which is centrifuged off and discarded. The supernatant is then dialyzed with stirring for two days or more against an ammonium sulfate solution of the same concentration. The crystalline precipitate B which forms is centrifuged off and suspended in 15% saturated ammonium sulfate solution.

The suspension is allowed to stand at room temperature for about 3 hr. and is centrifuged again. The supernatant obtained from this process contains impurities which have dissolved leaving as the precipitate highly purified fumarase. This precipitate can be spun down and dissolved in 0.01 *M* phosphate buffer (*p*H 7.3), spun again to clear and then dialyzed against 50% ammonium sulfate to precipitate the purified fumarase. More purification can be achieved by repeating the crystallization step. The crystalline fumarase obtained by this process has a specific activity of 336,000 and is considered to be pure. The purity, therefore, is defined as the ratio of the specific activity to 336,000.

The enzyme can be stored at 5° under 50% saturated ammonium sulfate or frozen in dilute buffer for several months without losing appreciable activity. The yield from the extracted material is about 20%.

Fumarase crystals which form in 50% saturated ammonium sulfate are rod-shaped as shown in Fig. 1. They dissolve extremely slowly in water, but are fairly soluble in dilute (0.01 *M*) phosphate buffer (*p*H 7.3). The stability of fumarase solutions increases as the *p*H is raised and is greatest at about *p*H 9.5. The stability of these solutions in glycine buffer at this *p*H is even greater than in phosphate buffer.

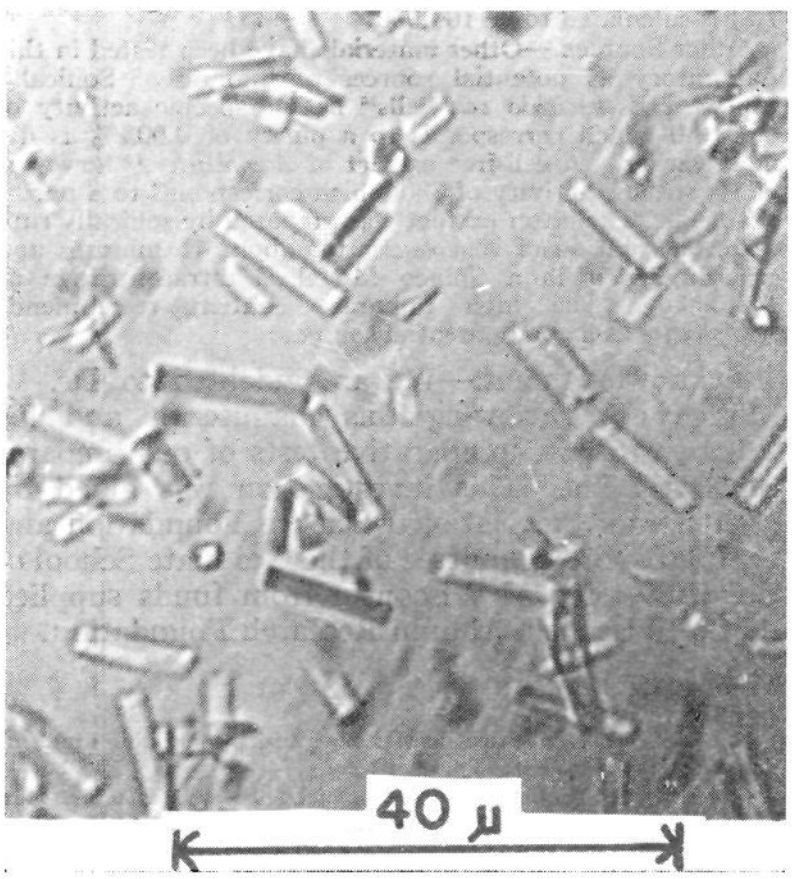

Fig. 1.—Photomicrograph of fumarase crystallized from 50% saturated ammonium sulfate and washed with distilled water.

(7) Unsaturated ammonium sulfate solutions were made up by volume using saturated (at 5°) ammonium sulfate solution and 0.01 *M* (*p*H 7.3) phosphate buffer.

Physical Properties

Electrophoresis and Ultracentrifuge Experiments.—The mobility of fumarase in phosphate buffer of *p*H 6.83 and 0.15 ionic strength is -1.95×10^{-5} cm.^{2} volt^{-1} sec.$^{-1}$ which is in excellent agreement with values reported by Massey.[6] As shown by the schlieren pattern in Fig. 2, there is a single moving boundary. At *p*H 8.01 in tris-(hydroxymethyl)-aminomethane chloride buffer of 0.05 ionic strength, the mobility is -1.2×10^{-5} cm.^{2} volt^{-1} sec.$^{-1}$. This lower value indicates that phosphate ions are bound to the enzyme to a greater extent than are chloride ions.

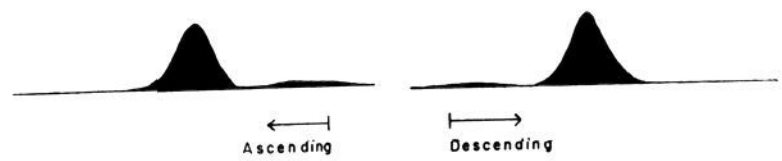

Fig. 2.—Electrophoretic pattern for crystalline fumarase in phosphate buffer of 0.15 ionic strength, *pH* 6.83, at 14,900 sec. and a potential gradient of 4 volts cm.$^{-1}$.

Ultracentrifugal studies of the crystalline fumarase have been made with both the Spinco Model E[8] and Svedberg oil turbine ultracentrifuges at several concentrations. In all experiments a single boundary was obtained as illustrated in Fig. 3. In the calculation of sedimentation coefficients from experiments with the Spinco ultracentrifuge, 1° was subtracted from the average temperature of the rotor in

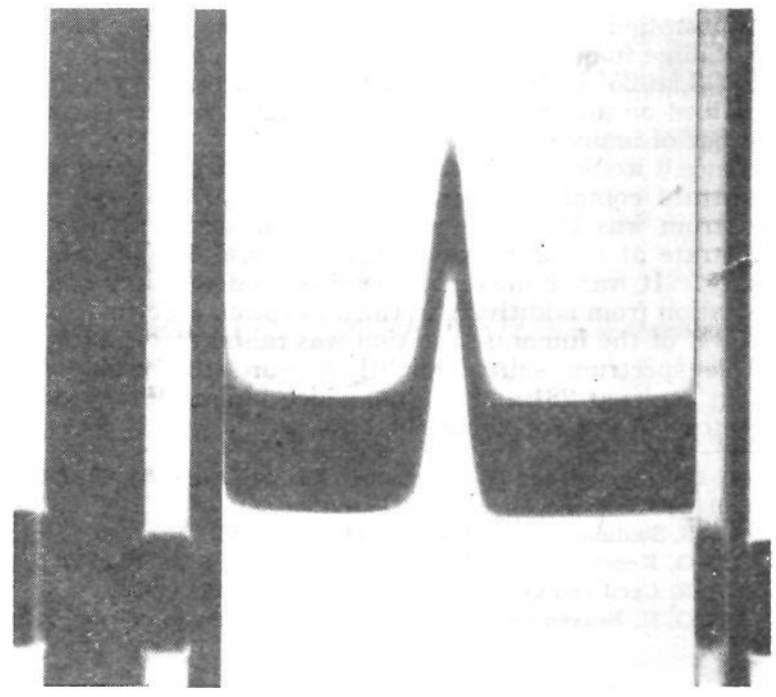

Fig. 3.—Schlieren pattern for a 0.58% fumarase solution in phosphate buffer *pH* 6.4, 0.15 ionic strength using a Spinco Model E velocity ultracentrifuge, 2880 sec. after reaching a constant speed of 59,700 r.p.m.

(8) Made available by the courtesy of Dr. P. P. Cohen and Dr. H. F. Deutsch.

order to allow for the decrease in the temperature of the rotor during acceleration as determined by Waugh and Yphantis[9] and Kegeles.[10] In the calculation of sedimentation coefficients from experiments with the oil turbine ultracentrifuge, the temperature correction of the thermocouple reading determined by Shulman[11] was used. A 0.74% solution of Bovine Plasma Albumin, Lot Number R370295A was run in the Spinco ultracentrifuge in order to correlate the area under the peak with concentration. The sedimentation coefficient obtained for this solution of albumin (4.10×10^{-13} sec.$^{-1}$ at *p*H 4.4, 0.15 *M* NaCl, 0.05 *M* acetate buffer) is in excellent agreement with that reported by Kegeles and Gutter[12] after correcting their temperature by 1°.

Assuming the same refractive index increment for both fumarase and albumin, it was possible to calculate the concentration of fumarase at the middle of the ultracentrifuge experiment by measuring the area under the peak at that particular time.

Table II gives values of $s_{20,w}$ at various fumarase concentrations. The determinations were made in phosphate buffer of *p*H 6.7, 0.15 ionic strength. The relative viscosity of this buffer was measured separately and found to be 1.019 at 25°. Below 0.4%, the data may be represented by $s = s_0/(1 + kc)$ where s_0 is the sedimentation coefficient at infinite dilution, c the concentration in g./100 ml. and k a constant. In this way, values of 9.09×10^{-13} sec.$^{-1}$ for s_0 and 0.08 for k are obtained.

The molecular weight may be calculated since Cecil and Ogston[13] have obtained a diffusion coefficient of 4.05×10^{-7} cm.^{2} sec.$^{-1}$. This value and s_0 equal to 9.09×10^{-13} sec.$^{-1}$ assuming that 0.75 ml./g. is the partial specific volume, gives a molecular weight of 220,000.

TABLE II

SEDIMENTATION COEFFICIENTS ($s_{20,w}$) FOR FUMARASE

Phosphate buffer of *p*H 6.7, 0.15 ionic strength

Concentration, g./100 ml.	$s_{20,w} \times 10^{13}$ sec.$^{-1}$
0.07	9.03
.16	8.96
.34	8.87
.52	8.90[a]
.58	8.88[b]
.78	8.86[c]

[a] Enzyme prepared by Dr. Massey. [b] In phosphate buffer of *p*H 6.4, 0.15 ionic strength. [c] Value obtained on Svedberg oil turbine ultracentrifuge.

Absorption Spectrum.—The ultraviolet absorption of crystalline fumarase dissolved in 0.005 *M* phosphate buffer, *p*H 7.3, at 25° is shown in Fig. 4. The spectrum, which was obtained on a Cary recording spectrophotometer, is similar to that of many other proteins.[14]

Since it would be desirable to be able to detect an enzyme-substrate complex spectrophotometrically, the absorption spectrum was also obtained for mixtures of fumarase and substrate at molar ratios of 1:1, 1:2 and 1:1000, respectively. It was, however, impossible to detect any shift or deviation from additivity in the absorption spectra. When the *p*H of the fumarase solution was raised to *p*H 11.5, the whole spectrum shifted slightly toward the visible with slight peaks at 281 and 289 mμ which indicate[14] a tyrosine-tryptophan ratio of about three to two.

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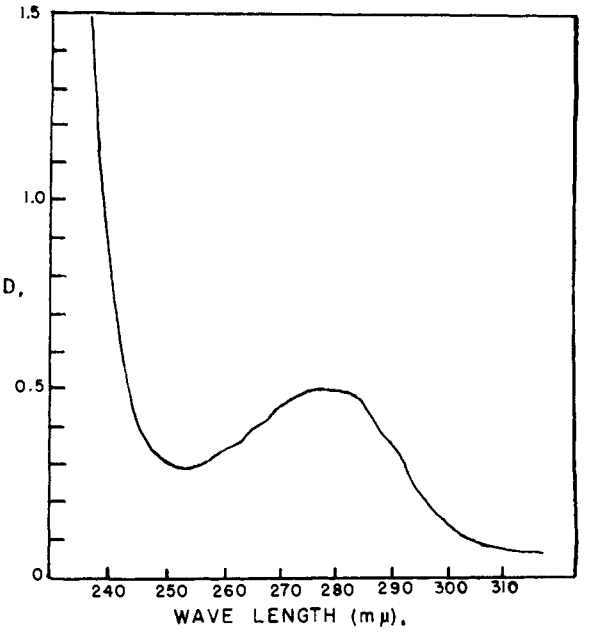

Fig. 4.—Absorption spectrum of fumarase (1 mg./ml.) at 25°, in 0.005 *M* phosphate buffer, *p*H 7.2.

The extinction coefficient[15] at 280 mμ as determined by dry weight is 0.53 ml. mg.$^{-1}$ cm.$^{-1}$.

Turnover Number.—Since the initial velocity obtained using 0.05 *M* *l*-malate under the conditions of the assay is about 6% lower than the maximum initial velocity obtained by extrapolation for these conditions, 0.46 mole of *l*-malate is converted to fumarate per minute per g. of enzyme at 25°, *p*H 7.3, and in 0.05 *M* phosphate buffer.

Thus the turnover number (that is, the number of *l*-malate molecules converted to fumarate per molecule of enzyme per minute at 25° in 0.05 *M* phosphate buffer of *p*H 7.3) is calculated to be 100,000.

Other Sources.—Other materials have been tested in this Laboratory as potential sources of fumarase. Sonically ruptured *Escherichia coli* cells[16] had a specific activity of only 9.0 which corresponds to a purity of 0.003% as defined earlier. A cell-free extract of *Azotobacter*,[16] however, had a specific activity of 330 which corresponds to a purity of 0.1%. The latter extract was prepared by sonically rupturing a solution of *Azotobacter* for about 15 minutes and centrifuging it in a Spinco Model E ultracentrifuge at 145,000 *g*. The high fumarase content recommends *Azotobacter* as a good potential source.

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(15) Defined as the optical density of a solution containing 1 mg./ml. in a cuvette with an optical path of 1 cm.

(16) Obtained through the courtesy of Dr. Perry Wilson and Martin Alexander, Department of Bacteriology University of Wisconsin.