Further Studies on N^5-Formyltetrahydrofolic Acid Cyclodehydrase*

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ABSTRACT: A method for purifying N^5-formyltetrahydrofolic acid cyclodehydrase from sheep liver has been developed using ammonium sulfate precipitation and carboxymethyl-cellulose and diethylaminoethyl-cellulose chromatography. This enzyme catalyzes the conversion of N^5-formyltetrahydrofolic acid in the presence of adenosine triphosphate to N^5,N^{10}-methenyltetrahydrofolic acid with the formation of adenosine diphosphate and inorganic phosphate. The product appears to be identical with anhydroleucovorin prepared by acidification of calcium leucovorin. The enzyme was shown to have maximum activity at about *p*H 4.8 and is inhibited by compounds reacting with free sulfhydryl groups.

The inhibition is prevented or reversed by reduced glutathione. The Michaelis constant of the enzyme for N^5-formyltetrahydrofolate was found to be 1.4×10^{-4} M at *p*H 6.0.

This communication reports work in which the enzyme N^5-formyltetrahydrofolic acid cyclodehydrase (citrovorum factor cyclodehydrase) from sheep liver has been more thoroughly purified and its properties and the nature of the reaction catalyzed reinvestigated. Peters and Greenberg (1957, 1958), working with cruder preparations, had suggested that a compound similar to, but distinguishable from, methenyl-FH_4[1] spectrophotometrically was formed in the reaction. Furthermore, their data suggested that ATP was converted to AMP and pyrophosphate in the reaction. The present work has shown that this is incorrect and that the reaction proceeds according to equation (1):

$$N^5\text{-formyl-}FH_4 + ATP \xrightarrow{Mg^{2+}} N^{5,10}\text{-methenyl-}FH_4 + ADP + P_i \quad (1)$$

A second enzyme, $N^{5,10}$-methenyl-FH_4 cyclohydrolase, catalyzes the hydrolysis of methenyl-FH_4 to N^{10}-formyl-FH_4 (equation 2). This enzyme was studied by Tabor and Wyngarden (1959) and found to be extremely labile.

$$N^{5,10}\text{-methenyl-}FH_4 + H_2O \rightleftharpoons N^{10}\text{-formyl-}FH_4 \quad (2)$$

Experimental

Materials. Calcium leucovorin (5-formyl-FH_4, Ca salt) was generously supplied by the Lederle Laboratories and purified as described below. ATP was obtained from the Sigma Chemical Co. and the cellulose ion exchangers were obtained from the Brown Company (Boston). Other chemicals were purchased from commercial firms.

Purification of N^5-Formyl-FH_4.[2] The calcium salt of the commercial product (calcium leucovorin) was purified by chromatography on a TEAE-cellulose column. About 100 mg of the material dissolved in 10 ml of 0.005 M ammonium acetate buffer, *p*H 5.3, was introduced onto the column (1.25 × 12 cm) and eluted stepwise with increasing concentrations of the buffer. The purified material emerged with 1.0 M ammonium acetate buffer in nine fractions of 8.0 ml each.

Further purification was accomplished, and salts were removed by filtering the eluate through a bed of acid-washed charcoal (Darco G-60) formed over a bed of Celite in a glass cylinder (2.5 cm diam) fitted with a sintered glass disk. The N^5-formyl-FH_4 was eluted with 1500 ml of a mixture of butanol-ethanol-water (100:40:50 by volume) adjusted to *p*H 8.1 with ammonia. The solution was evaporated to dryness and sealed in ampoules for use.

Enzyme Assay. Incubations were performed by incubating 0.2 μmole formyl-FH_4, 2.0 μmoles ATP, 2.0 μmoles $MgSO_4$, and 100 μmoles of sodium citrate buffer, *p*H 6.0, in 3.0 ml total volume at 30° in a cuvet of 1-cm light path in a Beckman spectrophotometer.

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[1] Abbreviations used are: formyl-FH_4, N^5-formyltetrahydrofolate (also f^5-FH_4); methenyl-FH_4, N^5,N^{10}-methenyltetrahydrofolate; FH_4, tetrahydrofolic acid; AMP, adenosine monophosphate; ADP, adenosine diphosphate; GTP, guanosine triphosphate; ATP, adenosine triphosphate; UTP, uridine triphosphate; CTP, cytidine triphosphate; DPNH, diphosphopyridine nucleotide.

[2] This procedure for purification of formyl-FH_4 was developed by Dr. Y.-C. Yeh.

Optical measurements were made at 343 mμ.[3] The validity of this assay was established by determining that the methenyl-FH_4 prepared in acid solution and then adjusted rapidly to *p*H 6.0 with the citrate buffer showed only a very slight shift in the absorption spectrum, with the maximum being at 350–353 mμ and the extinction coefficient remaining virtually the same. The methenyl-FH_4 is comparatively stable in the citrate buffer, *p*H 6.0, with a half-life of about 20 minutes, which compares favorably with the half-life of the compound in maleate solution at *p*H 7.0 reported by Rabinovitz (1960).

Enzyme Unit. One unit of N^5-formyl-FH_4 cyclodehydrase activity is defined as the amount of enzyme which will bring about a change in the absorbancy at 343 mμ of 0.001 unit/min. The molar extinction coefficient at 343 mμ, *p*H 6.0, of methenyl-FH_4 was estimated to be 23,600, based on the absorption ratio at 343 mμ to 350 mμ and the figure of 24,900 by Rabinovitz and Pricer (1957) at 350 mμ in 0.17 M HCl. Therefore, a change in absorbancy of 0.001 corresponds to the production of 0.042 mμmole of methenyl-FH_4 per 3 ml. Specific activity of the enzyme is expressed as enzyme units per mg of protein. Protein concentrations were determined by the method of Lowry *et al.* (1951), or, in purified solutions, by the absorption in the ultraviolet (cf. Layne, 1957).

Purification of Sheep Liver Cyclodehydrase. Fresh or freshly frozen livers were homogenized with acetone at −15° to prepare a highly stable acetone powder containing formyl-FH_4 cyclodehydrase activity (Peters and Greenberg, 1957).

Approximately 130–150 g of liver were homogenized for 1 minute with 2 liters of cold acetone at medium speed in a Waring Blendor. The homogenate was filtered on a Buchner funnel and washed with 5 liters of cold acetone to remove most of the pigment. The powder was then dried on the filter paper in a vacuum desiccator. Enzyme activity was extracted from the acetone powder by stirring for 2 hours with cold 0.05 M sodium citrate buffer, *p*H 6.5, using 1500 ml of buffer/100 g of powder. To the supernatant liquid obtained after centrifugation of the suspension at 10,000 × *g* for 30 minutes, solid ammonium sulfate was added to reach 30% saturation (16.8 g/100 ml). The *p*H was maintained at 6.0–6.5 by the addition of dilute ammonium hydroxide. The precipitate which formed was removed by centrifugation and more ammonium sulfate was added to reach 50% saturation (12.1 g/100 ml). After standing overnight at 4°, the suspension was centrifuged at 10,000 × *g* for 30 minutes and the supernatant liquid was discarded. The paste thus obtained retains enzyme activity indefinitely when frozen at −20°. Preliminary to further purification, the salts were removed by dialysis against five changes of 4 liters each of distilled water. At this stage, the specific activity of the enzyme preparation was 0.6–0.7. Lyophilization of the solution resulted in a slight decrease in specific activity.

Chromatography on CM-Cellulose. Further purification of the ammonium sulfate–treated cyclodehydrase preparation was carried out by column chromatography using CM-cellulose and DEAE-cellulose. Preliminary experiments showed that a much higher degree of purification was achieved by first chromatographing on CM-cellulose and then on DEAE-cellulose, rather than using the DEAE-cellulose first. Prior to their use, the celluloses were first purified by washing with sodium hydroxide and hydrochloric acid (Peterson and Sober, 1956). This procedure was also used for regeneration of the cellulose ion exchanger after its use.

Chromatography on the CM-cellulose was performed by adjusting the dialyzed protein from the ammonium sulfate treatment with citrate buffer to *p*H 5.4 and removing the precipitate that formed by centrifugation. About 200 ml of the above solution, containing 4–5 g of protein, was introduced onto a 2.5- × 21-cm column of the CM-cellulose which had been equilibrated to *p*H 5.4 with sodium citrate buffer and washed with 0.005 M buffer at the same *p*H. Chromatography was carried out in the cold room at 4°. Fractions of 15 ml were collected at a rate of 3 ml/min. The enzyme was eluted with 0.005 M sodium citrate at *p*H 5.4. A pink zone remained at the top of the column, and the enzyme activity was eluted in a greenish zone which migrated with the solvent front. Protein determinations were carried out on aliquots of the samples. The results of such a run are shown in Figure 1. Formyl-FH_4 cyclodehydrase activity was highest in the leading edge of the peak which emerged with the solvent front.

Chromatography on DEAE-Cellulose. Active material obtained from CM-cellulose chromatography, after dialysis against distilled water and 0.001 M potassium phosphate buffer, *p*H 7.5, was concentrated on a rotary evaporator and chromatographed on DEAE-cellulose. The concentrated enzyme solution was introduced onto a 2.0- × 30-cm column previously equilibrated to *p*H 7.5 and washed with 0.005 M potassium phosphate buffer. Chromatography was carried out at 4° and 10-ml fractions were collected. Elution was begun with 0.005 M buffer and later changed to 0.035 M. The active material thus obtained, assaying about 11 units/mg, was used for most of the studies of the properties of the enzyme, as this preparation was relatively stable when stored frozen at −20°.

A higher purification was obtained by rechromatography on DEAE-cellulose. Samples of 70 to 155 mg of protein were adsorbed on a 2.0- × 23-cm column at *p*H 7.5 with 0.005 M buffer. Elution was begun with 0.005 M buffer, then raised to 0.02 M, and finally to 0.035 M, with the *p*H maintained at 7.5. The results of such a run are shown in Figure 2. The enzyme obtained on rechromatography, assaying 33–38 units/mg protein, was unstable and lost activity when concen-

[3] The wavelength of 343 mμ was maintained in the enzyme assay procedure because this was used by Peters and Greenberg (1957, 1958) and in the initial studies in the present work, although as is indicated below at *p*H 6.0 the adsorption peak is shifted to about 353 mμ.

TABLE I: Purification of Sheep Liver N^5-Formyltetrahydrofolate Cyclodehydrase.[a]

	Volume (ml)	Total Protein (mg)	Total Enzyme (units)	Specific Activity	Yield (%)
Acetone powder extract	2640	55,440	4580	0.083	100
$(NH_4)_2SO_4$ treatment	210	5,580	3315	0.625	73
CM-cellulose	50	1,780	2840	1.60	62
First DEAE-cellulose		155	1558	10.7	34
Second DEAE-cellulose		18	596	33.1	13

[a] Sheep liver acetone powder (200 g) extracted with 3000 ml of 0.05 M sodium citrate buffer, *p*H 6.5, and centrifuged to remove insoluble material.

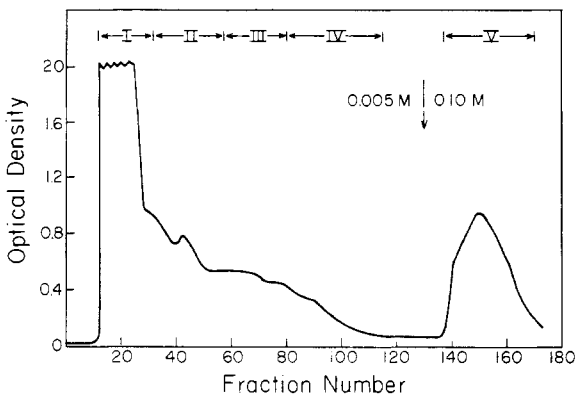

FIGURE 1: Chromatography of sheep liver N^5-formyltetrahydrofolic acid cyclodehydrase on CM-cellulose. Protein concentration: solid line. Chromatography was carried out as described in the text using sodium citrate buffer, *p*H 5.4. Fraction size: 12 ml. The arrow indicates the point at which the concentration of eluting buffer was changed. Specific activities of eluate fractions: I, 1.67; II, 1.0; others, zero.

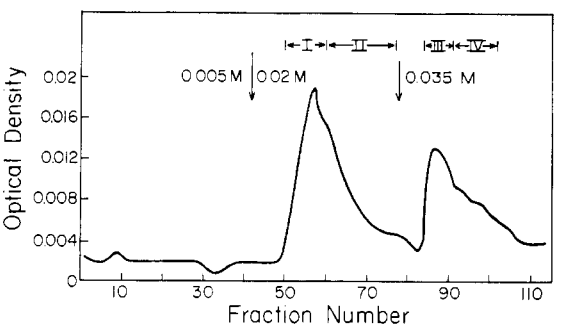

FIGURE 2: Rechromatography of N^5-formyltetrahydrofolic acid cyclodehydrase on DEAE-cellulose. Protein concentration: solid line. Chromatography was carried out using potassium phosphate buffer, *p*H 7.5. The left-hand arrow indicates a change in eluting buffer from 0.005 to 0.020 M. The right-hand arrow indicates a change to 0.035 M. Fraction size: 12 ml. Specific activities of eluate fractions: I, 10; II, 38; others, zero.

trated on a rotary evaporator at temperatures below 20° or when lyophilized.

Activities of the enzyme in a typical preparation at various levels of purification are shown in Table I. The total procedure has been repeated at different times by several workers and yielded enzyme of comparable specific activity.

The activity of the N^5-formyl-FH_4 cyclodehydrase either is very low in sheep liver or it has a very low turnover number. The best purified preparations obtained had an estimated conversion rate of only 84 mμmoles/hour per mg of protein. Since formyl-FH_4 apparently is a storage form of the folic acid derivatives and is metabolically comparatively inert, high enzyme activity may not be an essential for the metabolism of the organism.

$N^{5,10}$-Methenyl-FH_4 Cyclohydrolase Activity. The absence of any hydrolase activity in the cyclodehydrase preparation was demonstrated in the following manner. Synthetic formyl-FH_4 was converted to methenyl-FH_4 by acidification with HCl to *p*H 1 (May *et al.*, 1951). The progress of the conversion reaction was followed by measuring the increase in light absorption at 358 mμ in a Beckman Model DU spectrophotometer. At the *p*H employed for the enzyme reaction, 6.0, methenyl-FH_4 is progressively converted to N^{10}-formyl-FH_4. A control containing no enzyme was run in order to measure this spontaneous conversion. The rates of decrease in absorbancy at 358 mμ for the control and enzyme-containing reaction mixtures are shown in Figure 3. The conversion of methenyl-FH_4 to N^{10}-formyl-FH_4 is not significantly changed by the addition of the cyclodehydrase preparation.

Effect of Cations on Enzyme Activity. Previous studies (Peters and Greenberg, 1957) had shown the importance of the choice of anions in the buffers used to extract the biological activity from the acetone powder. In this work, a comparison was made between

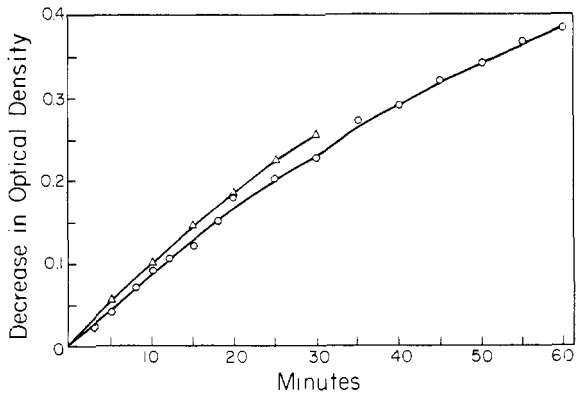

FIGURE 3: Conversion of N^5,N^{10}-methenyltetrahydrofolic acid to N^{10}-formyltetrahydrofolic acid. (Δ—Δ), optical density of control run containing 0.276 μmole of methenyl-FH_4, 11.0 μmoles of mercaptoethanol, and 150 μmoles of *p*H 6.0 sodium citrate buffer in a total volume of 3.0 ml. (O—O), optical density of experimental run containing, in addition to the above, 0.40 mg of cyclodehydrase.

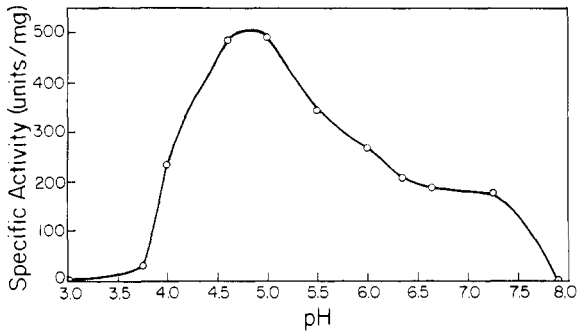

FIGURE 4: *p*H-activity curve of N^5-formyltetrahydrofolic acid cyclodehydrase. Cuvet contents: 2.0 μmoles of ATP, 2.0 μmoles of $MgSO_4$, 100 μmoles of sodium citrate buffer, 125 μg of formyl-FH_4, and 0.2 mg of enzyme in a total volume of 3.0 ml. Specific activities are corrected for spontaneous conversion to methenyl-FH_4 at low *p*H values.

Na^+ and K^+ citrate buffers at *p*H 6.0 utilizing the usual assay conditions. No significant difference between the two cations was found. The potassium citrate-containing reaction mixture assayed 7% higher than that containing sodium citrate. This is within the experimental error of the assay.

Temperature Stability. The effect of temperature on the stability of the enzyme was studied by preincubating the enzyme at a given temperature for 5 or 10 minutes before assaying it under the normal assay conditions of 30°. The results of this experiment are shown in Table II. The enzyme maintains full activity up to 45° and is progressively inactivated at higher temperatures.

TABLE II: Temperature Stability of N^5-Formyltetrahydrofolic Acid Cyclodehydrase.

Preincubation Temperature (°C.)	Preincubation Time (minutes)	Enzyme Activity (units/mg)	Original Activity Remaining (%)
30		4.3	100
40	5	4.6	109
40	10	4.8	113
45	5	4.4	104
45	10	4.4	104
50	5	3.3	77
50	10	2.1	50
55	5	0.8	18
55	10	0.4	9

Optimum pH of Cyclodehydrase Reaction. Previous studies on a cruder enzyme preparation indicated that the optimum *p*H for the reaction was at 3.8 (Peters and Greenberg, 1958). This has been repeated using a preparation which assayed 9.2 units/mg. The results obtained, corrected for spontaneous conversion of formyl-FH_4 to methenyl-FH_4 at low *p*H, are shown in Figure 4. The optimum reaction rate appears to be at about *p*H 4.8.

Michaelis-Menten Constant. The Michaelis-Menten constant for the substrate, formyl-FH_4, was estimated at *p*H 6.0, under standard assay conditions with the substrate concentration being varied over the concentration range of 9.0×10^{-7} to 1.8×10^{-4} M. A K_m value of 1.43×10^{-4} M was calculated from the double reciprocal plot of the data thus obtained.

The Michaelis constant of ATP was similarly determined with the formyl-FH_4 concentration increased to 0.2 mM. The K_m value obtained was 4.5×10^{-4} M.

Enzyme Inhibitors. It was shown by Peters and Greenberg (1958) that the N^5-formyl-FH_4 cyclodehydrase was inhibited by the sulfhydryl agents, *p*-mercuribenzoate and *o*-iodosobenzoate. The inhibition could be prevented by the presence of reduced glutathione. In additional experiments, it was found that the enzyme also was inhibited by *N*-ethylmaleimide (50% at 1.7×10^{-3} M) and silver nitrate (100% at 3.3×10^{-4} M), but not by iodoacetate (at 10^{-3} M).

To determine the reversibility of the inhibition further, an experiment was performed under standard assay conditions with 1×10^{-5} M *p*-mercuribenzoate. This reduced the conversion of formyl-FH_4 to 5% of the control level. The reaction rate was returned to 90% of the control value upon addition of 1×10^{-3} M reduced glutathione to the latter.

Identification of Reaction Products, Methenyl-FH_4. Repetition of the experiment of Peters and Greenberg (1958) on the fractionation of the enzyme reaction products by chromatography on Dowex 1 (Cl^- form)

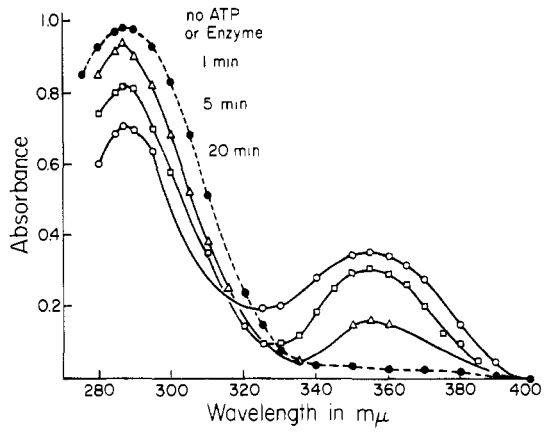

FIGURE 5: Spectrophotometric determination of enzymatic formation of methenyltetrahydrofolate from N^5-formyltetrahydrofolate. The incubation mixture in a 3-ml volume contained citrate buffer, *p*H 6.0 (100 μmoles), $MgSO_4$ (200 μmoles), ATP (2 μmoles), mercaptoethanol (20 μmoles), and enzyme (4 mg). The reaction was started by the addition of 0.05 ml of buffer containing the formyl-FH_4. The blank cuvet contained all components except the substrate.

failed to confirm the previous results. Double amounts of the constituents of a normal enzyme assay reaction were introduced onto a 1.0- × 13.5-cm column of Dowex 1X8 (200–400 mesh) washed with 0.005 M ammonium choride, *p*H 6.1. The formyl-FH_4 was first converted to methenyl-FH_4 by acidification. Contrary to the reports of Peters and Greenberg, no methenyl-FH_4 was eluted with 0.1 N HCl. The fraction emerging with 1.0 N HCl was found to be identical with synthetic methenyl-FH_4 under identical conditions in a Cary recording spectrophotometer, Model 14. No other absorbing material appeared in the 1.0 N HCl fraction, and the presence of a phosphorylated intermediate was further discounted by the absence of any phosphate in the fraction identified as methenyl-FH_4.

Additional evidence for the enzymatic formation of methenyl-FH_4 from formyl-FH_4 was obtained spectrophotometrically by scanning the changes in the absorption spectra of the enzyme incubation mixture between 280 and 380 mμ in a Bausch and Lomb Spectronic 505 recording spectrophotometer. The time taken for one complete sweep of the spectrum was about 1 minute. The conditions of the experiment are given in the legend to Figure 5.

Figure 5 shows the parallelism in the disappearance of formyl-FH_4 with the formation of methenyl-FH_4, by the changes in their characteristic spectral maxima. As mentioned earlier, it was determined that methenyl-FH_4 in citrate buffer, *p*H 6.0, has an absorption peak at 350–353 mμ, and the half-life of the compound at this *p*H is about 20 minutes. That the change in the spectra was enzymatically induced was shown by the absence of the alterations in spectra when enzyme or ATP were omitted.

Products from ATP. It had been previously postulated that the ATP required for the enzymatic conversion of formyl-FH_4 to methenyl-FH_4 was broken down to form AMP and pyrophosphate (Peters and Greenberg, 1957). This reaction was studied using highly purified enzyme. The reaction mixture was fractionated by descending paper chromatography using a 1-propanol–trichloroacetic acid–ammonia system (Cerletti *et al.*, 1957) with Whatman No. 1 paper. The compounds found were unreacted ATP, ADP, and P_i. The nucleotides were detected by ultraviolet light, the phosphates by an ammonium molybdate–ascorbic acid reaction.

The stoichiometry of ATP cleavage was established by measuring the amounts of ADP and ATP present after completion of the reaction by the method of Munch-Peterson and Kalckar (1957), employing 5′-adenylate deaminase, adenylate kinase (myokinase), and potato apyrase. The 5′-adenylate deaminase was prepared by the procedure of Nikiforuk and Colowick (1955); adenylate kinase was obtained commercially and potato apyrase was prepared according to the method of Krishnan (1955).

The reaction mixture, containing 100 μmoles of sodium citrate buffer, *p*H 6.0, 2 μmoles each of ATP and $MgSO_4$, and 1 μmole of formyl-FH_4 in a total volume of 3.0 ml, was incubated with 3.5 units of enzyme at 30° for 30 minutes. At the end of the incubation period, the reaction mixture was chilled in ice and the ADP and ATP contents were assayed.

Reaction mixture (0.05 ml) was transferred to a 3-ml cuvet containing 2.5 ml of 0.3 M succinate buffer, *p*H 6.1, and 0.25 ml of 0.1 M $MgCl_2$. To this 0.2 ml of adenylate deaminase (50 mg/ml) was added. This resulted in no significant change in absorbancy at 265 mμ until adenylate kinase was added (resulting in the conversion of ADP to AMP and ATP). Measurement of the decrease in absorbancy was determined until this ceased (5 minutes). At this point, apyrase (0.02 ml, 300 μg/ml) was added and readings were taken until the absorbance became constant. The ADP and ATP were calculated from the data and yielded the following results:

ATP remaining (μmoles)	ATP decomposed (μmole)	ADP formed (μmole)
1.35	0.65	0.58
1.39	0.61	0.64

The virtual absence of ATPase in the enzyme preparation was established by test with the method of Lowry and Lopez (1946).[4] Ten mg of enzyme was incubated in citrate buffer, *p*H 6.0, with 40 mμmoles of ATP and 20 μmoles of $MgSO_4$ at 30°. Inorganic phosphate liberated was determined at several time intervals up to 17 minutes. No increase in P_i occurred in the presence of the ATP.

Stoichiometry of Disappearance of Formyl-FH_4 and Formation of ADP. This was determined by measuring

[4] Test kindly performed by D. Kasbekar.

the disappearance of the formyl-FH_4 by the decrease in absorbance at 287 mμ and the simultaneous increase in ADP by means of the combined pyruvate kinase–lactic dehydrogenase method of Kornberg and Pricer (1951). The results shown in Table III are consonant with the interpretation that 1 mole of ATP is required to secure the dehydration of 1 mole of formyl-FH_4 and that the products are ADP and P_i. Absence of adenylate kinase in the enzyme preparation was shown by the above procedure for ADP estimation, using ADP as the substrate in the incubation. When radioactive P_i was added to the cyclodehydrase reaction mixture and the products were separated by paper chromatography, it was not possible to demonstrate the incorporation of P^{32} into the ATP present under conditions which would detect an exchange of greater than 0.1%.

TABLE III: Stoichiometry between Disappearance of 5-Formylfolate-H_4 and ADP Formation.[a]

f_5-FH_4 added (mμmoles)	$-\Delta A$ at 287 mμ	f^5-FH_4 Disappearing (mμmoles)	$-\Delta A$ at 340 mμ DPNH Disappearing (mμmoles)	ADP Formed (mμmoles)
200	0.51	65	0.105	58
200	0.58	72	0.110	61
100	0.26	32	0.070	35
100	0.24	30	0.045	25

[a] Contents of the incubation mixtures were the same as in Figure 5, with the differences that the ATP content was 5 μmoles and the 5-formyl-FH_4 was 0.1 or 0.2 μmole; 0.5 ml enzyme was added (10 mg/ml). The decrease in absorbancy at 287 mμ was followed for 30 minutes; then there were pipetted into the cuvet, successively, 0.44 ml of 0.5 M potassium phosphate buffer, *p*H 7.2, 0.02 ml of DPNH (0.2 μmole), 0.02 ml. of lactic dehydrogenase (0.1 mg protein/ml), and 0.02 ml. of pyruvate kinase (0.5 mg protein/ml). The decrease in absorbancy was followed at 340 mμ until a constant value was reached (about 4 min). The ADP was calculated from the amount of DPNH oxidized.

Kay *et al.* (1960), working with a chicken liver enzyme which catalyzes the conversion of formyl-FH_4 to N^{10}-formyl-FH_4, reported that other nucleoside triphosphates could be used to replace ATP, although not all were equally effective. The ability of GTP, UTP, and CTP to replace ATP was also observed with the sheep liver enzyme system.

Discussion

The ATP-dependent conversion of formyl-FH_4 to N^{10}-formyl-FH_4 was first observed by Greenberg (1954) in pigeon liver extracts. This author inferred the intermediate formation of methenyl-FH_4. Subsequently, Peters and Greenberg (1958) separated N^5-formyl tetrahydrofolate cyclodehydrase, which catalyzes the reaction of equation (1). Kay *et al.* (1960) reported the presence of an enzyme in chicken liver which isomerizes formyl-FH_4 to N^{10}-formyl-FH_4 without the intermediation of methenyl-FH_4, although the reaction is ATP dependent. However, with a similar isomerase from *Micrococcus aerogenes* (Kay *et al.*, 1960), there was an accumulation of methenyl-FH_4.

As pointed out by Greenberg (1954), it would be expected that the formation of the onium structure in methenyl-FH_4 from formyl-FH_4 would require energy. This explains the dependency of the reaction on ATP. It is not implausible to presume that a transitory intermediate would be formed in the reaction, either a phosphate or adenylate adduct with a high energy bond. Peters and Greenberg (1958) presumed that they had obtained evidence for such an intermediate based on differences in the absorption spectra of methenyl-FH_4 and the product obtained by chromatography of the enzymatic incubation products on Dowex 1 (Cl^-). Also, the incubation product appeared to be more stable in neutral solution than methenyl-FH_4. This could not be confirmed in the present work, and methenyl-FH_4 is the only detectable product formed in the enzymatic reaction.

An interaction between ATP and the substrate, mediated by the enzyme, would confidently be expected to be involved in the reaction mechanism. Experimental evidence for this was sought in experiments on the exchange between $^{32}P_i$ and ATP. No exchange could be demonstrated. We have no explanation at this time for the negative results. Jaenicke and Brode (1961) also observed no exchange between $^{32}P_i$ and ATP with FH_4 formylase.

In a still earlier work by Peters and Greenberg (1957), it was provisionally concluded that AMP and PP were reaction products, based on the finding that roughly 2 moles of P_i appeared per mole of ATP utilized. In view of the crudeness of enzyme preparation used in that work, decomposition of ATP and of ADP by apyrase in the material could account for this result. Kay *et al.* (1960) demonstrated the formation of ADP in the reaction catalyzed by chicken liver isomerase by utilization of a pyruvic kinase–lactic dehydrogenase assay system. The estimated amount was less than that expected from the stoichiometry of the reaction. The authors also noted that analysis for P_i and PP were obscured by the presence of folic acid materials. With the purified enzyme employed in the present work, it has been established that ADP and P_i are the only reaction products.

This investigation has resulted in the isolation of a more highly purified preparation of the cyclodehydrase enzyme and in the clarification of certain discrepancies concerning the reaction, bringing it in harmony with related work of other investigators.

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Countercurrent Distribution of Chemically Reacting Systems. IV. Kinetically Controlled Dimerization in a Boundary*

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ABSTRACT: Countercurrent distribution is a discrete separation process for which any theoretical treatment is handled conveniently by digital computer methods. Useful information about other separation methods can be derived, by analogy, from the predictions obtained from such calculations. In particular, it is possible to treat the difficult problem of the separation of chemically reacting systems by this method (Bethune, J. L., and Kegeles, G., 1961, *J. Phys. Chem. 65*, 1761). This investigation includes the effects of kinetics and diffusion in a dimerizing system subjected to transport. When diffusion effects are taken into account, the distinctive features of such systems, found by solution of asymptotic equations (Belford, G. G., and Belford, R. L., 1963, *J. Chem. Phys. 37*, 1926), are altered. For example, alterations are found in the functional dependence of peak height and area upon time as well as in the range of the relevant parameters over which three boundaries are observed. In addition, it is found that either monomer or dimer can give bimodal boundaries, while the total pattern shows either one, two, or three boundaries.

The theory for moving-boundary experiments involving a single species in a countercurrent distribution apparatus has been rigorously developed by Bethune and Kegeles (1961c). In this paper, it was shown that first differences of concentration for a moving-boundary process in countercurrent distribution were identical with the corresponding concentration values, as described by binomial coefficients, for the zone process. When the number of transfers is large, the pattern described by the binomial coefficient expressions is very nearly equal to a Gaussian error function (Craig and Craig, 1950). In the case of electrophoresis, the solution of the continuity equation, starting with an infinitely sharp zone, gives the Gaussian error function for concentration, while starting with an infinite volume of solution results in the Gaussian error function for the derivative of the concentration curve. If an analogy is drawn between certain parameters in one process (for example, number of transfers), and corresponding parameters in the other process (for example, time), the results become formally identical. It is on the basis of such a correspondence that the theoretical results obtained for countercurrent distribution may be

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