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ACETOACETATE DECARBOXYLASE ACTIVITY OF FRACTION V HUMAN SERUM ALBUMIN

by

Robert R. Rosecrans

A Dissertation Submitted to the Faculty of the Graduate School of Loyola University of Chicago in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

January 1980

[illegible]

ACKNOWLEDGMENTS

The author is indebted to Dr. Maurice V. L'Heureux for his continued guidance, support and encouragement extending from the beginning of this study to its completion. His integrity as a teacher, advisor and a citizen have made a indelible impression upon the author.

The author would also like to thank all the members of the Department of Biochemistry and Biophysics of Loyola University of Chicago for their comments and exchange of ideas during the course of this study.

The author is indebted to Dr. Frank H. Westheimer, Harvard University, for a sample of the bacterial enzyme acetoacetate decarboxylase, and to Dr. William E. Dulin, the Upjohn Company, Kalamazoo, Michigan, for a one gram sample of streptozotocin for use in this study.

Finally, the author wishes to express his sincerest gratitude to his wife, Marlene, for her encouragement and support during this investigation.

VITA

The author, Robert Richard Rosecrans, is the son of Robert Matthew Rosecrans and Dolores (Testa) Rosecrans. He was born August 8, 1948, in Chicago, Illinois.

His secondary education was obtained at Lane Technical High School, Chicago, Illinois, from which he graduated in 1966. In September 1966, he entered Northeastern Illinois University, and in August 1970, received the degree of Bachelor of Arts with a major in Chemistry. From January 1971, to December 1973, he served on active duty in the United States Army.

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American Association for the Advancement of Science

American Association for Clinical Chemistry

Sigma Xi.

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CHAPTER I

INTRODUCTION

World War I caused a great increase in the demand for acetone which was needed in the manufacture of cordite and airplane wing dope. The available supplies of acetone were inadequate and researchers began to investigate fermentation processes for the conversion of carbohydrate to acetone. Northrup et. al., (1919) found, that during the fermentation of potatoes, high levels of acetone and ethyl alcohol were produced. From the fermenting potatoes, the workers isolated a microorganism responsible for the production of acetone and ethyl alcohol. Microscopic morphology showed the organism to be gram negative and rod-shaped. Northrup et. al., (1919) named the microorganism Bacillus acetoethylicum on the basis of the cell morphology and end products produced during the fermentation of potatoes. Reilly et. al., (1920) isolated a microorganism from the fermentation of corn mash. The workers found that during the fermentation of corn mash, there are high levels of acetone, butyl alcohol and ethyl alcohol produced. As the pH of the culture medium began to drop, there were further increases in the production of acetone. If the acidification of the culture medium was prevented, the production of acetone was curtailed. On the other hand, the addition of acetic acid to the culture medium resulted in increased production of acetone by the microorganism. Johnson et. al., (1933) studied the intermediate compounds in the acetone-butyl alcohol fermentation of corn mash. These workers discovered that the acetone produced in the culture medium and

from centrifuged washed cells of Clostridium acetobutylicum arose from the decarboxylation of acetoacetic acid. The production of acetic acid in the culture medium was found to be responsible for the decreasing pH. Wood et. al. (1945) studied the effect of adding (1-^{13}C-) acetate to the culture medium, and found that the labeled carbon was incorporated exclusively into acetone. To account for this, a mechanism was proposed in which two moles of acetate condense to form one mole of acetoacetate; the acetoacetate, in turn, is decarboxylated to form one mole each of acetone and carbon dioxide.

Early work on cell free extracts of Clostridium acetobutylicum was carried out by Davies (1943). Partial purification of acetoacetate decarboxylase (E. C. 4.1.1.4 acetoacetate carboxylase) was attained by extraction of acetone powder of washed cells of Clostridium acetobutylicum. The enzyme has an optimum pH of 5.0 and a K_m of 8mM. The enzyme is heat stable and is inhibited by heavy metal ions such as mercuric, silver, cupric and ferric. Davies also demonstrated that the enzyme associated with Clostridium acetobutylicum is very specific for acetoacetate and it will not decarboxylate either alpha or other beta keto acids. Since its initial partial purification by Davies (1943), the enzyme has been extensively purified (Hamilton and Westheimer 1959a) and subsequently crystallized (Zerner et. al., 1966). These procedures were described as follows:

> The spores of Clostridium acetobutylicum are harvested from sterile sand and grown in test tubes containing a growth medium of L (+) arabinose, trypticase, yeast extract, L-asparagine, and ascorbic acid. The salt composition of the medium comprises magnesium sulfate heptahydrate, manganous sulfate monohydrate, ferrous sulfate heptahydrate and sodium chloride. Growth is

evident by gas evolution after approximately 24 hours. After 4-7 days, the bacteria are used to inoculate 20 liter carboys of the growth medium. After 3 days, the cells are harvested by centrifugation and used to prepare an acetone powder. The packed cells are homogenized with cold acetone in a chilled Waring blender. The dehydrated cells are collected on a Buchner funnel and washed with cold acetone. The filter cake is dried in vacuo and ground with a mortar and pestle. Each 100 g of acetone powder is extracted for 2 hours at 37°C on a rotary shaker with 1500 ml of 50 mM phosphate buffer, pH 5.9. The most important step in the purification procedure is the precipitation of the active material from the clear extract by acidification to pH 3.8-3.9 with 2 M acetic acid. The protein is collected by centrifugation for 2 hours at 3900 rpm at 4°C. The assay of this material reveals a 20-67 fold increase in the specific activity of the enzyme. The protein is collected, resuspended in 100 ml of 50 mM phosphate buffer, pH 5.9 and mixed to redissolve the material. The suspension is clarified by centrifugation at 10,000 rpm. The enzyme is precipitated out of the clarified solution at 60% saturation with solid ammonium sulfate at room temperature. The enzyme is collected by centrifugation, dissolved in 10-15 ml of 50 mM phosphate buffer and desalted by dialysis against phosphate buffer, pH 5.9. The solution is heat-activated at 55°C for 1 hour and purified by passing the protein through a DEAE cellulose column equilibrated with the phosphate buffer. The buffer is passed through the column until no more material which absorbs at 280 nm is observed. The column is then subjected to an ammonium sulfate salt gradient from 0-0.1 M. The enzyme is eluted in a well defined, sharp peak. Fractions which exhibit a 280 nm: 260 nm ratio of 1.9 or greater are pooled and brought to 75% saturation with ammonium sulfate. The precipitated protein is collected by centrifugation at 10,000 rpm. Once again the material is dissolved in 5-10 ml of 50 mM phosphate buffer, pH 5.9 and crystallized at room temperature by the successive addition of small amounts of solid ammonium sulfate on a stirring rod. The growth of thin hexagonal plates are observed after about 12 hours.

An interesting property of the bacterial enzyme is that of latent enzymatic activity. Latent activity is exposed after the enzyme is heated at 55°C for 1 hour. The activation by heat causes a two-fold

increase in the enzymatic activity. The activation is first order and effectively irreversible (Neece and Fridovich, 1967). They reported that the rate of activation is not affected by dilution of the enzyme solution, dialysis or extensive purification. The latent decarboxylase will co-sediment with the expressed activity in a sucrose density gradient. Neece and Fridovich (1967) postulated that the latent material is converted to active enzyme by a conformational change within the latent enzyme. Once the latent enzyme sites are activated, they cannot be distinguished from the original active enzyme with respect to inhibition by monovalent anions, to substrates and to known competitive inhibitors of the active enzyme. Attempts to activate latent sites by the use of denaturants such as urea and ethanol, or with the addition of the proteolytic enzymes trypsin, α-chymotryspin and pronase were unsuccessful. Nothing is known about chemical nature of the latent site or the reason why the native enzyme contains latent enzyme sites.

The molecular weight of the bacterial enzyme is 340,000 $\pm$ 10,000 as determined by equilibrium sedimentation (Tagaki and Westheimer (1968a). After exposure to pH 2.5, 4.0 M urea or 6.0 M guanidine hydrochloride, the enzyme dissociates into its subunits. Each subunit weighs 29,000 $\pm$ 1,000 and it appears that the native enzyme is a dodecamer. If the native enzyme is subjected to a pH of 8 and 4.0 M urea, the enzyme will dissociate into dimers weighing 60,000. Once the urea has been removed, the dimers will reassociate to form the native dodecamer. However, if the dodecamer is dissociated into the monomers, these will not reassociate to form the dodecamer or any intermediate molecular species.

The amino acid content of the subunit has been determined by Lederer et al., (1966). Methionine is the only N-terminal amino acid present and lysine appears at the C-terminal. The amino acid composition shows 20 lysine residues, 12-13 arginine residues and 9 histidine residues. The positive charges are countered by 29 aspartic acid residues and 20 glutamic acid residues; 17 of the glutamic acid residues are bound as amides. At pH 5.0 it would be expected that the molecule would possess a strong positive charge yet its isoelectric point is 4.9 (Lederer et al., 1966).

The bacterial enzyme is stable at 25°C in the pH range of 4-9 (Lederer et. al., 1966). The enzyme is inactivated by denaturants such as urea, guanidine hydrochloride and sodium dodecyl sulfate (Tagaki, 1968). The enzyme is resistant to heat; heating at 70°C in a 50 m*M* phosphate buffer, pH 5.9, results in no perceptible loss of activity (Autor and Fridovich, 1970b). When the enzyme is subjected to temperatures above 70°C, there is an irreversible loss of activity. The loss of activity appears to be biphasic; there is a rapidly inactivated component (within 10 minutes) and a slowly inactivated component (within 60 minutes). Autor and Fridovich (1970b) postulated that during heat inactivation, the native enzyme is converted either to an inactive enzyme or a modified enzyme which contains less activity than the native enzyme. The modified enzyme is then converted at a very slow rate to the inactive species. The presence of acetylacetone will prevent the thermal inactivation of the enzyme up to a temperature of 85°C. It is not known how acetylacetone performs this action.

Hamilton and Westheimer (1959b) first proposed that the mechanism of action for the bacterial enzyme occurred through a Schiff base inter-

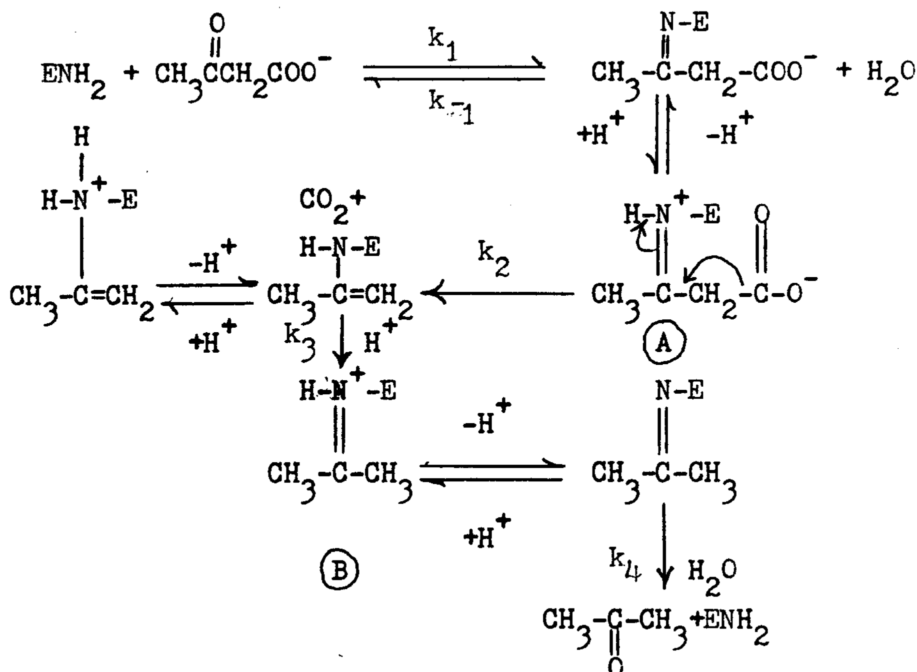
mediate formed between the beta carbonyl group of acetoacetate and a specific amino group in the active site. The proposed mechanism was based upon the observation that there occurs an obligatory oxygen exchange between labeled oxygen in the carbonyl position and the solvent water. The oxygen exchange is a necessary step in the formation of a Schiff base. Schiff bases are extremely susceptible to reduction by sodium borohydride. Fridovich and Westheimer (1962) used this information to demonstrate that sodium borohydride will inhibit the bacterial enzymatic activity. Sodium borohydride will reduce the ketimine intermediate which is formed between the enzyme and substrate. The reduction produces a complex which inactivates the enzyme by blocking the active site and acting as a non-competitive inhibitor. Fridovich and Westheimer (1962) were able to trap the Schiff base intermediate and to detect it radiochemically. Acetoacetate labeled in the carbonyl position with carbon-14 was reacted with the enzyme and sodium borohydride. The borohydride reduced an intermediate and formed a stable radioactively-labeled enzyme complex. Unreacted acetoacetate and labeled acetone produced by nonenzymatic decarboxylation of acetoacetate were removed by dialysis and the radioactivity of the enzyme complex was counted by liquid scintillation. The borohydride reduction with labeled substrate resulted in an 18-fold increase in the radioactivity incorporated into the enzyme. The labeled enzyme was subjected to hydrolysis with hydrochloric acid. The hydrolysate was subjected to two dimensional paper chromatography and radioautography. The radioautograph showed a single spot responsible for the radioactivity. Fridovich and Westheimer (1962) had thus trapped the Schiff base intermediate with the sodium borohydride reaction and given further evidence for its existence. These results

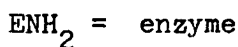
indicated that borohydride reduces a compound formed between the enzyme and acetoacetate and that this reduction forms a bond stable to acid hydrolysis. The available evidence supported the concept that the enzymatic decarboxylation of acetoacetate proceeds by way of a Schiff base intermediate.

The amino acid sequence of the active site has been determined by Laursen and Westheimer (1966). The workers subjected the borohydride-reduced radiochemically trapped enzyme to the proteolytic actions of trypsin and chymotrypsin. The peptides were separated by column chromatography and the radioactive peptide was sequenced. The sequence of the peptide was identified as follows:

- GLU - LEU - SER - ALA - TYR - PRO - LYS* - LYS - LEU

The lysine bearing the asterisk represents the site of the Schiff base formation. A single one lysine residue in the active site seems to account for the catalytic activity of acetoacetate decarboxylase. Currently, the proposed scheme for the mechanism of action of acetoacetate decarboxylation is as follows (Warren et. al., 1966):



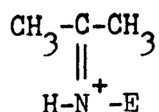


In the first step of the mechanistic scheme, there is a nucleophilic attack by the episilon amino group of the reactive lysine on the beta carbonyl group of acetoacetate. The nucleophilic attack results in the formation of a Schiff base with the obligatory oxygen exchange described by Hamilton and Westheimer (1959b). The Schiff base in the first reaction is referred to as the enzyme-substrate complex. The enzyme substrate complex is reduced to form a Schiff base salt or a ketimine (labeled A in the Scheme). The ketimine has a localized positive charge which pulls electrons from the carboxylate terminal by a process known as induction. The induced electrons stabilize the complex and the first product carbon dioxide is released. The resulting complex then undergoes reduction to form a Schiff base salt of the enzyme-pro-

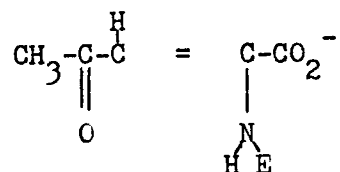
duct complex (labeled B in the Scheme). Both Schiff base salts (A and B) are susceptible to reduction by borohydride; however only the ketimine B salt has been isolated. Tagaki et al., (1968d) has suggested that the conversion of ketimine A to B is very rapid and, therefore, little or no reduction occurs at complex A. The ketimine B is oxidized to form the Schiff base of acetone and this complex subsequently undergoes hydrolysis to yield the final product acetone and releases the free enzyme.

Acetopyruvate, a **competitive** inhibitor of the bacterial enzyme, will protect the enzyme against reduction by borohydride (Tagaki, et. al., 1968). Acetopyruvate is a beta diketone and thus is an analogue of the substrate acetoacetate. Acetopyruvate has been shown to bind to the active site of the enzyme; however, the acetopyruvate enzyme complex will not go on to form a product or any other intermediate. The acetopyruvate enzyme complex will not support the borohydride reduction because the complex formed between the analogue is electron-rich. Borohydride will reduce carbon to nitrogen double bonds of Schiff bases and Schiff base salts which are relatively electron-deficient. The enamine double bond formed between acetopyruvate and enzyme is electron-rich, and therefore, not reducible by borohydride.

Below is an example of the electron-deficient complex formed between acetoacetate and the bacterial enzyme and the electron-rich bond formed between acetopyruvate and the enzyme.



Electron-deficient Schiff base salt of acetone



Electron-rich acetopyruvate enzyme complex

The formation of the acetopyruvate enzyme complex gives evidence for the existence of ketimine A in the mechanistic scheme. The acetopyruvate enzyme complex is an analogue of the enzyme substrate complex. If complex A is formed, its conversion to complex B must be very rapid because it has not been detected with the native substrate.

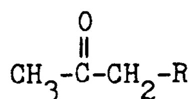
It is possible that the oxidation and hydrolysis of ketimine B is the rate limiting step in the mechanism, and therefore, the concentration of $B \gg A$. This could account for the fact that a borohydride reduced ketimine A complex has not been isolated.

The bacterial enzyme has a K_m of 8 m$\underline{M}$ with an optimum activity at pH 5.9 (Westheimer, 1963). The enzyme has a high specificity for acetoacetate; it has no action on alpha or other beta keto acids. The compounds so tested include acetopyruvate, acetone dicarboxylate, oxaloacetate, α-ketoglutrate and pyruvate. Phenylacetoacetate will serve as a substrate, but the enzyme has a poorer K_m (10 m$\underline{M}$), than the native substrate acetoacetate (Fridovich, 1972).

Acetoacetate decarboxylase is inhibited by structural analogues of the substrate, by compounds that interfere with the formation of a Schiff base and by numerous monovalent anions. Acetone sulfonate, an analogue of acetoacetate, has a K_I of 10 m$\underline{M}$. The analogue reacts with the enzyme to form an enzyme-substrate complex, but it will not be converted to an enzyme-product complex (Fridovich, 1968). The inhibitor

will support the reduction of the analogue-enzyme complex. This is very interesting because the borohydride reduction of labeled acetoacetate in the presence of acetoacetate decarboxylase occurs only in the enzyme-product complex. This indicates two things, first, the Schiff base intermediate is confirmed, and secondly, the decarboxylation step is very rapid. Thus, in the mechanistic scheme shown earlier, the steady state conditions must favor very high levels of the enzyme-product complex as compared to the enzyme-substrate complex (B >> A).

Beta diketones are very potent inhibitors of acetoacetate decarboxylase. They will protect the enzyme against reduction by borohydride in the presence of acetoacetate. The ability of beta diketones to inhibit the enzyme develops rather slowly. This indicates that there is an association of the diketone with the active site. The binding of diketone to the active site prevents the borohydride reduction. Beta diketones form carbon to nitrogen bonds which are electron-rich and, therefore, not reducible by borohydride. The essential feature of inhibitory beta diketones is:



$$CH_3-\overset{\overset{\displaystyle O}{\|}}{C}-CH_2-R$$

where R is some electron withdrawing group. Acetylacetone and acetopyruvate, two of the simplest diketones, inhibit the enzyme by forming a ketimine between the epsilon amino group of lysine and the carbonyl group adjacent to the methyl group. Both inhibitors will prevent the borohydride reduction. Removal of the inhibitors by dialysis results in a recovery of the enzymatic activity. Lineweaver-Burk plots demonstrate

that the inhibition by acetylacetone and acetopyruvate is competitive (Tagaki et. al., 1968; Autor and Fridovich, 1970a).

Monovalent anions will inhibit the activity of acetoacetate decarboxylase. The inhibition is due to the binding of the anions to a cationic Schiff base salt. Four characteristics of the inhibition include: (a) The cationic site behaves such that the pKa is 5.8 or that consistent with an ammonium salt residue; (b) Binding sites indicate that one anion per site is sufficient to cause a loss of activity. An increase in temperature will cause a decrease in the potency of inhibition by bromide; (c) There appears to be a critical ionic volume necessary for the inhibition by monovalent anions. As the ionic size increases from F^- to Cl^- to Br^-, the entropy for the reaction of the anion with the sensitive enzymic site becomes increasingly negative; further increase in ionic size going from I^- to ClO_3^- to BrO_3^- to IO_3^- causes a reversal of the trend; (d) Divalent anions do not appreciably inhibit the enzyme (Fridovich, 1963). Table 1 lists a series of anions and the concentration of each anion required to cause 50% inhibition of the decarboxylation of 0.027 M acetoacetate at pH 5.2 and at 30°C.

Acetoacetate is produced chiefly in the liver and to some extent in the kidneys by a reaction in which acetyl coenzyme A reacts with acetoacetyl coenzyme A to form 3-hydroxy-3methyl glutaryl coenzyme A (Figure 1). The acetyl coenzyme A and acetoacetyl coenzyme A arise from the catabolism of fatty acids and ketogenic amino acids. 3-Hydroxy-3 methyl-glutaryl coenzyme A is cleaved to produce acetyl coenzyme A and acetoacetate. Acetoacetate can undergo one of two

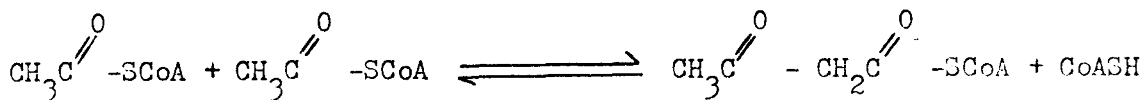
Table 1

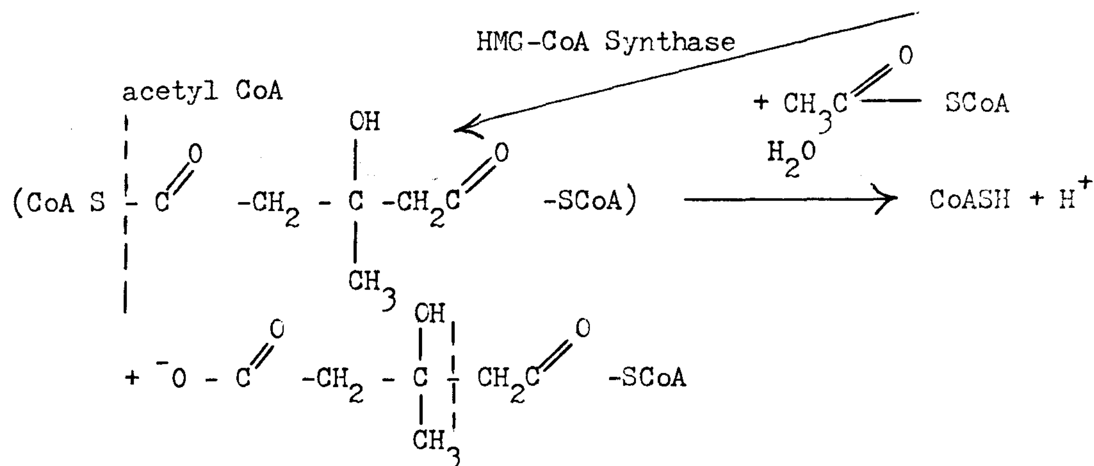
Inhibition Of Acetoacetate Decarboxylase By Monovalent Anions.*

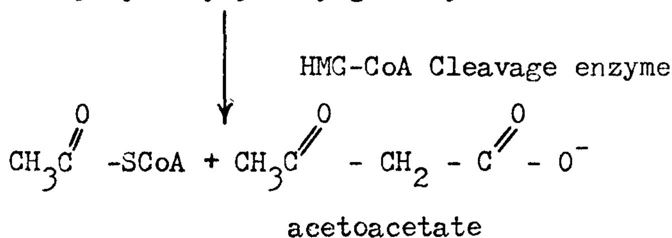
Anion	Concentration for 50% Inhibition
HSO_3^-	7×10^{-5} M
SCN^-	11×10^{-5}
ClO_4^-	73×10^{-5}
I^-	100×10^{-5}
NO_3^-	126×10^{-5}
ClO_3^-	400×10^{-5}
Br^-	$1{,}000 \times 10^{-5}$
Cl^-	$5{,}000 \times 10^{-5}$
BrO_3^-	$7{,}600 \times 10^{-5}$
F^-	$10{,}000 \times 10^{-5}$
IO_3^-	$10{,}000 \times 10^{-5}$
Tricholoroacetate	$33{,}000 \times 10^{-5}$

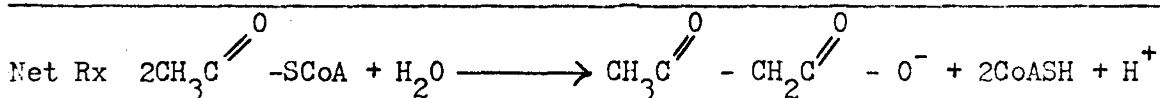
Fridovich, I. (1963)

Formation of Acetoacetate

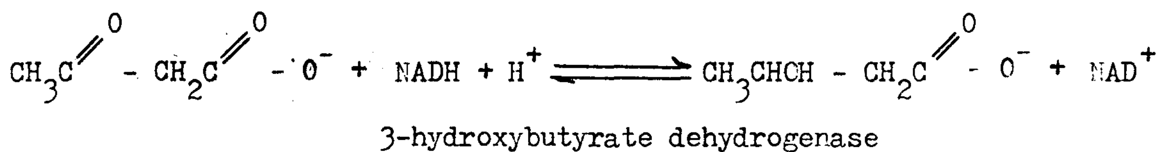








Formation of 3-hydroxybutyrate



Formation of Acetone

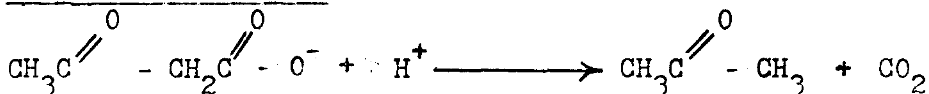


Figure 1. Ketogenesis

reactions; it can be reduced to 3-hydroxybutyrate or decarboxylated to acetone and carbon dioxide. The oxybutyrates, acetoacetate and 3-hydroxybutyrate are transported in the blood to target tissues chiefly skeletal and cardiac muscle (Figure 2). In the target tissues 3-hydroxybutyrate is oxidized to acetoacetate. Acetoacetate is converted to acetoacetyl coenzyme A in the presence of succinate-acetoacetate CoA transferase, a pathway that appears to be minimal or absent in the liver. The acetoacetyl coenzyme A thus formed is cleaved by yielding 2 molecules of acetyl coenzyme A which then enter the citric acid cycle.

Many biochemistry textbooks cite the decarboxylation of acetoacetate to occur nonenzymatically or spontaneously (West, Todd, Mason, van Bruggen, 1966; Bhagvan, 1974; Karlson, 1975; Stryer, 1975; Harper, 1977; Montgomery, Dryer, Conway, Spector, 1977). Several textbooks make no comments on the specifics of the reaction (Mahler and Cordes, 1966; McGilvery, 1979; Bohinski, 1973; Cantarow and Trumper, 1975; Orten and Neuhaus, 1975; Conn and Stumpf, 1976). Only a few textbooks cite the reaction to occur enzymatically (Rafelson, Binkley, Hayashi, 1971; White, Handler, Smith, Hill, Lehman, 1978).

Acetoacetic acid is a weak acid and during the overproduction of oxybutyrates as in the ketotic state, it will contribute to lowering the blood pH. The lowered blood pH causes vascular damage often associated with the uncontrolled diabetic. During the decarboxylation of acetoacetate, there is the uptake of a proton, lowering the proton pool. The decarboxylation of acetoacetate thus helps combat the falling blood pH.

Van Stekelenburg and Koorevaar (1972) have presented evidence that human blood serum has acetoacetate decarboxylase activity. All studies

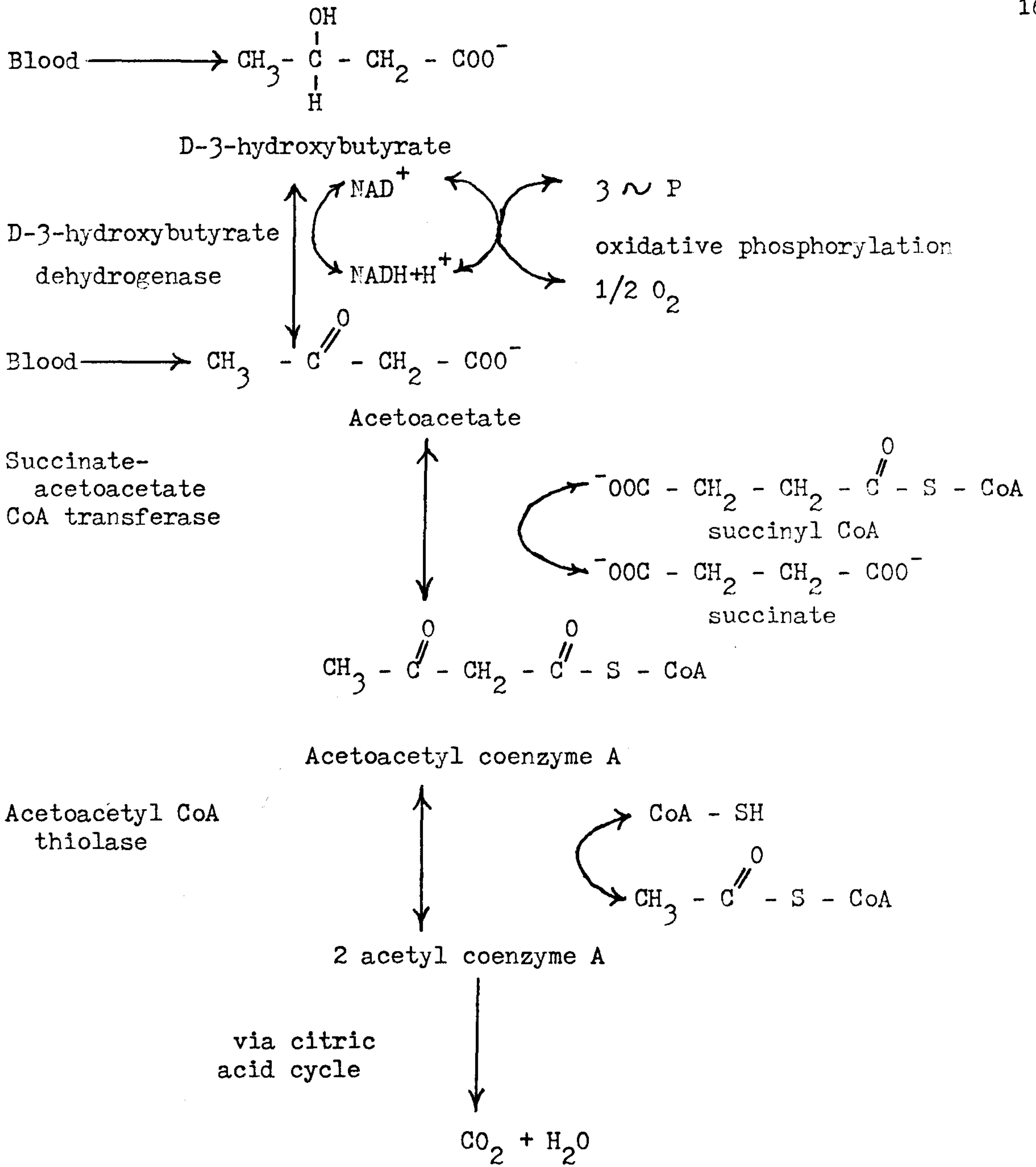


Figure 2. Ketone body utilization.

by Van Stekelenburg and Koorevaar were conducted by measuring the rate of acetone production by head space vapor gas chromatography. Blank or control solutions containing only acetoacetate in Tris buffer, pH 7.4, were always run to determine the extent of nonenzymic decarboxylation. Fifty-six random serum samples were collected and assayed for their acetoacetate decarboxylase activity. The mean activity present was 62.2 $\pm$ 12.9 (S.D.) μmole min^{-1} l^{-1} of acetone formed. Interestingly, all fifty-six samples demonstrated acetoacetate decarboxylase activity.

Characterization of acetoacetate decarboxylase activity in human serum was first based upon a molecular weight study (Van Stekelenburg and Koorevaar, 1972). Serum samples were subjected to ultrafiltration accelerated by centrifugal force. The molecular weight cut-off of the membrane was 30,000. After separation, the retentate was washed with physiological saline and the initial volume reestablished. The filtrate and retentate were assayed for acetoacetate decarboxylase activity. The filtrate did not demonstrate any acetoacetate decarboxylase activity, while the retentate contained all the activity initially observed. Thus, the activity in human serum is not due to any ions, small proteins or peptides; the molecular weight of the active component is greater than 30,000.

The acetoacetate decarboxylase activities of different human serum protein fractions have been tested (Van Stekelenburg and Koorevaar, 1972). The albumin fraction showed by far the greatest amount of activity. The globulins demonstrated activity but only a fraction of that seen for albumin. The albumin contents of the different globulin fractions were

determined immunochemically by the Ouchterlony plate technique. By assuming the activator present in human serum is albumin-like or albumin itself, the activities present in the globulin fractions could be corrected for the albumin impurities present. By determining the amount of albumin and calculating the activity associated with that amount of albumin, these workers concluded that the globulins do not exhibit significant acetoacetate decarboxylase activity.

The influence of pH of the incubation medium upon the acetoacetate decarboxylase activity of purified human albumin was also investigated (Van Stekelenburg and Koorevaar, 1972). Optimal activity occurs at pH 4.5; however, a considerable fraction of the activity is still present at pH 7.4.

The influence of substrate concentration was studied by evaluating the K_m at pH 5.0 and pH 7.4. Albumin demonstrates a very poor binding constant which is not affected by the change in pH (Van Stekelenburg and Koorevaar, 1972). The K_m was estimated to be 0.4 M.

The effect of temperature upon the acetoacetate decarboxylase activity of albumin was also studied by Van Stekelenburg and Koorevaar (1972). Purified albumin diluted with physiological saline was heated at 80°C for 15, 30 and 60 minutes respectively at pH 7.4. After 15 minutes, there was a 35% loss of activity, at 30 minutes a 45% loss and at 60 minutes a 55% loss of activity. The active component of the albumin fraction is not heat stable. Autor and Fridovich (1970b) reported the thermal inactivation of bacterial acetoacetate decarboxylase to occur in the temperature range of 74-82°C. Heating at 80°C for 30 minutes will cause a 50% decrease in the activity of the bacterial enzyme. Thus,

there is a good correlation between the heat inactivation of bacterial and mammalian acetoacetate decarboxylase activity.

Human serum albumin has been subjected to various inhibitors of bacterial acetoacetate decarboxylase. Davies (1943) showed that the bacterial acetoacetate decarboxylase would be totally inactivated in the presence of a 10^{-5} M concentration of $HgCl_2$. Van Stekelenburg and Koorevaar (1972) observed no loss in activity with $HgCl_2$ at 10^{-5}, 10^{-4} or 10^{-3} M; however, at 10^{-2} M, the decarboxylase activity of albumin was inhibited by 76%.

The influence of monoiodoacetate upon the acetoacetate decarboxylase activity of purified albumin has been studied. At a concentration of the inhibitor of 10 mM, 74% inhibition was found (Van Stekelenburg and Koorevaar, 1972); at the same concentration, the bacterial enzyme activity is inhibited by 63% (Davies, 1943).

Urea will also inhibit the acetoacetate decarboxylase activity of the albumin fraction (Van Stekelenburg and Koorevaar, 1972). In the concentration range of 0-10 M, the highest inhibition attained was 45% and at 10 M. The active component can be denatured and inhibited to some extent, but very high concentrations of urea are necessary. At a concentration of 10 M urea, 45% inhibition of activity was attained.

The effect of increasing concentrations of EDTA (ethylenediaminetetraacetic acid) upon the activity of purified albumin has been studied (Van Stekelenburg and Koorevaar, 1972). Interestingly, at low concentrations, 1-3 mM, there is an activation of the decarboxylase activity. Above 3 mM, inhibition is observed; this reaches a maximum of 30% at

9 mM. No explanation has been given as to why a chelating agent will increase the acetoacetate decarboxylase activity of the purified albumin.

In contrast to the findings of Davies (1943), Van Stekelenburg and Koorevaar (1972) observed no inhibition of acetoacetate decarboxylase by pyruvic acid. The concentration range employed for pyruvic acid was similar to that used for the bacterial enzyme studies. In all previous inhibition studies by these workers, the concentration needed to inhibit the decarboxylase activity of albumin was always greater than that required to inhibit the bacterial enzyme. It is not known why Van Stekelenburg did not increase the concentration of pyruvic acid.

Vitamin B_2 (lactoflavin) acts as an activator for the decarboxylase activity observed in purified human albumin. The activation is much greater than that observed with EDTA. However, like EDTA, as the concentration increases, there is a reversal in the role of vitamin B_2. At a lactoflavin concentration of 1 μg/ml, the original activity is doubled. Above 1 μg/ml, the activity declines until at 6 μg/ml of lactoflavin the reaction is inhibited by 40%.

Koorevaar and Van Stekelenburg (1976) performed an extensive study of the reaction kinetics for the decarboxylation of acetoacetate in the presence of Fraction V human serum albumin. The relationship between the enzymatic activity and substrate concentration was found to be sigmoidal in nature. The reaction deviates from normal Michaelis-Menten kinetics and, therefore, the K_m has been estimated to be equal to or less than 0.1 M. The binding constant is better than reported earlier (Van Stekelenburg and Koorevaar, 1972); however, it is still very poor.

Even though the binding constant is poor, albumin still demonstrates reaction kinetics.

Seeley (1955) reported that rates of decarboxylation of acetoacetate are increased in the presence of organ extracts. Koorevaar and Van Stekelenburg (1976) investigated whether this effect could be due to the presence of mild reducing substances in the incubation medium. Decarboxylase activity was determined in the presence and in the absence of glutathione and cysteine. It was found that the reducing agents did increase the rate of non-enzymatic decarboxylation of acetoacetate, but had no effect upon the activity associated with the albumin fraction tested. Thus, the increased decarboxylation rates observed by Seeley (1955) could be due to the presence of small organic SH-group containing substances in the organ extracts.

Because of the well known microheterogeneity of albumin (Hughes, 1964), Koorevaar and Van Stekelenburg investigated whether or not there is a localization of acetoacetate decarboxylase activity within one of the various albumin subfractions obtained by column chromatography. Human albumin (Mann Research Laboratories, cat #1702) was passed through a Sephadex G-200 column. Three peaks containing protein were resolved. The peaks were reported to represent the monomer, dimer and trimer (and higher polymers) of albumin. Each peak was divided into subfractions and assayed for decarboxylase activity. The specific activity of the subfractions was found to be homogeneous. It was concluded that the acetoacetate decarboxylase activity is bound tightly to the albumin molecule and that the enzymatic activity does not depend upon the degree of polymerization of the albumin molecule.

To obtain further evidence on the coupling of the decarboxylase activity to albumin, Koorevaar and Van Stekelenburg (1976) subjected Fraction V human serum albumin to ion exchange chromatography with DEAE Sephadex A-50. Two peaks were obtained from the elution. One peak consists of mercaptalbumin, while the other was considered to be a mixture of non-mercaptalbumin and polymers of albumin. The mercaptalbumin peak was found to exhibit low specific activity. It is possible that the activity present in the mercaptalbumin peak is due to the contamination of non-mercaptalbumin and polymers of albumin. The non-mercaptalbumin peak was divided into two subfractions and shown to contain a high specific activity. It was concluded that the free SH-group of mercaptalbumin interferes in some way with the acetoacetate decarboxylase activity.

Koorevaar and Van Stekelenburg (1976) assayed fifty-six serum samples obtained from patients chosen at random and found a positive correlation between the acetoacetate decarboxylase activity and the corresponding albumin concentration of the patient. In the study of the fifty-six sera, 43 specimens showed specific activities within the range found for purified human albumin (1.49 ± 0.18 (S.D.) μmole min^{-1} g albumin $^{-1}$), with a mean value of 1.48 umole min^{-1} g $albumin^{-1}$. The remaining sera showed significant deviations from the mean. Some of the extreme values obtained were: an oligophrenic patient, with hypertelorisma, 0.63; a patient with a mild compensated, metabolic acidosis 0.80; a ketotic diabetes mellitus patient, with decreased kidney function 0.94; a patient with collagenosis 1.11; a patient who died from a hemolytic uremic syndrome 2.13; and a ketotic diabetes mellitus patient with a nephrotic syndrome 3.85 umole min^{-1}g albumin $^{-1}$. It is not known why differences

exist for the patients in the disease states mentioned above.

Acetoacetate decarboxylase activity has also been observed in mammalian organ extracts (Koorevaar and Van Stekelenburg, 1976). Liver, kidney and brain from adult male rats were homogenized and subsequently fractionated. Five fractions were obtained: 1. the original homogenate; 2. the nuclear fraction; 3. the mitochonodria; 4. the lysosome fraction and 5. the supernatant. The specific activity (μmole min^{-1}g $protein^{-1}$) of the original brain homogenate was found to exceed that of kidney and liver. With respect to the liver, the specific enzyme activities do not differ much for the tissue fractions except for the nuclear fraction whose specific activity was very low. The kidney had approximately the same specific activity in all fractions with the exception of that of the mitochondrial fraction which was very low. Brain tissue was found to contain its highest specific activity in the nuclear fraction, and higher as well when compared to the values obtained for the tissue fractions of liver and kidney. No explanation has been given for the different activities observed among the subfractions of the organ extracts.

Generally, in the schemes of mammalian biochemical pathways, the decarboxylation of acetoacetate is said to occur nonenzymatically, From experiments by Koorevaar and Van Stekelenburg (1976) the nonenzymatic decarboxylation of acetoacetate at pH 7.4 and 37°C appears to be a first order reaction. In a blank or control sample, it has been calculated that 0.65% of the original acetoacetate present is decarboxylated in one hour. In the presence of a normal serum, a decarboxylation velocity of 13.5% of the original substrate concentration per hour was found. Based

upon this fact, these workers believe that the nonenzymatic decarboxylation of acetoacetate is of no, or minor, biochemical significance.

Peters (1970), in a lengthy review of serum, states that albumin has many functions and raises the question, are there undetected functions? Koorevaar and Van Stekelenburg (1976) believe that albumin has another function; to help defend the body against ketosis.

Statement of the Problem

Van Stekelenburg and Koorevaar (1972) have given evidence that the decarboxylation of acetoacetate in mammalian systems may occur enzymatically. Human serum albumin seems to possess enzymatic properties which are similar to those observed for the bacterial enzyme acetoacetate decarboxylase. The human serum albumin fraction demonstrates activity that: (1) is not ultrafiltrable, (2) has an optimum pH of 4.5, (3) has enzyme-like substrate dependency, (4) is inhibited by monoiodoacetate, urea and $HgCl_2$, (5) exhibits heat instability at 80°C and (6) is activated by lactoflavin. From fractionation studies of human serum albumin by means of gel filtration with Sephadex G-200, it has been concluded that the degree of polymerization has no effect upon the acetoacetate decarboxylase activity (Koorevaar and Van Stekelenburg, 1976). Homogenates and the subfractions of rat brain, liver and kidney tissues were shown to possess acetoacetate decarboxylase activity.

The dissertation study has investigated four aspects of the decarboxylation reaction. First, Van Stekelenburg reported in 1972 that rat liver homogenates possessed acetoacetate decarboxylase activity. To follow this aspect, a partial purification of acetoacetate decarboxylase was attempted from bovine liver. Second, the inducibility of the enzymatic activity was determined by placing rats under the stress of ketosis. Rats were made diabetic with streptozotocin and their acetoacetate decarboxylase activity was monitored in plasma samples. Third, further characterization of the acetoacetate decarboxylase activity was performed on samples of Fraction V human serum albumin. Fourth,

experiments were performed to gain insight into the mechanism of decarboxylation of acetoacetate by albumin.

CHAPTER II

MATERIALS AND METHODS

Assay of Acetoacetate Decarboxylase.

Acetoacetate decarboxylase activity was measured by following the production of acetone from the lithium salt of acetoacetate by head space vapor gas chromatography (Van Stekelenburg and De Bruyn, 1970). To control extraneous activity or breakdown of acetoacetate, recommendations suggested by Van Stekelenburg and Koorevaar (1972) were taken into consideration. (1) Acetoacetate is chemically unstable; therefore, it is necessary to measure the nonenzymatic decarboxylation of substrate in each experiment (blank experiments). (2) To maintain conditions of equilibrium between the acetone concentration in the vapor and in the aqueous phase, all experiments were carried out in a shaking waterbath thermostated at 37°C. (3) The acetone concentration in the vapor phase depends not only upon the concentration of acetone in the aqueous phase, but it also depends upon the osmolality of the aqueous phase. (4) In general, the acetoacetate decarboxylase assays were run for four hours and the concentration of acetone was determined five or six times during the incubation period.

The acetoacetate decarboxylase reaction was carried out in 40 ml rubber-stoppered vials. The reaction mixture contained 2.7 ml of 50 m<u>M</u> Tris buffer, pH 7.4, 0.1 ml of 0.45 <u>M</u> lithium acetoacetate and 0.2 ml of the test material. Head space samples were withdrawn by puncturing the

rubber stopper with a 1 ml air-tight syringe (Pressure LOK, Series A-2 Precision Sampling, Baton Rouge, Louisiana). The 1 ml head space sample was compressed to 0.1 ml and injected into the gas chromatograph. The syringe must be purged with extra-dry nitrogen after each injection to ensure that there is no build-up of residual acetone.

The gas chromatograph used was either a Varian 2440 or a Barber-Colman Model 5320. Both gas chromatographs were equipped with a flame ionization detector. The Barber-Colman gas chromatograph had an aluminum column, 6 ft. in length with an internal diameter of 1/4 inch. The injection port temperature was 220°C, the column oven temperature was 160°C, and the detector temperature was 220°C. The Varian model had a glass column, 6 ft. in length with an internal diameter of 1/8 inch. The injection port temperature was 250°C, the column oven temperature was 170°C, and the detector temperature was 290°C. The columns of both gas chromatographs were packed with Porapak QS resin, 80-100 mesh (Anspec Company, Inc. Ann Arbor, Michigan). The carrier gas for both columns was zero grade nitrogen (Linde). The gas flow of nitrogen, hydrogen and air was 30 ml, 20 ml and 300 ml/min, respectively.

Standard solutions of reagent grade acetone (Mallinckrodt, St. Louis, Missouri) were prepared containing 15, 30, 60 and 90 μg of acetone respectively in 3 ml of aqueous solution. The relationship between the concentration of acetone and the peak height in mm was found to be linear over the range described.

Before an assay was performed, the validity of the standard curve was checked by three injections of the 90 μg standard. The peak

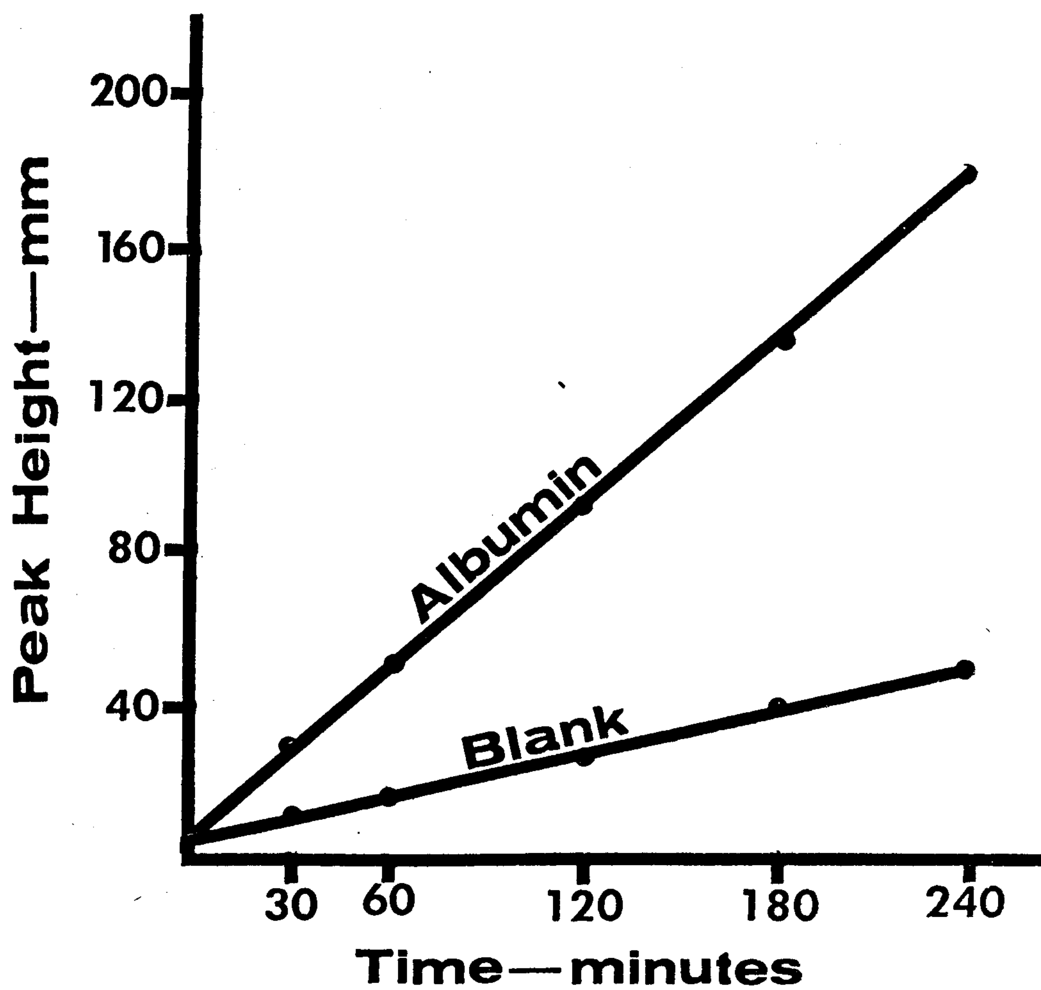
heights must be monitored closely since the columns lose sensitivity with time. A new standard curve was prepared when the peak height determined after an incubation time of four hours for the 90 μg standard varied more than 5 mm from the previously determined value.

Porapak QS is a pure white resin. After two months of use, the resin begins to turn brown. The brown color is readily observed through the glass column; and when seen, the column is repacked. The status of the aluminum column is checked by monitoring the retention time of the acetone. When the retention time increased by thirty seconds, the column was emptied and repacked with fresh resin.

For the assay of experimental samples for acetoacetate decarboxylase activity, the total change of the acetone concentration in the vapor phase expressed in mm of peak height per unit of time minus the change of the acetone concentration in the vapor phase of the corresponding blank experiment is a measure of the enzyme activity. The acetoacetate decarboxylase activity was expressed in terms of units. One unit of activity is defined as the production of 1 μmole of acetone per minute per gram of protein. An example of a typical plot for the determination of acetoacetate decarboxylase activity of Fraction V human serum albumin is presented in Figure 3. In all experiments described in the dissertation, the source of Fraction V human serum albumin was Miles Laboratories, Elkhart, Indiana (Code no. 82-302, Lot no. 141). The purity of the albumin was stated to be 96% as determined by cellulose acetate electrophoresis in a 75 m*M* baribital buffer, pH 8.6.

Figure 3. Acetoacetate decarboxylase assay by head space vapor gas chromatography.

The graph shows the increase in the acetone concentration (in mm peak height) of head space vapor during a typical acetoacetate decarboxylase assay. The blank reaction mixture contains 2.7 ml of 50 mM Tris buffer, pH 7.4, 0.1 ml of 0.45 M lithium acetoacetate and 0.2 ml of deionized water. The sample reaction mixture contains 2.7 ml of 50 mM Tris buffer, pH 7.4, 0.1 ml of 0.45 M lithium acetoacetate and 10 mg of Fraction V human serum albumin in 0.2 ml of deionized water. The difference in peak height between the blank and albumin sample is related to the acetone concentration by use of a predetermined standard curve.



Peak Height—mm
200
160
120
80
40
Albumin
Blank
30
60
120
180
240
Time—minutes

Preparation of Labeled and Unlabeled Lithium Acetoacetate.

Lithium acetoacetate, the substrate for the acetoacetate decarboxylase assay was prepared according to the method of Hall (1962). The unlabeled substrate was prepared from ethyl acetoacetate (Fisher Scientific Company, Fair Lawn, New Jersey) while the labeled substrate was prepared from ethyl-(3-^{14}C)-acetoacetate (Amersham Corporation, Arlington Heights, Illinois). The labeled and unlabeled substrates are prepared in the same way. A mixture of 5.2 g (0.04 mole) ethyl acetoacetate, 25 ml water and 10.0 ml of 4.00 M LiOH was placed in a cork-stoppered Erlenmeyer flask and incubated at 40°C for four hours. A pale yellow solution resulted, and it was evaporated to dryness under reduced pressure at a bath temperature of 40°C. It is essential that the flask contents be completely dry, otherwise the subsequent crystalization of the compound from a methanol-ether mixture is difficult. The crude lithium acetoacetate was extracted at room temperature from the dry crystalline mass with three successive 15 ml portions of absolute methanol. Insoluble material was removed by passage of the mixture through a Whatman no. 4 filter paper. Absolute ether was added slowly to the clear methanol filtrate until the solution became slightly turbid. The mixture was allowed to stand at room temperature for 30 minutes. Five volumes of ether were added slowly to the mixture which was then chilled to 4°C. The crystals were collected by suction filtration, washed with ether and dried *in vacuo*. The product was recrystallized twice by redissolving the material in absolute methanol (6 ml per gram) removing insoluble material by filtration and adding absolute ether as before. The yield based on ethyl acetoacetate was 50-60%.

The purity of the lithium acetoacetate was determined from absorption spectra. The measurements were taken in water, in 0.1 M NaOH and in 0.1 M HCl. The spectra were obtained with the use of a Model DB-G Beckman Spectrophotometer. The concentration of the lithium acetoacetate in all three media was 4.00 mM; the light path was 1.00 cm. The molar extinction coefficients at the peak absorptions of 273, 267 and 247 nm were respectively 47,0, 147.5 and 72.5 cm^2. Hall (1962) reported molar extinction coefficients at the same peak absorptions of 50.0, 148.0 and 73.0 cm^2 respectively.

The crystalline lithium salt of acetoacetate is non-hygroscopic and is stable for several months when kept under vacuum at 4°C. Neutral solutions of the lithium salt may be frozen at -10°C for 2-3 weeks without significant decomposition.

Protein Determination.

Protein was determined by utilizing the colorimetric method of Lowry et. al., (1951) as modified by Oyama and Eagle (1951). There are two distinct steps which lead to the final color development with protein: (1) a reaction with copper in alkali, and (2) a reduction of the phosphomolydic-phosphotungstic acid reagent by the copper-treated protein. One ml of reagent "C" was placed in each of a series of 10 X 75 mm polystyrene test tubes. Two hundred µl of sample was added to appropriately marked tube and two hundred µl of deionized water was added to a blank tube. The contents of the tubes were mixed and allowed to stand for ten minutes. One hundred µl of Folin-Ciocalteu reagent (Hartman-Leddon Company, Philadelphia, Pa.) was added to each tube and the tubes

were mixed thoroughly. The reaction mixtures were allowed to stand for 30 minutes to allow for full color development. The absorbance of the sample solutions was determined with a Beckman DU spectrophotometer at a wavelength setting of 750 nm. The blank tube was used to zero the instrument. A standard curve was prepared following this procedure using Fraction V bovine albumin powder (Pentex Biochemicals, Kankakee, Illinois) as the standard.

The concentration of the unknown sample is calculated by using the formula:

$$\frac{\mu g \text{ protein}}{\text{ml of unknown sample}} = \frac{\text{absorbance of unknown}}{\text{absorbance of standard}} \times \text{concentration of standard in } \mu g \times 5$$

The data for the calibration curve are given in Table 2 and the standard curve is shown in Figure 4.

Solutions

1. Reagent A

   20 g of Na_2CO_3, 4 g of NaOH and 200 mg of KNa $C_4H_4O_6 \cdot 4H_2O$ were dissolved in one liter of deionized water.

2. Reagent B

   5 g of $CuSO_4 \cdot 5H_2O$ was dissolved in one liter of deionized water.

3. Folin-Ciocalteu Reagent

   Folin-Ciocalteu Reagent was obtained from the Hartman-Leddon Company and diluted with deionized water in the ratio of 5 parts of the phenol reagent to 7 parts of deionized water as recommended by Oyama and Eagle (1956).

Table 2

Standard Curve Data For Protein Determination

Albumin (μg)	Number of Determinations	Absorbance* 750 nm
5	6	0.066 ± 0.002
10	6	0.124 ± 0.001
20	6	0.231 ± 0.003
30	6	0.323 ± 0.003
40	6	0.408 ± 0.002
50	6	0.497 ± 0.006

* Mean ± standard deviation.

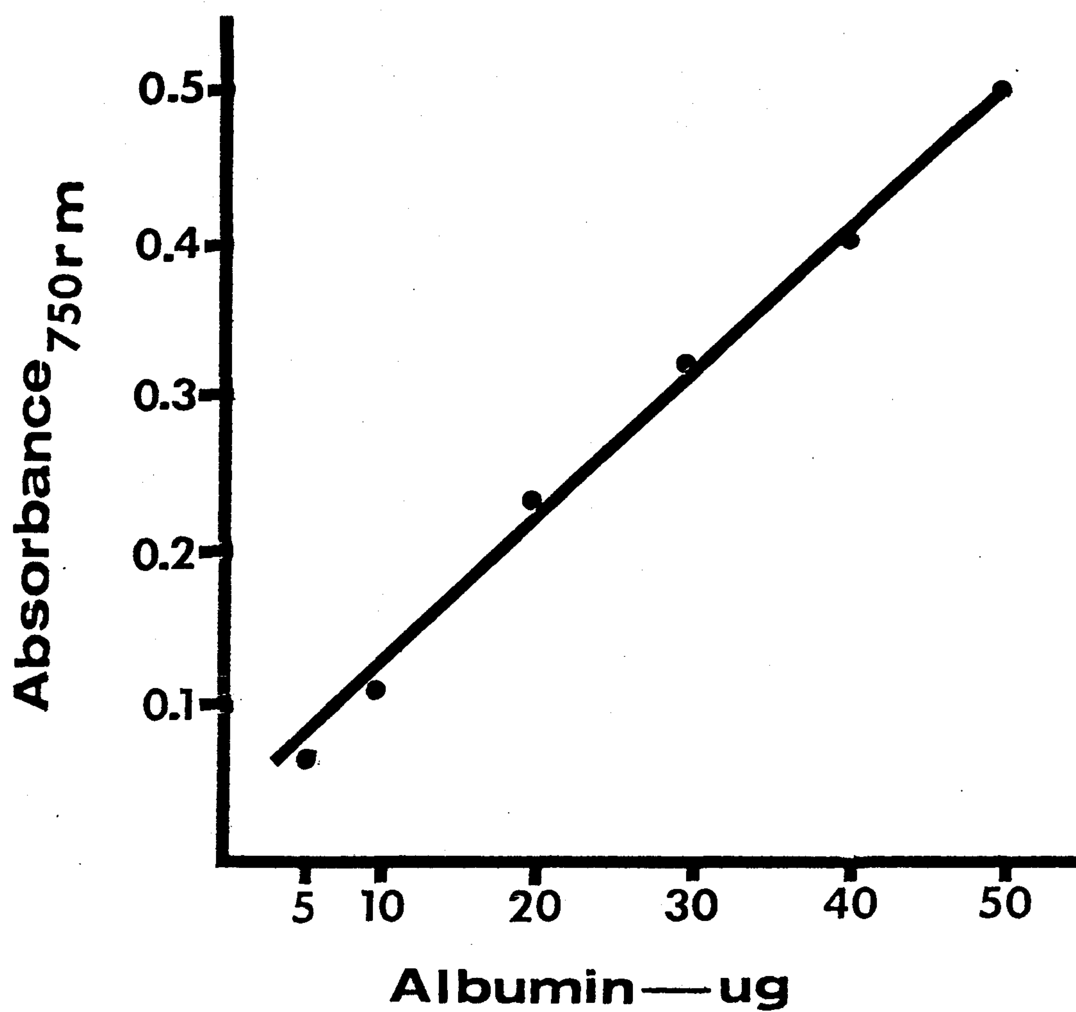


Figure 4. Standard curve for protein determination

4. Reagent C

Fifty parts of reagent A and one part of reagent B were mixed freshly prior to use.

Determination of Plasma Glucose

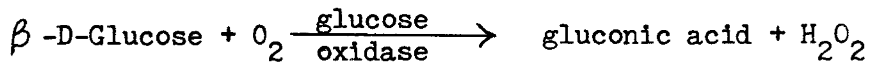
During the course of this study, it was of interest to determine whether acetoacetate decarboxylase activity was inducible during starvation or in the diabetic state. For the latter purpose, streptozotocin (Upjohn Company, Kalamazoo, Michigan) was used to induce diabetes mellitus in fed adult male rats.

The blood was collected from the tail vein while the animals were lightly anesthetized with sodium pentobarbital, 32.5 mg/kg of body weight, injected intraperitoneally. The animals were placed on their backs and taped into position. The tip of the tail was snipped and the blood was collected in Natelson heparinized micropipettes. The tail was milked to facilitate collection of the blood and prevent the formation of a clot. Three Natelson pipettes were filled before the bleeding was stopped.

The plasma was obtained from the Natelson micropipettes by flaming shut the opposite end of the pipette used to introduce the blood. The closure allows the blood to be centrifuged in the pipettes without unnecessary transfer and possible loss of plasma. The pipettes were centrifuged at 2000 rpm for 15 minutes. After centrifugation, the pipettes were cut near the plasma/RBC junction. The plasma was transferred to test tubes and frozen until assayed.

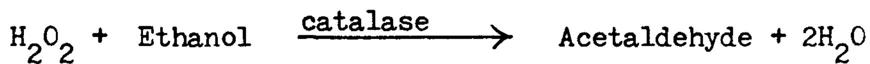
The glucose levels were monitored with a Beckman Glucose Analyzer (Beckman Instruments, Inc., Fullerton, California). The analyzer

employs an oxygen sensitive electrode and an electronic system that measures the rate of change in oxygen concentration when a sample is injected into an enzyme solution. Plasma is introduced into a sample cup containing glucose oxidase. β -D-glucose in the plasma combines with the dissolved oxygen in the solution according to the reaction:



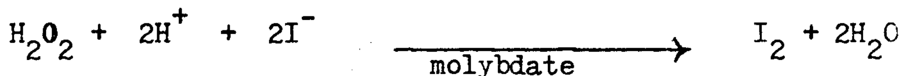
$$\beta\text{-D-Glucose} + O_2 \xrightarrow[\text{oxidase}]{\text{glucose}} \text{gluconic acid} + H_2O_2$$

The oxygen is consumed at the same rate as the glucose reacts and, therefore, the rate of oxygen consumption is directly proportional to the glucose concentration. Because oxygen consuption rather than peroxide formation is measured, peroxide must be destroyed by a path not leading back to oxygen. The destruction of peroxide can be accomplished in one of two ways. In the presence of catalase, ethanol reacts with hydrogen peroxide according to the following reaction:



$$H_2O_2 + \text{Ethanol} \xrightarrow{\text{catalase}} \text{Acetaldehyde} + 2H_2O$$

Also, peroxide may be decomposed in the presence of iodide and molybdate ions according to the equation:



$$H_2O_2 + 2H^+ + 2I^- \xrightarrow[\text{molybdate}]{} I_2 + 2H_2O$$

The enzyme reagent solution contains, in addition to the enzyme glucose, oxidase, ethanol, catalase, iodide and molybdate to ensure that a complete destruction of the generated peroxide is accomplished.

Isoelectric Focusing

In electrophoresis, the pH of the stationary phase is constant

throughout. Thus, proteins migrate according to the charge they possess at that pH. In isoelectric focusing, there is a pH gradient established by commercially available ampholytes. When current is applied to an isoelectric focusing column, proteins will migrate to a position in the pH gradient where the net charge they possess is zero. Proteins layer themselves in distinct bands. At the end of the electrophoretic run, the effluents from the column are passed through a monitoring device and collected with a fraction collector.

The pH gradient, which is the basis for isoelectric focusing, is established by aliphatic polyamino-polycarboxylic acids. These compounds have small differences in pI when compared to each other. At the beginning of a run, the pH of the column is the average value for all the carrier ampholytes in solution. When current is applied to the column, the ampholytes migrate to a position where their net charge is zero.

To prevent disruption of the pH gradient by convection, the column is stabilized by a sucrose density gradient. The column is prepared by mixing the carrier ampholytes and sample proteins in the sucrose solution. The sucrose solution is then layered on the column using a gradient mixer.

After current is applied to the column, it is necessary to protect the carrier ampholytes located near the electrodes from anodic oxidation and cathodic reduction. This is done by surrounding the anode with an acid solution and the cathode with an alkaline solution. The carrier ampholytes at the anode become positively charged and these are repelled from the anode. At the cathode, the ampholytes become negatively charged and are repelled away from the cathode.

Partially purified bovine liver extracts were applied to isoelectric focusing columns. The entire electrofocusing column (LKB 8100 Ampholine Column, LKB Instruments, Inc., Rockville, Maryland) was thermostated at 4°C by utilizing a cryostat (Tamson Thermostatic Bath (TM-9) and circulator (PBC-4, Neslab Instruments, Inc., Portsmouth, New Hampshire), which circulated coolant around both the inner and outer electrofocusing columns. The coolant consisted of equal volumes of distilled water and ethylene glycol. The anode solution consisted of 0.2 ml of 14.6 M phosphoric acid, 14 ml of distilled water and 12 grams of sucrose. The anode solution was introduced through a side port in the column. It is important to ensure that the anode is covered by approximately one inch of solution. The sucrose gradient was prepared by dissolving 28 grams of sucrose in 42 mls of water and adding 3.75 ml of an LKB Ampholine with a pH range of 3.5-10 (LKB Instruments, Inc., Rockville, Maryland) to the solution. The solution was heated gently and stirred to facilitate the dissolution of the sucrose. This solution is called the dense sucrose solution. A light solution was prepared by dissolving 1.25 mls of the LKB Ampholine to 60 ml of distilled water. Five ml of this solution was discarded (to accommodate the column volume) and 20 ml of the bovine liver extract was added to the light solution. The sucrose gradient was established through the use of an LKB Gradient Mixer, Model 8121. After the dense and light solutions were applied to the column, the cathode electrode solution was added. The cathode solution was prepared by dissolving 0.20 grams of sodium hydroxide in 20 ml of distilled water. The cathode solution was layered on the sucrose solution until the platinum cathode was covered by approximately 1 inch of solution. A

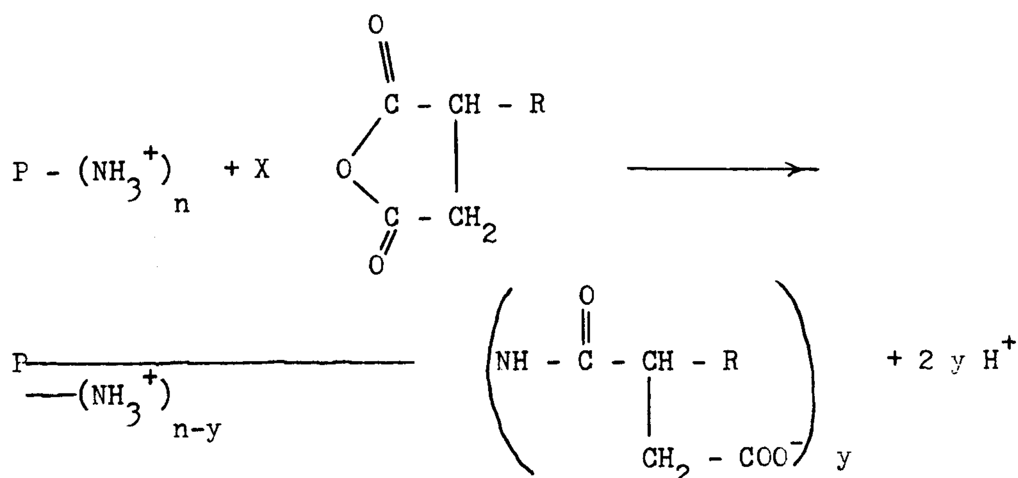
constant voltage of 500 volts was applied to the column for a period of 48 hours. The power source was shut off and the bottom of the column opened so that the effluent could be monitored and collected. Monitoring was performed with a Uvicord Absorptionmeter and Recorder (LKB Instruments, Inc., Rockville, Maryland) and fractions were collected with a Unifrac Fraction Collector (Savant Instrument Inc., Hicksville, New York). Fifty drop aliquots (3.8 ml) were collected until the column was completely emptied.

The pH of the fractions was measured while cold with a Corning pH meter, Model 12-B, (Corning Scientific Instruments, Corning, New York) equipped with a Markson pH electrode (Markson Science Inc., Del Mar, California).

Acylation of Human Serum Albumin

The mechanism of action for the bacterial enzyme occurs through the formation of Schiff base between the carbonyl group of the acetoacetate and a reactive lysine residue in the active site of the enzyme, (Warren et. al., 1966). To investigate whether albumin has a similar mechanism, acylating agents such as succinic and citraconic anhydrides, were used to block the epsilon amino groups of lysine.

Fraction V human serum albumin (Miles Laboratories, Inc., Elkhart, Indiana) was acylated with succinic or citraconic anhydride (2-methylmaleic anhydride). Klotz (1967) represents the acylation reaction to occur as follows:



Jonas and Weber (1970) have demonstrated from fluorescence polarization measurements that the dicarboxylic anhydrides will expand the albumin molecule, due to electrostatic repulsion, but that the attachments of acylating groups have no effect on the protein structure. Thus, modification of albumin by acylating agents would not be expected to alter the acetoacetate decarboxylase activity.

The acylation of albumin by succinic or citraconic anhydride was carried out as described by Jonas and Weber (1970). Solid succinyl anhydride (Eastman Kodak Company, Rochester, New York) was added slowly in small amounts to a 1% solution of the human serum albumin with continuous stirring at room temperature. During the reaction of succinic anhydride with the epsilon amino group of lysine, there is a release of protons. The pH of the mixture is kept constant at 7 by the drop-wise addition of 1 M NaOH. The reaction was deemed complete when there occurred no further drop in the pH of the solution upon the addition of succinic anhydride. The unreacted succinic anhydride, which in water is largely hydrolyzed to succinate, was removed by exhaustive dialysis. After

dialysis, the succinated albumin solution was lyophilized, reconstituted with 2.0 ml of 50 mM Tris buffer, pH 7.4 and assayed for acetoacetate decarboxylase activity. The succinated albumin is a stable complex and can be stored in the refrigerator for several days without decomposition.

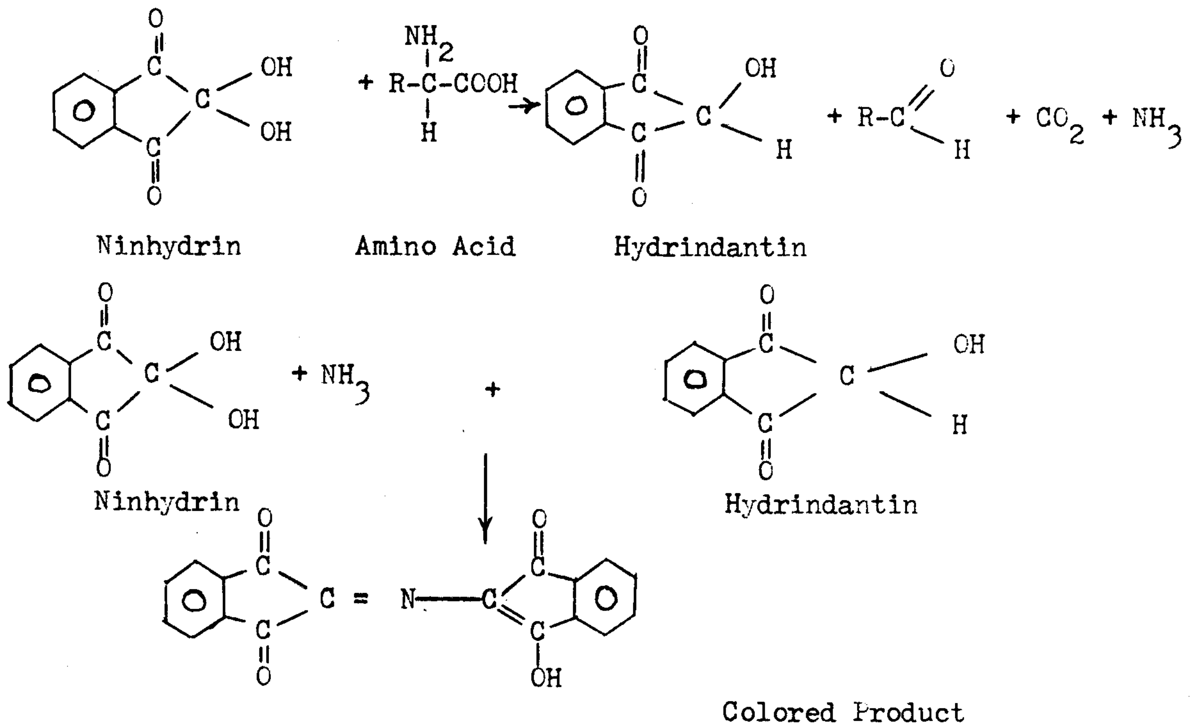
The reaction of Fraction V human serum albumin with citraconic anhydride (Aldrich Chemical Company, Inc., Milwaukee, Wisconsin) was also carried out at room temperature with continuous stirring and manual adjustment of pH with 1 M NaOH to pH 8.0. The citraconyl protein is hydrolyzed easily at pH values as high as 7 after several hours at room temperature, but when kept cold at pH 8.0 is stable for a few days (Jonas and Weber, 1970). To speed the removal of the unreacted citraconic anhydride and restrict undesired hydrolysis, the acylated protein was passed through a Sephadex G-25 column (Pharmacia Fine Chemicals, Inc., Piscataway, New Jersey) 2.5 X 30 cm, equilibrated with 50 mM Tris buffer, pH 8.0. Volume fractions of 130 drops (approximately 10 ml per fraction) were collected with a Unifrac Fraction Collector (Savant Instruments, Inc., Hicksville, New York). The absorbance of the effluent was monitored at 280 mm with a Uvicord absorptionmeter and recorder (LKB Instruments, Inc., Rockville, Maryland). The albumin complex was eluted in the void volume and in a broad peak. Fractions containing the acylated albumin were pooled and concentrated by lyophilization. Each albumin-complex sample to be tested was reconstituted with 2 ml of 50 mM Tris buffer, pH 8.0 and assayed for acetoacetate decarboxylase activity.

After assay, the citraconyl albumin samples were hydrolyzed to remove the acylating agent. The reason for this approach was an attempt to recover activity lost during the acylation. If successful, this

procedure would lend further support for a role of the free epsilon amino group of lysine in acetoacetate decarboxylation. Hydrolysis was performed by placing the samples in 20 ml screw-capped vials with a stirring bar and lowering the pH to 2.5 with 0.5 M HCl. The samples were allowed to mix overnight at 4°C. After hydrolysis, the samples were again passed through the Sephadex column to remove the 2-methylmaleic acid. Ten ml fractions were collected as previously described, concentrated by lyophilization and assayed for acetoacetate decarboxylase activity.

Determination of the Free Amino Group Content of Human Serum Albumin

The extent of acylation of Fraction V human serum albumin by succinic and citraconic anhydrides was followed by utilizing the colorimetric ninhydrin assay of Matthews et. al., (1964) for the determinations of free amino groups. Ninhydrin reacts with amino acids as follows:



In this reaction, the amino acid is oxidized to an aldehyde containing one less carbon atom. Ammonia and carbon dioxide are released. All true amino acids, peptides, proteins, or any compound containing free amino groups undergo this reaction. In an acidic environment, one residue each of reduced and oxidized ninhydrin condense with ammonia to form a bluish colored reaction product. The color developed differs with the character of the protein, the amino acid or mixtures of the two.

The ninhydrin method is simple to perform and it is as specific as the gasometric method for α-amino nitrogen (Matthews et al., 1964). The colored product formed during the reaction is measured spectrophotometrically at 570 nm. The following reagents are needed for the assay:

Stock cyanide, 0.01 M NaCN. - Dissolve 490 mg in 1 liter of water.

Cyanide - acetate buffer, pH 5.2. - To 190 g $CH_3COONa \cdot 3H_2O$ add 500 ml water and dissolve. Add 36 ml glacial acetic acid and 5 g disodium EDTA. Mix well, then add 20 ml of the stock cyanide solution and make up to 1 liter with water. Store at 4°C.

Methyl cellosolve (ethylene glycol monomethyl ether) - The reagent should give a negative peroxide reaction with 10% KI, and a clear solution on mixing with water.

Ninhydrin reagent - Ninhydrin 3% (w/v) in methyl cellosolve. Store at 4°C.

Stock standard amino acid solution - Dissolve 536 mg glycine in 100 ml water (= 100 mg of NH_2N per 100 ml). Preserve with chloroform and store at 4°C.

Working standard - Dilute 5 ml of stock standard to 100 ml with water on the day of the estimation.

Diluent - Isopropanol, mixed with an equal volume of water.

Procedure:

1. Into a series of 18 X 150 mm pyrex test tubes was added 0.9 ml of deionized water.
2. To the appropriate tubes was added 100 µl of unknown solution or of deionized water. Next 0.5 ml of the cyanide-acetate buffer and 0.5 ml of ninhydrin reagent were added. The solutions were mixed, capped with aluminum foil and placed in a boiling water bath for 15 minutes. The tubes were then removed and cooled by immersion in cold water. To each tube, 15 ml of diluent was added. The tube contents were mixed by inversion and read against water at 570 nm in 1 cm cuvettes at a Beckman DU Spectrophotometer.

A standard curve was prepared following this procedure. The data for the standard curve are given in Table 3 and the standard curve is shown in Figure 5.

Borohydride Reduction of a Postulated Schiff Base Intermediate Formed Between Acetoacetate and Human Serum Albumin

The bacterial acetoacetate decarboxylase enzyme catalyzes the decomposition of acetoacetate by forming a Schiff base with the substrate. Schiff base intermediates formed between the bacterial enzyme and acetoacetate can be reduced by borohydride (Fridovich and Westheimer, 1962). The enzyme is inactivated by this treatment. To ascertain whether the mechanism of action of the acetoacetate decarboxylase activity associated with Fraction V human serum albumin involves Schiff base formation, a borohydride reduction was performed with a radioactively labeled substrate

TABLE 3

Standard Curve Data For The

Determination Of Free Amino Groups

NH_2-N (μg)	Number of Determinations	Absorbance* 570 nm
2.5	3	0.228 ± 0.003
5.0	3	0.453 ± 0.003
7.5	3	0.680 ± 0.000
10.0	3	0.900 ± 0.000

* Mean ± standard deviation.

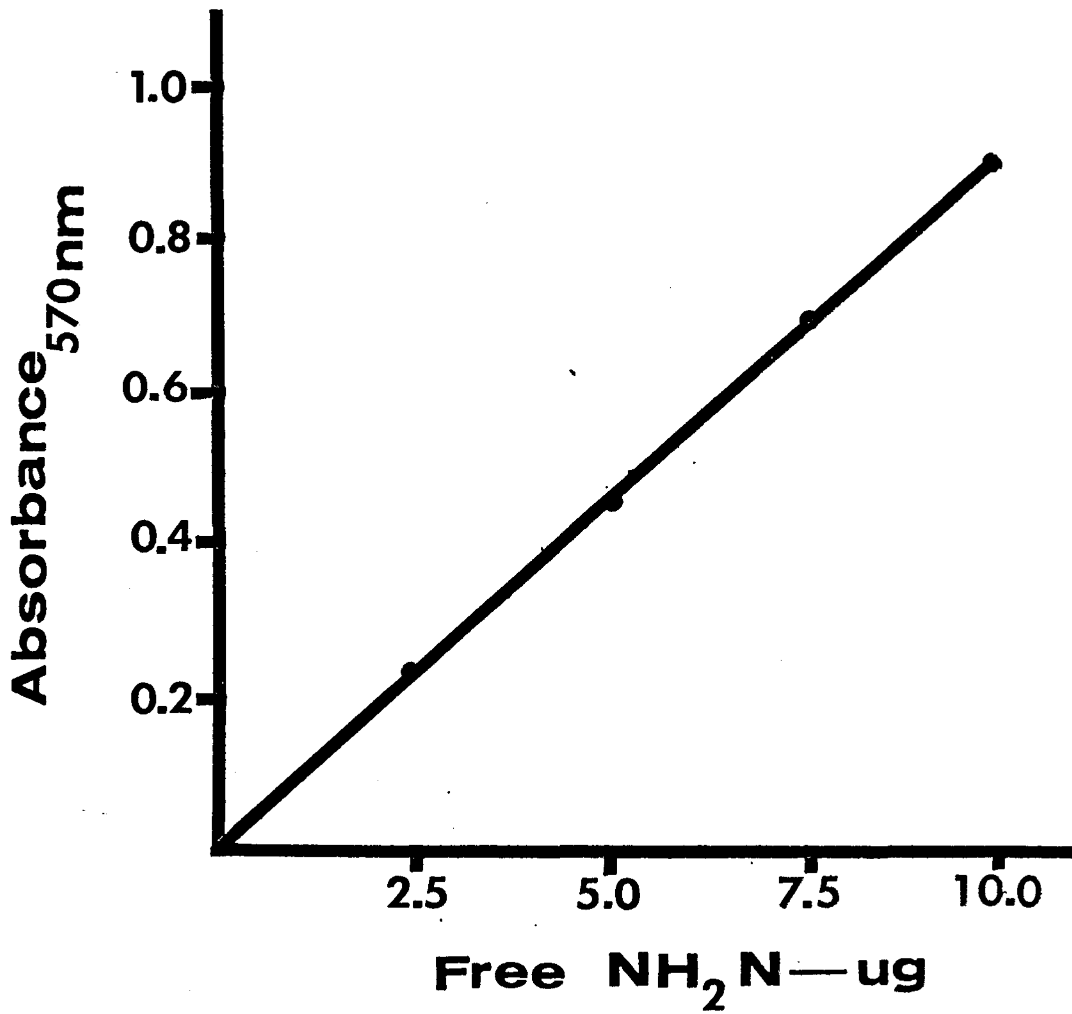


Figure 5. Standard curve for alpha amino nitrogen determination

($3-^{14}C$)-acetoacetate. Reduction occurs if a carbon-14 labeled albumin complex is obtained from the reaction mixture. Formation of a labeled albumin complex offers evidence for the involvement of a Schiff base intermediate in the mechanism of action of the enzyme.

Ten mg samples of Fraction V human serum albumin (Miles Laboratories, Elkhart, Indiana) were dissolved in 2.9 ml of 50 m*M* Tris buffer, pH 7.4 and placed in 25 ml Erlenmeyer flasks. The contents of the flasks were brought to 37°C in a shaker water bath. One hundred micro-liters of 0.45 *M* ($3-^{14}C$) acetoacetate (5.06×10^5 dpm) (New England Nuclear, Boston, Massachusetts) was added to each flask. All flasks were incubated at 37°C for 30 minutes. During the incubation period, 30 and 60 m*M* solutions of sodium borohydride (J. T. Baker Chemical Co., Phillipsburg, New Jersey) were prepared in 10 ml of 1 m*M* sodium hydroxide. Sodium borohydride is unstable in aqueous solution and must be kept at pH 13 to prevent its acid-catalyzed decomposition. After the 30 minute incubation period, 100 µl of the appropriate borohydride solution was added to the flasks to achieve a final borohydride concentration of either 1 m*M* or 2m*M* as desired. The flasks were restoppered, the contents swirled and the flasks were replaced in the shaker water bath.

Davies et al., (1962) have reported that borohydride is rapidly hydrolyzed below pH 9. Thus, at the pH of the assay mixture, the reduction of an albumin complex will occur very rapidly. The borohydride addition was repeated at 60, 90 and 120 minutes to ensure that a detectable level of the reduced albumin complex was attained. Following the last borohydride reduction step, the flask contents were exhaustively dialyzed against water to remove unreacted labeled substrate from the

mixture. Aliquots of 500 μl of the dialysands were removed, added to a scintillation cocktail and the radioactivity of each sample was determined with a Beckman LS-250 scintillation spectrometer.

Decarboxylation of Alpha Keto Acids

An investigation of the effect that albumin may have on the decarboxylation of alpha keto acids was conducted. The assay for acetoacetate decarboxylase activity which measures the production of acetone in the head space vapor by flame ionization gas chromatography is reproducible and extremely sensitive. However, to measure the decarboxylation of alpha keto acids, another method was needed since acetone would not be the product. Unfortunately, carbon dioxide cannot be measured by flame ionization gas chromatography. An alternative method was tried, that of thermal conductivity gas chromatography (Bollman and Mortimore, 1972). Carbon dioxide can be measured by this method; however, the sensitivity was very poor and the results were not reproducible.

A method was developed in which radioactively labeled alpha keto acids (1-^{14}C)-pyruvate and (1-^{14}C) alpha ketoglutarate were utilized. During the decarboxylation, labeled carbon dioxide is released. It is trapped in dilute sodium hydroxide and its radioactivity counted by liquid scintillation. The method proved useful for studying the effect of albumin on the decarboxylation of alpha keto acids.

The decarboxylation reaction was carried out in a 25 ml Erlenmeyer flask stoppered with a rubber septum. In the center of the septum there was a small hole which accommodates a long stemmed plastic collection bucket. The collection bucket was positioned approximately one cm

above the reaction mixture and contained 0.3 ml of 0.1 N sodium hydroxide. The reaction mixture contained 10 mg of Fraction V human serum albumin (Miles Laboratories, Elkhart, Indiana) in 2.9 ml of 50 mM Tris buffer, pH 7.4 and 0.1 ml of the (1-^{14}C)- labeled alpha keto acid. The mixture of albumin and Tris buffer was brought to 37°C in a shaking water bath. The collection buckets containing the sodium hydroxide were inserted into the flasks. At appropriate time intervals, 100 μl of labeled keto acid was added to each flask; the flask was restoppered and replaced in the 37°C water bath. After two hours of incubation, the flask was opened carefully and the collection bucket was removed. The long stem was cut from the collection bucket and the bucket was dropped into 10 ml of a scintillation cocktail (PPO-toluene-BBS-3). The scintillation vials were sealed, shaken and the radioactivity of the sample was counted with a Beckman LS-250 liquid scintillation counter to determine the degree of decarboxylation.

Measurement of Radioactivity

A Beckman LS-250 Liquid Scintillation Spectrometer was utilized for all measurements of radioactivity. The samples were counted in 20 ml low-potassium borosilicate glass vials fitted with tin-foil-lined plastic screw caps. Ten ml of scintillation fluor was added to each vial. The fluor was prepared by dissolving 5 gm of PPO in 1 L of scintillation grade toluene and to every 100 milliliters of this solution was added 20 ml of a solubilizing agent BBS-3 (Beckman Instruments, Fullerton, California). Liquid samples were introduced into the counting fluor by the use of a Gilson Pipetman automatic pipettor (Rainen Instrument Co., Brighton, Massachusetts). The scintillation vials were capped and

shaken vigorously to dissolve the test material. Dark adaptation of the samples was complete after 30 minutes.

The efficiency of counting was determined by the method of internal standardization. After all the samples were counted, 100 microliters of (C-14)-toluene standard (40,000 dpm, Beckman Instruments, Fullerton, California) was added to the vials. The samples were recounted. The mean value for the counting efficiency was determined to be 85.6% and this mean counting efficiency was used to convert counts per minute as recorded by the instrument to disintegrations per minute (dpm). Quenching was not a problem in the measurement of the radioactivity of the samples.

CHAPTER III

RESULTS

Partial Purification of Acetoacetate Decarboxylase Activity from Bovine Liver Homogenates

Van Stekelenburg and Koorevaar (1972) reported that rat liver homogenates exhibited acetoacetate decarboxylase activity and they postulated the existence of a mammalian enzyme. The initial experiments in this study centered on an attempted partial purification of acetoacetate decarboxylase activity from bovine liver. The purification of the bacterial acetoacetate decarboxylase from *Clostridium acetobutylicum* was reported by Hamilton and Westheimer (1959) and some of the steps of their procedure were used as guidelines.

Fresh beef liver, (1700 grams) was obtained from the slaughter house (Lincoln Meat Company, Chicago, Illinois), packed in ice for transport and homogenized without undue delay at the laboratory in 2 liters of cold acetone. All steps in the preparation of an acetone powder were carried out in the cold room at 4°C. The homogenate was mixed for 1 hour and filtered with a Buchner funnel. The residue was defatted by resuspending it in 2 liters of cold chloroform and mixing it for 1 hour. The mixture was filtered as before. The residue was resuspended in 2 liters of cold acetone, stirred for 1 hour, filtered and allowed to air dry. The weight of dried acetone powder was 400 grams. The acetone powder was extracted with 2 liters of 50 m*M* phosphate buffer, pH 5.9 with continuous stirring for 4 hours at 4°C. The solid material was then removed by centrifugation in a refrigerated centrifuge at 2400 X *g* for 30 minutes.

The supernatant was acidified to pH 3.8 with 2<u>M</u> acetic acid and allowed to stand overnight at 4°C. The resultant precipitate was collected by centrifugation at 3900 rpm (2400 X g) for 30 minutes and redissolved in 200 ml of 50 m<u>M</u> phosphate buffer, pH 5.9. Following the Hamilton and Westheimer (1959) procedure, a heat-activation step at 55°C for 1 hour was performed. Protein denaturation occurs at this step and the mixture was clarified by centrifugation as before. The supernatant was subjected to various approaches of purification. These included precipitation with ammonium sulfate at numerous concentrations, ultrafiltration, molecular exclusion chromatography, cation and anion chromatography, isoelectric focusing and isotachophoresis. Only ultrafiltration and isoelectric focusing techniques were useful in the characterization of the acetoacetate decarboxylase activity. Ultrafiltration of the heat-activated material was performed with an Amicon Ultrafiltration Cell, Model 52 (Amicon Corporation, Lexington, Mass.) fitted with an XM-50 Diaflo Ultrafiltration Membrane (Amicon Corporation, Lexington, Mass.) which has a molecular weight cutoff of 50,000. Ultrafiltration was carried out with continuous stirring under a nitrogen pressure of 60 lbs per square inch in the cold room at 4°C. Assay of the retentate and the ultrafiltrate showed that the bulk of the acetoacetate decarboxylase activity was found in the ultrafiltrate. Although ultrafiltration membranes do not yield quantitative molecular weight fractionation, it appears that the active material has a molecular weight range less than 50,000. The active filtrate was ultrafiltered as before with a Diaflo PM-30 membrane which has a molecular weight cutoff of 30,000. Assay of the retentate and filtrate showed that the bulk of the activity was associated with the PM-30 ultrafiltrate although the specific activity

of the PM-30 retentate was considerable. Table 4 lists the acetoacetate decarboxylase activities associated with the fractions obtained from ultrafiltration of the heat-activated bovine liver extract.

The retentate and filtrate from ultrafiltration with the PM-30 membrane were pooled and twenty mg of protein from the mixture was subjected to isoelectric focusing in the pH range of 3.5-10 for 48 hours at 500 volts. Fractions of 2.5 ml each were collected from the column and the protein content of the effluent was monitored at 280 nm. Figure 6 depicts the UV absorption curve and the superimposed pH curve. The contents of tubes 1 through 10 comprise the anode solution and were discarded. The contents of tubes 11 through 17 were pooled and lyophilized (Fraction I). The contents of tubes 18 through 45 were pooled and lyophilized (Fraction II). Fraction I and II were reconstituted in one ml of 50 mM Tris buffer, pH 7.4 and aliquots were assayed for acetoacetate decarboxylase activity. Fraction II was found to produce acetone at the same rate as the reagent blank, 2×10^{-4} mole min^{-1}. Fraction I was found to produce acetone at the rate of 4×10^{-4} mole min^{-1}. The active acetoacetate decarboxylase component of bovine liver extracts has a molecular weight less than 30,000 and a pI of approximately 4.

In 1976, Koorevaar and van Stekelenburg presented further evidence that mammalian acetoacetate decarboxylase activity is associated with the albumin fraction of human serum. As a result of this report, further attempts of characterization of the enzymic activity from bovine liver were discontinued and attention was soon focused on the decarboxylase activity associated with human serum albumin.

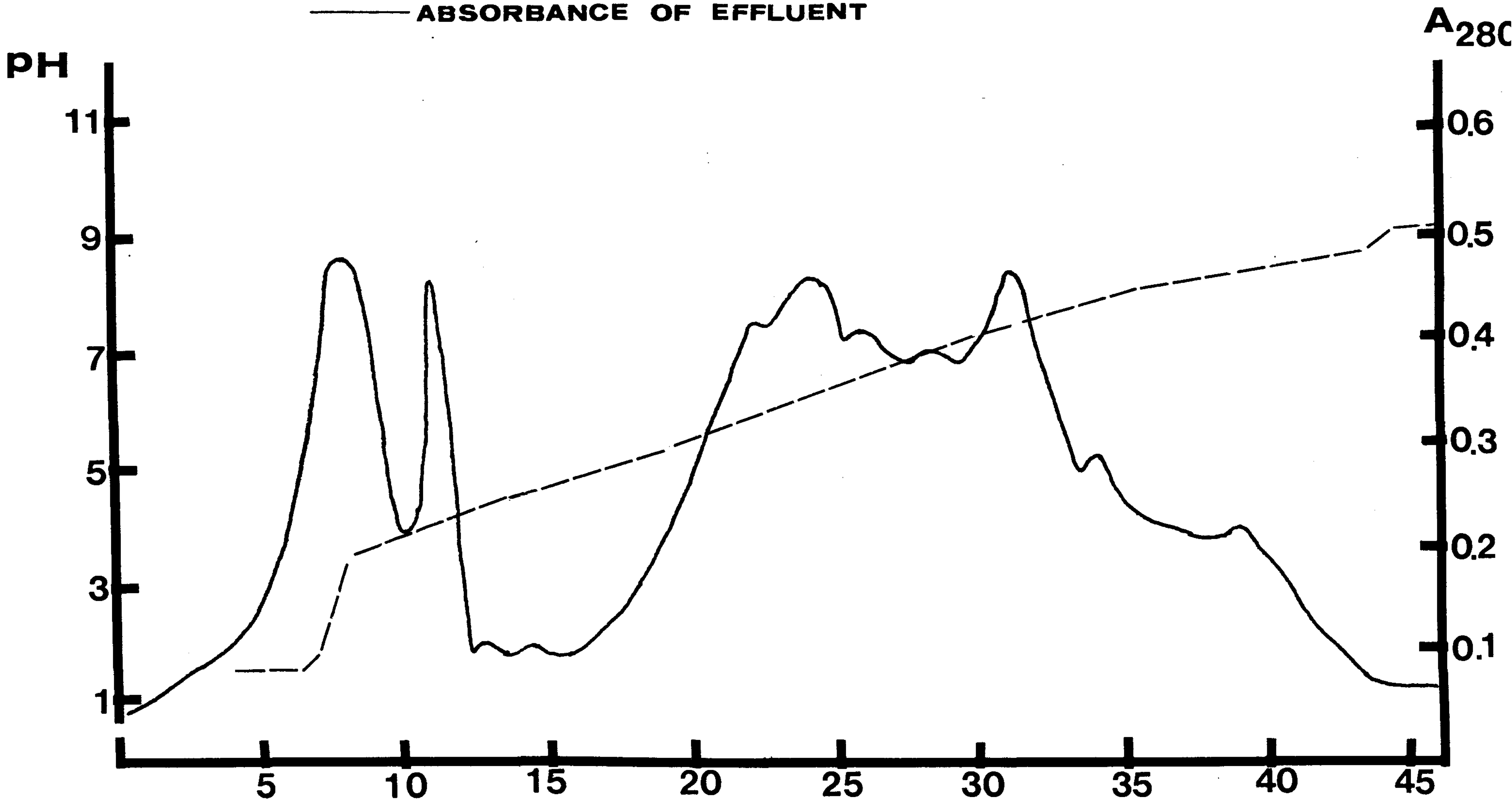
Table 4

Acetoacetate Decarboxylase Activities of Fractions Obtained from Ultrafiltration of Heat-Activated Bovine Liver Extract

Fraction	Acetoacetate Decarboxylase Activity (Units)	Total Activity Units x gms of protein
Protein Collected After Acidification with Acetic Acid	0.86	437
Heat Activated Material	1.2	736
Retentate from XM-50 (> 50,000)	0.77	55
Ultrafiltrate from XM-50 (< 50,000)	4.1	259
Retentate from PM-30 (> 30,000)	2.1	43
Ultrafiltrate from PM-30 (< 30,000)	4.1	252

Figure 6. Isoelectric resolution of the less than 50,000 molecular weight fraction from an ultrafiltrate of bovine liver homogenate.

Electrophoretic resolution of a 21 mg pool of active protein of molecular weight less than 50,000 in a LKB 8100 electrofocusing column. The pH gradient extends from 3.5 to 10.0. Each tube contains 2.5 ml. Tubes 1 through 10 contain anode electrode solution; Fraction I (tubes 11 through 17) exhibited acetoacetate decarboxylase activity; Fraction II (tubes 18 through 45) contained no detectable acetoacetate decarboxylase activity. Resolved peak of Fraction I has a pI of 4.



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Effects of Experimentally-Induced Diabetes Mellitus and of Starvation on the Acetoacetate Decarboxylase Activity of Rat Plasma

In the first paper by Van Stekelenburg and Koorevaar (1972), it is inferred that an enzyme is responsible for the decarboxylation of acetoacetate in mammalian systems. To ascertain whether the acetoacetate decarboxylase activity is inducible during the metabolic stress of diabetes, a group of rats were made diabetic to increase their plasma concentration of oxybutyrates. Assay for acetoacetate decarboxylase activity during the diabetic state will determine whether the activity is inducible or constitutive.

Adult male rats (Locke-Erickson, Maywood, Illinois) were used in this study and were kept in individual cages. They were acclimated in the animal quarters for four days before use. Laboratory chow and water were provided ad libitum. At time of use, the rats had a mean body weight of 344 grams. Six rats were divided into two groups of three rats each. One group served as control animals. Streptozotocin (Upjohn Company, Kalamazoo, Michigan) was used to induce diabetes mellitus in the second group of rats.

Solutions of streptozotocin are very unstable and must be injected within ten minutes of their preparation. The concentration of streptozotocin used to induce diabetes was 65 mg/kg body weight. The animals were weighed and the appropriate amount of drug to be administered was weighed in small test tubes and kept at 4°C until the animals were prepared for the injection.

To facilitate handling, the animals were placed under a light anesthesia with an intraperitoneal injection of sodium pentobarbital of a concentration of 32.5 mg/kg of body weight. Blood samples were collected

from the animals to obtain initial levels for acetoacetate decarboxylase activity and for plasma glucose. The animals were weighed and blood samples were taken between 9 a.m. and 10 a.m. on the day of the testing. Once the blood samples were obtained, the appropriate test tube containing the streptozotocin was diluted with 0.2 ml of 50 m<u>M</u> citrate buffer, pH 4.5 and the solution was injected intraperitoneally. The control animals were handled in the same manner except that a placebo of 0.2 ml of the citrate buffer was used in place of the streptozotocin.

After 24 hours, blood samples were collected from all the animals and the appropriate groups were reinjected with streptozotocin or the placebo. Body weights and blood samples were obtained again after 72, 120 and 144 hours. Glucose and acetoacetate decarboxylase levels were determined as previously described for all animals.

In Table 5 are presented the changes in body weights, plasma glucose concentration and plasma acetoacetate decarboxylase activity during the experimental regime. Over the six day experimental period, the control animals maintained their weight. The streptozotocin-treated animals lost weight but the weight loss was not striking. Over the same time period, the glucose levels of the control animals varied little. The glucose levels of the streptozotocin-treated animals increased markedly, a mean increase of 249% was noted after six days. The acetoacetate decarboxylase activity of the plasma of both the control and the streptozotocin-treated animals remained fairly constant. There was no evidence of a definite increase in the acetoacetate decarboxylase activity of the diabetic animals. Thus, the onset of the diabetic state did not induce acetoacetate decarboxylase activity.

Table 5

Changes in Body Weight, Plasma Glucose Concentration and Plasma Acetoacetate Decarboxylase Activity of Control and Streptozotocin-treated Rats over a Period of Six Days and for the Streptozotocin-treated Animals, After a Subsequent Four Day Fast.

0 Hours

	Weight (g)	Glucose (mg/dl)	Acetoacetate Decarboxylase (Units)
Control #1	320	124	0.37
#2	314	116	0.27
#3	334	122	0.23
Test #1	333	122	0.30
#2	349	141	0.28
#3	414	128	0.26

24 Hours

	Weight (g)	Glucose (mg/dl)	Acetoacetate Decarboxylase (Units)
Control #1	314	136	0.36
#2	310	134	0.34
#3	325	127	0.34
Test #1	328	208	0.23
#2	339	311	0.21
#3	398	369	0.20

Table 5 (Continued)

72 Hours

	Weight (g)	Glucose (mg/dl)	Acetoacetate Decarboxylase (Units)
Control #1	321	114	0.25
#2	317	124	0.23
#3	327	117	0.25
Test #1	313	388	0.23
#2	335	383	0.25
#3	383	408	0.27

120 Hours

	Weight (g)	Glucose (mg/dl)	Acetoacetate Decarboxylase (Units)
Control #1	322	158	0.30
#2	325	133	0.40
#3	340	124	0.43
Test #1	318	428	0.31
#2	331	-	-
#3	395	548	0.32

144 Hours

	Weight (g)	Glucose (mg/dl)	Acetoacetate Decarboxylase (Units)
Control #1	322	134	0.58
#2	318	117	0.32
#3	334	123	0.32
Test #1	308	422	0.28
#2	334	422	0.31
#3	403	513	0.29

Table 5 (Continued)

After a subsequent 4 day fast

	Weight (g)	Glucose (mg/dl)	Acetoacetate Decarboxylase (Units)
Test #1	258	118	0.20
#2	280	144	0.22
#3	333	152	0.32

To place more stress upon the diabetic animals, their laboratory chow was removed after 144 hours. The diabetic animals were starved for four days and blood samples were collected once again so that the acetoacetate decarboxylase activity could be assessed further. However, even starvation secondary to the diabetes did not induce an increase in the level of acetoacetate decarboxylase activity.

The Effect of Acylating Agents on the Acetoacetate Decarboxylase Activity of Human Serum Albumin

The mechanism of action for the decarboxylation of acetoacetate by the bacterial enzyme acetoacetate decarboxylase, occurs through a Schiff base intermediate which forms between the carbonyl group of acetoacetate and the epsilon amino terminal of a lysine residue in the active site. The formation of a Schiff base in the mechanism for decarboxylation requires the presence of a free amino group. The primary source of free amino groups in human serum albumin would be the epsilon amino terminals of lysine residues. Jonas and Weber (1970) found that the epsilon amino groups of lysine in the bovine serum albumin molecule can be blocked after acylating the site with a dicarboxylic anhydride such as succinic and citraconic anhydride (2-methylmaleic anhydride). Maximum reaction with the amino groups of bovine serum albumin and succinic anhydride occur at a molar ratio of 15:1, anhydride to amino groups and a 70-80% modification of bovine serum albumin with citraconic anhydride occurs at a molar ratio of 1:1, anhydride to amino groups (Jonas and Weber, 1970).

On the basis of these consideration, it was decided to study the effect of acylation agents on the acetoacetate decarboxylase activity of human serum albumin to obtain further evidence for the formation of a Schiff base intermediate in the mechanism of action.

The free amino group content of Fraction V human serum albumin was determined by the method of Matthews et. al., (1964). A 1% solution of Fraction V human serum albumin (Miles Laboratories, Elkhart, Indiana), was found to have a free amino group content of 7.6 mM. The molar ratios of succinic and citraconic anhydride used in the acylations of albumin were based on this value. The acylations were carried out as described in the chapter on Materials and Methods. The succinated albumin solutions were lyophilized and reconstituted with 2.0 ml of 50 mM Tris buffer, pH 7.4. For the assay of acetoacetate decarboxylase activity, 200 μl of the reconstituted succinated albumin solution and 0.1 ml of 0.45 M lithium acetoacetate solution were placed in the test reaction flasks. The control reaction flasks contained 200 μl of reconstituted unacylated human serum albumin and 0.1 ml of 0.45 M lithium acetoacetate solution. Table 6 shows the inhibitory effect on the acetoacetate decarboxylase activity of the human serum albumin of the acylating agent succinic anhydride in the range of molar ratios of 0.025:1 to 0.25:1, anhydride to free amino group. Figure 7 presents the dose dependent response of inhibition observed for the acetoacetate decarboxylase activity of succinated albumin.

Minimal acylation of human serum albumin by succinate will inhibit the acetoacetate decarboxylase activity. This is evident by the fact that the free amino group content of succinated albumin remains fairly constant at low levels of acylation. It appears that very few sites on the albumin molecule are involved in the reaction with acetoacetate.

The second acylating agent, citraconic anhydride, has a distinct advantage over succinic anhydride in that the ligand can be readily hydrolyzed from albumin (Jonas and Weber, 1970). Human serum albumin

Table 6

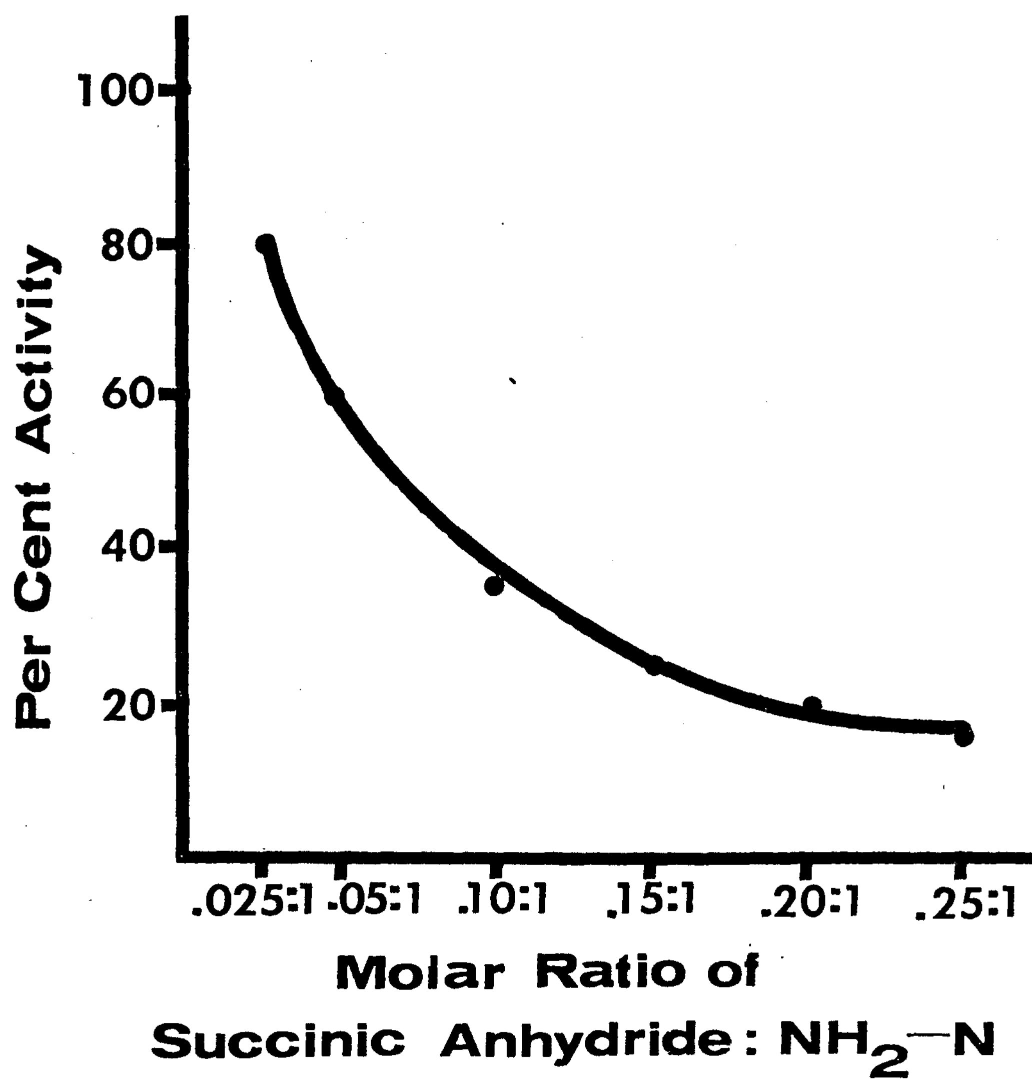
Acetoacetate Decarboxylase Activity of Succinyl Human Serum Albumin

Succinic Anhydride: αNH_2 Group Molar Ratio	Number of Determinations	Acetoacetate* Decarboxylase (Units)	Per cent* of Control Activity	Free Amino Group Content µg/mg Protein
Control	5	1.09 ± 0.11	-	6.73 ± 0.29
0.025:1	4	0.94 ± 0.15	79.9 ± 10.5	6.52 ± 0.24
0.05:1	5	0.64 ± 0.13	57.5 ± 6.9	6.68 ± 0.16
0.10:1	5	0.40 ± 0.17	35.4 ± 5.0	6.15 ± 0.28
0.15:1	5	0.26 ± 0.04	24.2 ± 2.8	5.88 ± 0.23
0.20:1	5	0.21 ± 0.03	18.7 ± 2.0	5.97 ± 0.29
0.25:1	5	0.17 ± 0.02	14.8 ± 1.7	5.81 ± 0.13

* Mean ± Standard Error of the Mean

Figure 7. Dose dependent response of inhibition observed for the acetoacetate decarboxylase activity of human serum albumin after acylation with succinic anhydride.

1% solutions of Fraction V human serum albumin were reacted with succinic anhydride. The reaction was carried out at room temperature with continuous stirring and the pH maintained at 7.0 by the dropwise addition of 1 M NaOH. The reaction was deemed complete when no further addition of NaOH was necessary to keep the solution at pH 7.0. Unreacted succinate was removed by dialysis against water. The acetoacetate decarboxylase activity was determined as previously described. The sample reaction mixture contained 2.7 ml of 50 mM Tris buffer, pH 7.4, 0.1 ml of 0.45 M lithium acetoacetate and 0.2 ml of the succinated albumin complex. For the blank reaction mixture, 0.2 ml of deionized water was substituted for the test sample.


100
80
60
40
20
Per Cent Activity
.025:1
.05:1
.10:1
.15:1
.20:1
.25:1
Molar Ratio of
Succinic Anhydride : NH2—N

can be acylated with citraconic anhydride and the acetoacetate decarboxylase activity of the complex can be determined. After the determination of the catalytic activity, the citraconyl group can be removed and the albumin reassayed for acetoacetate decarboxylase activity. Subsequent recovery of acetoacetate decarboxylase activity upon reassay would demonstrate the essential need of a free epsilon amino group for catalytic activity of albumin.

At a molar ratio of 1:1 citraconic anhydride to free amino content, the epsilon amino terminals of albumin are maximally acylated. At molar ratios between 0.25:1 and 1:1, the acetoacetate decarboxylase activity of human serum albumin was maximally inhibited. Ninhydrin assay of the citraconyl albumin samples demonstrated marked decreases in the free amino content between molar ratios of 0.25:1 and 1:1. Two molar ratios of citraconic anhydride to free amino group were used in the acylation, 0.10:1 and 0.25:1. These concentrations were chosen because at a molar ratio of 0.25:1 the acetoacetate decarboxylase activity is maximally inhibited while at a molar ratio of 0.10:1 the activity is inhibited by 60%. Unreacted citraconate was removed by passage of the acylated albumin through a Sephadex G-25 column. After assaying the acetoacetate decarboxylase activity of the citraconyl albumin samples, the ligand was removed by hydrolysis. Hydrolysis was performed by lowering the pH of the citraconyl albumin samples to 2.5 and stirring the resultant solution continuously at 4°C for 18 hours. The citraconate released was removed by passage of the samples through a Sephadex G-25 column. The acetoacetate decarboxylase assays for this set of experiments were carried out in the same manner as were those with succinated albumin. Table 7 lists the acetoacetate decarboxylase activity of human serum albumin after

acylation with citraconic anhydride. Table 8 presents the acetoacetate decarboxylase activity of human serum albumin after the removal of citraconyl groups from the citraconyl albumin complex.

The acetoacetate decarboxylase activity of human serum albumin is markedly decreased by its acylation with citraconic anhydride. Inhibition of the decarboxylase activity by the acylating agent further demonstrates the essentiality of the free amino groups for the catalytic activity associated with albumin. The inhibition of acetoacetate decarboxylase activity resulting from the attachment of the citraconyl group to the epsilon amino groups of albumin is reversible as shown by the recovery of activity from the hydrolyzed citraconyl-albumin complex.

The acetoacetate decarboxylase activities of the albumin samples following removal of the citraconyl group are greater than those observed for the citraconyl albumin complex. This qualitative difference was also observed for the activities of the control albumin samples subjected to the same conditions of hydrolysis. These phenomena may be explained in terms of the microheterogeneity of albumin. During the hydrolysis of the citraconyl albumin complex the pH of the solution is lowered to 2.5. Below pH 4 albumin undergoes a transition to another molecular species called the expanded form (Foster et. al., 1965). In the expanded form, groups which were folded into the interior of the albumin molecule become available at the hydrophilic surface. Jonas and Weber (1970) estimate that after the acid expansion of bovine serum albumin, 20% of the buried lysine residues become uncovered. The acid expansion of albumin may account then for the increased acetoacetate decarboxylase activity observed after the acid hydrolysis of the citraconyl albumin complex. However, of more import, was the finding that catalytic activity was recovered

Table 7

Acetoacetate Decarboxylase Activity of Citraconyl Human Serum Albumin

Citraconic Anhydride: αNH_2 Group Molar Ratio	Number of Determinations	Acetoacetate* Decarboxylase (Units)	Per Cent* of Control Activity	Free Amino Group Content µg/mg Protein
Control	3	0.93 ± 0.14	-	6.65 ± 0.64
0.10:1	3	0.36 ± 0.08	37.6 ± 4.1	6.48 ± 0.45
0.25:1	3	0.11 ± 0.03	11.7 ± 1.8	5.33 ± 0.63

*Mean ± Standard Error of the Mean

Table 8

Acetoacetate Decarboxylase Activity of Human Serum Albumin After Removal of Citraconate from Citraconyl Albumin Complex

Citraconic Anhydride: αNH_2 Group Molar Ratio	Number of Determinations	Acetoacetate* Decarboxylase (Units)	Per Cent* of Control Activity	Free Amino* Group Content µg/mg Protein
Control	3	1.21 ± 0.15	-	6.11 ± 0.52
0.10:1	3	1.11 ± 0.15	92.0 ± 0.9	5.55 ± 0.57
0.25:1	3	0.83 ± 0.14	67.6 ± 2.8	5.55 ± 0.32

* Mean ± Standard Error of the Mean

from the removal of the acylating agent providing further support for a role of the free epsilon amino group of lysine in acetoacetate decarboxylation.

The Effect of Beta Diketones on the Acetoacetate Decarboxylase Activity of Human Serum Albumin.

To obtain further evidence of acetoacetate decarboxylase activity associated with Fraction V human serum albumin characterization of the activity is necessary. Beta diketones are powerful competitive inhibitors of the bacterial enzyme that interfere with the formation of the Schiff base intermediate. To demonstrate a similar relationship in the mechanistic scheme, the effect of two beta diketones, acetopyruvate and acetylacetone, on the acetoacetate decarboxylase activity of human serum albumin was performed. The concentration of acetopyruvate employed ranged from 0.10 m$\underline{M}$ to 2.0 m$\underline{M}$ and the albumin concentration in each reaction flask was 10 mg. Table 9 shows the per cent inhibition of acetoacetate decarboxylase activity of the albumin at various concentrations of acetopyruvate. Figure 8 demonstrates the relationship between the concentration of acetopyruvate and the extent of inhibition by the diketone.

The effect of acetylacetone was performed in the concentration range of 10 m$\underline{M}$-80 m$\underline{M}$. As with acetopyruvate, each reaction mixture contained 10 mg of Fraction V human serum albumin. Table 10 shows the per cent inhibition of acetoacetate decarboxylase activity of the albumin at various concentrations of acetylacetone. Figure 9 demonstrates the relationship between the concentration of acetylacetone and the extent of inhibition caused by the diketone.

Both acetopyruvate and acetylacetone have marked inhibitory effects on the acetoacetate decarboxylase activity of Fraction V human serum

Table 9

Effect of Acetopyruvate Upon the Acetoacetate Decarboxylase Activity of Fraction V Human Serum Albumin

Concentration of Acetopyruvate	Number of Determinations	Acetoacetate Decarboxylase Activity (Units)	Per Cent * Inhibition
Control	5	0.98 ± 0.03	
0.10 mM	5	0.86 ± 0.01	12.2 ± 1.7
0.25 mM	5	0.82 ± 0.01	15.9 ± 1.9
0.50 mM	5	0.72 ± 0.01	26.5 ± 0.9
1.00 mM	5	0.66 ± 0.02	32.2 ± 1.7
2.00 mM	5	0.55 ± 0.03	43.5 ± 2.6

* Mean ± Standard Error of the Mean

Figure 8. The effect of acetopyruvate on the acetoacetate decarboxylase activity of Fraction V human serum albumin.

The acetoacetate decarboxylase activity was determined as previously described. The sample reaction mixture contained 2.6 ml of 50 m*M* Tris buffer, pH 7.4, 0.1 ml of a stock acetopyruvate solution necessary to bring its concentration in the reaction mixture to the predetermined level, 0.1 ml of 0.45 *M* lithium acetoacetate and 10 mg of Fraction V human serum albumin in 0.2 ml of deionized water. For the blank reaction mixture 0.2 ml of deionized water was substituted for the test sample.

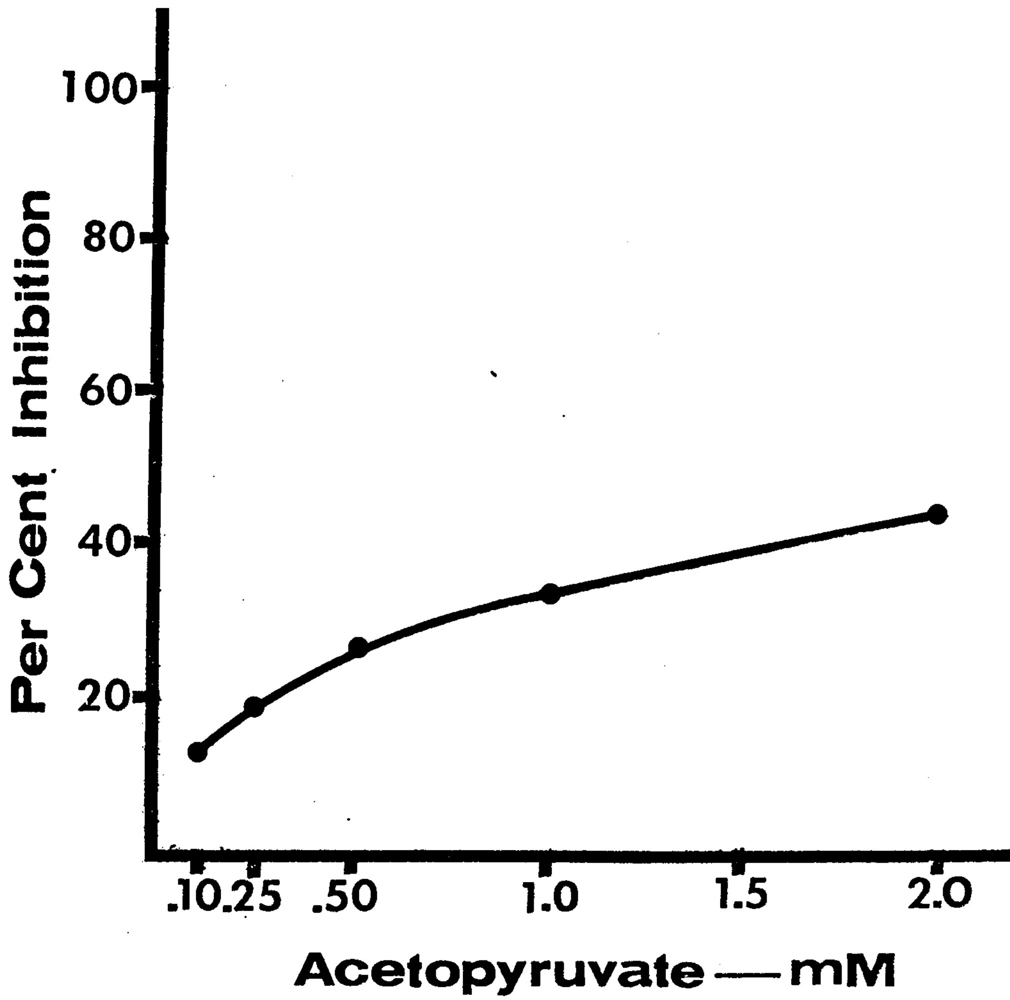

100
80
60
40
20
Per Cent Inhibition
.10
.25
.50
1.0
1.5
2.0
Acetopyruvate — mM

Table 10

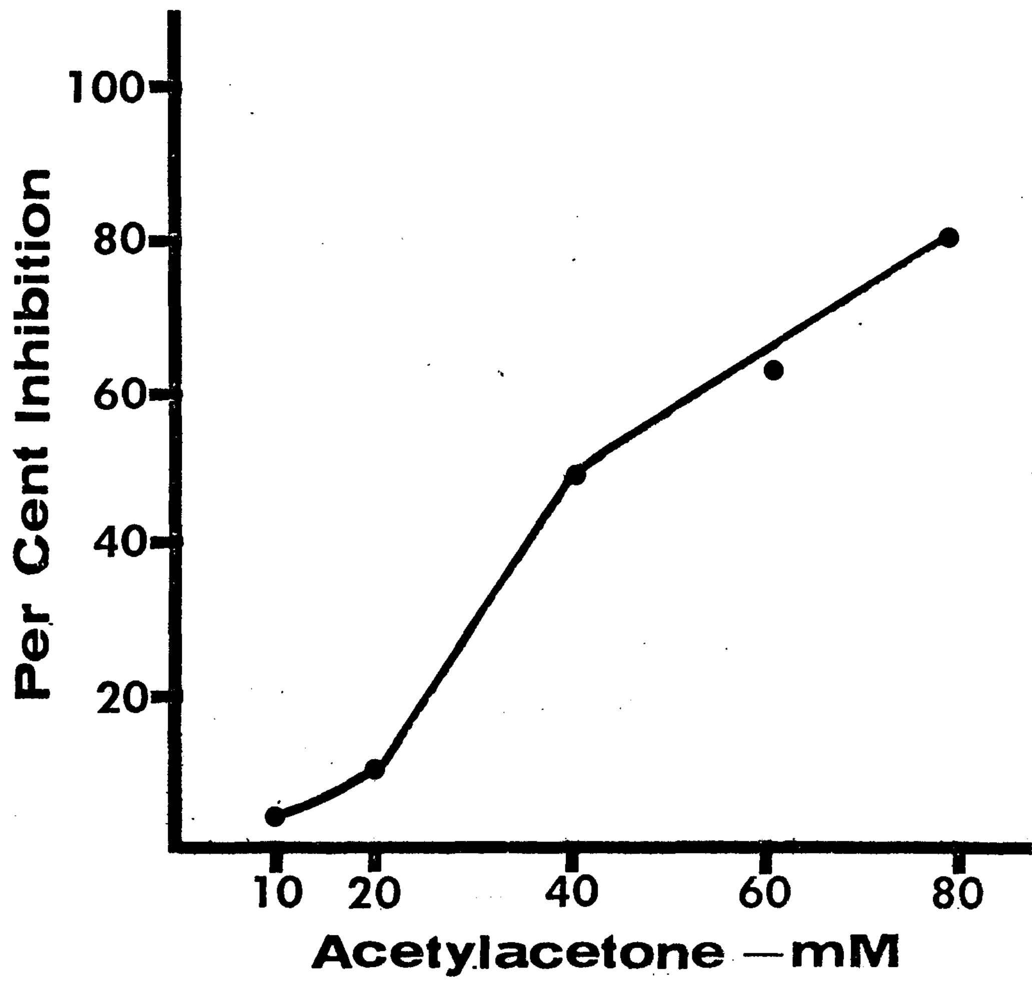
The Effect of Acetylacetone on the Acetoacetate Decarboxylase Activity of Fraction V Human Serum Albumin

Concentration of Acetylacetone	Number of Determinations	Acetoacetate Decarboxylase Activity (Units)	Per Cent * Inhibition
Control	10	0.99 ± 0.03	
10 m*M*	4	0.96 ± 0.00	3.5 ± 0.3
20 m*M*	4	0.90 ± 0.01	9.1 ± 1.1
40 m*M*	4	0.50 ± 0.00	49.0 ± 0.3
60 m*M*	4	0.46 ± 0.05	54.0 ± 6.2
80 m*M*	4	0.22 ± 0.02	78.2 ± 2.6

* Mean ± Standard Error of the Mean

Figure 9. The effect of acetylacetone on the acetoacetate decarboxylase activity of Fraction V human serum albumin.

The acetoacetate decarboxylase activity was determined as previously described. The sample reaction mixture contained 2.6 ml of 50 m*M* Tris buffer, pH 7.4, 0.1 ml of a stock acetylacetone solution necessary to bring its concentration in the reaction mixture to the predetermined level, 0.1 ml of 0.45 *M* lithium acetoacetate and and 10 mg of Fraction V human serum albumin in 0.2 ml of deionized water. For the blank reaction mixture 0.2 ml of deionized water was substituted for the test sample.


100
80
60
40
20
Per Cent Inhibition
10
20
40
60
80
Acetylacetone —mM

albumin. This observation is not unexpected if the mechanism of decarboxylase action by albumin occurs through a Schiff base intermediate. The beta diketone will react with the epsilon amino group of lysine rendering it inaccessible to the substrate acetoacetate. The higher the concentration of the diketone the more effective its competition for the active lysine residue and therefore, the inhibition is greater.

The Effect of Borohydride on the Acetoacetate Decarboxylase Activity of Human Serum Albumin

Sodium borohydride is a known reductant of enzymes whose mechanism occurs through a Schiff base intermediate (Fischer, et. al., 1958, Grazi et. al., 1962, Horecker et. al., 1961, Dempsey et. al, 1962 and Fridovich and Westheimer 1962). To gain information on the mode of action for the acetoacetate decarboxylase activity associated with human serum albumin, a borohydride reduction was performed.

Borohydride will reduce not only Schiff base intermediates but also the substrate acetoacetate and the product produced in the decarboxylation reaction, acetone. Thus, the assay for acetoacetate decarboxylase by head space analysis cannot be used in this experiment because of the inability to measure precisely the acetone release. To investigate the borohydride reduction of an albumin-acetoacetate complex the assay developed for trapping Schiff base intermediates (Fridovich and Westheimer 1962) was modified for use in this study. Incubation of 10 mg of Fraction V human serum albumin with ($3-^{14}C$) acetoacetate was carried out in 2.9 ml of 50 mM Tris buffer, pH 7.4. If the mechanism of action for the decarboxylation of acetoacetate occurs through a Schiff base intermediate, a ketimine will be formed which is reducible by borohydride. Reduction of the ketimine intermediate results in the formation of a

radiochemically labeled complex. The bacterial enzyme has a low K_m and, therefore, when borohydride is added to the reaction mixture, there is a considerable amount of reduction occurring at the enzyme-substrate level. This is not the case for albumin because the acetoacetate decarboxylase activity associated with the molecule has a high K_m, approximately 0.1 M (Koorevaar and Van Stekelenburg, 1976). The addition of borohydride to the albumin-reaction mixture results in a much lower level of reduction at the albumin complex site. To compensate for the high K_m associated with the albumin fraction, the reduction was performed every 30 minutes for 2 hours. Various concentrations of sodium borohydride were employed. The use of 1 mM and 2 mM sodium borohydride were found to be effective in the trapping of a radiochemically-labeled albumin complex (Table 11).

For each borohydride reduction of the solution containing albumin and (3-^{14}C) acetoacetate, there is a significant incorporation of label in the albumin molecule. The radiochemically-labeled albumin complex is stable; its radioactivity is not lost after exhaustive dialysis. The trapping of the radiochemically-labeled albumin offers evidence that the mechanism of action utilized by Fraction V human serum albumin occurs through a Schiff base intermediate.

The Effect of Acetopyruvate on the Borohydride Reduction of the Human Serum Albumin-(3-^{14}C) Acetoacetate Complex

Acetopyruvate acts as a competitive inhibitor of the bacterial enzyme acetoacetate decarboxylase; the carbonyl group adjacent to the methyl group forms a Schiff base with the epsilon amino group of lysine in the active site. The resultant ketimine complex forms a non-productive intermediate which has been isolated (Tagaki et. al., 1968). The non-productive binding of acetopyruvate to the enzyme interferes with the

Table 11

The Trapping of a Radiochemically-Labeled Albumin-Acetoacetate Complex by Borohydride Reduction

Experiment 1

Sample	DPM/mg Protein	Per Cent Increase
Control	515	-
1 mM BH_4^-	1616	214
2 mM BH_4^-	1250	143

Experiment 2

Sample	DPM/mg Protein	Per Cent Increase
Control	856	-
1 mM BH_4^-	1443	69
2 mM BH_4^-	1341	57

Experiment 3

Sample	DPM/mg Protein	Per Cent Increase
Control	1383	-
1 mM BH_4^-	2010	45

Experiment 4

Sample	DPM/mg Protein	Per Cent Increase
Control	450	-
1 mM BH_4^-	987	119
2 mM BH_4^-	1061	136

Experiment 5

Sample	DPM/mg Protein	Per Cent Increase
Control	519	-
1 mM BH_4^-	949	83
2 mM BH_4^-	1293	149

Table 11 (Continued)

Experiment 6

Sample	DPM/mg Protein	Per Cent Increase
Control	768	-
1 m*M* BH_4^-	1000	30
1 m*M* BH_4^-	975	27
2 m*M* BH_4^-	1072	40
2 m*M* BH_4^-	982	28

productive binding of acetoacetate to the active site.

It has been shown in this study that acetopyruvate will inhibit the acetoacetate decarboxylase activity of Fraction V human serum albumin. Experiments on the borohydride reduction of the albumin-(3-^{14}C) acetoacetate complex have given evidence that the mechanism of action of albumin in the decarboxylation of acetoacetate occurs through a Schiff base intermediate. To gain further support for the mechanism of action, the borohydride reduction of the albumin-(3-^{14}C) acetoacetate complex was conducted in the presence of acetopyruvate. If a Schiff base intermediate is formed between the albumin and acetoacetate, acetopyruvate will interfere with the binding at the active site. The competition between acetopyruvate and acetoacetate for the site will become evident by a decrease in the radioactivity of the borohydride-reduced albumin-acetoacetate complex. Preliminary studies showed that 6 m$\underline{M}$ acetopyruvate inhibits the acetoacetate decarboxylase activity of human serum albumin by approximately 80% and this concentration of the inhibitor was used in this study. The borohydride reduction of the albumin-(3-^{14}C) acetoacetate complex, the trapping of the radiochemically labeled albumin complex and the measurement of radioactivity were performed as previously described. Table 12 presents the extent of incorporation of the label in the albumin-acetoacetate complex after performing the borohydride reduction in the presence and in the absence of acetopyruvate. Acetopyruvate when incubated with (3-^{14}C) acetoacetate and Fraction V human serum albumin was found to inhibit the formation of a labeled acetoacetate-albumin complex. In three of the experiments (2,3,4), the per cent of label incorporated into the complex was decreased by 55 $\pm$ 1.0% (S.E.M.).

Table 12

The Effect of Acetopyruvate Upon the Borohydride Reduction of Albumin (3-^{14}C) Acetoacetate

Experiment 1	DPM/mg Protein	Incorporation of Label Percent of Control
(3-^{14}C) Acetoacetate + Human Serum Albumin	1504	
(3-^{14}C) Acetoacetate + Human Serum Albumin + 6 m$\underline{M}$ Acetopyruvate	1327	88
(3-^{14}C) Acetoacetate + Human Serum Albumin + 1 m$\underline{M}$ BH_4^-	2447	
(3-^{14}C) Acetoacetate + Human Serum Albumin + 6 m$\underline{M}$ Acetopyruvate + 1 m$\underline{M}$ BH_4^-	841	34
Experiment 2		
(3-^{14}C) Acetoacetate + Human Serum Albumin	1630	
(3-^{14}C) Acetoacetate + Human Serum Albumin + 6 m$\underline{M}$ Acetopyruvate	755	46
(3-^{14}C) Acetoacetate + Human Serum Albumin + 1 m$\underline{M}$ BH_4^-	2591	
(3-^{14}C) Acetoacetate + Human Serum Albumin + 6 m$\underline{M}$ Acetopyruvate + 1 m$\underline{M}$ BH_4^-	771	30
Experiment 3		
(3-^{14}C) Acetoacetate + Human Serum Albumin	418	
(3-^{14}C) Acetoacetate + Human Serum Albumin + 6 m$\underline{M}$ Acetopyruvate	180	43
(3-^{14}C) Acetoacetate + Human Serum Albumin + 1 m$\underline{M}$ BH_4^-	1196	
(3-^{14}C) Acetoacetate + Human Serum Albumin + 6 m$\underline{M}$ Acetopyruvate + 1 m$\underline{M}$ BH_4^-	388	32

Table 12 (Continued)

Experiment 4	DPM/mg Protein	Incorporation of Label Percent of Control
(3-^{14}C) Acetoacetate + Human Serum Albumin	430	
(3-^{14}C) Acetoacetate + Human Serum Albumin + 6 m$\underline{M}$ Acetopyruvate	200	47
(3-^{14}C) Acetoacetate + Human Serum Albumin + 1 m$\underline{M}$ BH_4^-	1061	
(3-^{14}C) Acetoacetate + Human Serum Albumin + 6 m$\underline{M}$ Acetopyruvate + 1 m$\underline{M}$ BH_4^-	404	38

For each borohydride reduction of the solution containing albumin and (3-^{14}C) acetoacetate, there was, as expected, an increased incorporation of the label into the albumin complex in the absence of acetopyruvate. In all four experiments, when the borohydride reduction was carried out in the presence of 6 m_M_ acetopyruvate significant inhibition of incorporation of label occurred. The incorporation was decreased by $66 \pm 2\%$ (S.E.M.).

Acetopyruvate interferes with the borohydride reduction of the albumin-(3-^{14}C) acetoacetate complex and acts in a manner similar to that observed for the bacterial enzyme. It is postulated that acetopyruvate reacts with albumin forming a ketimine with an epsilon amino group of lysine. The acetopyruvate competes with acetoacetate for the active lysine residues on the albumin molecule and the competition results in fewer labeled ketimine intermediates available for reduction by borohydride. The observed data give supportive evidence that the mechanism of action for the decarboxylation of acetoacetate occurs through a Schiff base intermediate.

The Effect of Human Serum Albumin on the Decarboxylation of Alpha Ketoglutarate and Pyruvate

The bacterial enzyme acetoacetate decarboxylase is very specific for acetoacetate having no action on the decarboxylation of alpha or other beta keto acids. To investigate the effect of Fraction V human serum albumin on other substrates, two alpha keto acids were chosen, α-ketoglutarate and pyruvate. To measure the decarboxylation rate, the liberation of carbon dioxide was measured radiochemically. Fifty μCi of (1-^{14}C) sodium pyruvate and fifty μCi of (1-^{14}C) α-ketoglutarate (New England Nuclear, Boston, Mass.) were brought to a concentration of

0.45M with the corresponding unlabeled alpha keto acid. Each reaction mixture contained 10 mg of the albumin in 2.9 ml of 50 mM Tris buffer, pH 7.4 and 100μl (5μCi) of the labeled keto acid solution. The decarboxylation reaction was carried out at 37°C for two hours. The labeled carbon dioxide was trapped in the sodium hydroxide solution contained in a plastic collection bucket. The extent of decarboxylation was determined by measuring the trapped labeled carbon dioxide in the sodium hydroxide solution by liquid scintillation. Blank solutions containing 2.9 ml of 50 mM Tris buffer, pH 7.4 and 100μl of substrate were run to monitor the nonenzymatic decarboxylation. Table 13 shows the effect of human serum albumin (HSA) on the decarboxylation of alpha ketoglutarate and pyruvate in terms of the radioactivity of the trapped labeled carbon dioxide. The counts obtained from the decarboxylation of α-ketoglutarate and pyruvate in the presence of Fraction V human serum albumin were lower than those for the corresponding blank solutions. The data indicate that albumin will not stimulate the decarboxylation of α-ketoglutarate or pyruvate. The decarboxylation of the alpha keto acids in solution is actually decreased in some unknown manner by the albumin.

The Effect of Bound Fatty Acids on the Acetoacetate Decarboxylase Activity of Human Serum Albumin

Unlike other plasma proteins albumin has numerous functions; it is the principal material responsible for the oncotic pressure of the blood and it transports fatty acids and bilirubin. Albumin has other less defined functions such as the transport vehicle for tryptophan, various hormones and drugs; it also serves as a source of amino acids in the peripheral tissues.

Table 13

The Effect of Human Serum Albumin (HSA) on the Decarboxylation of Alpha Ketoglutarate and Pyruvate

Alpha ketoglutarate

Experiment No.	Sample	Radioactivity of the Trapped CO_2 DPM
1.	Reagent Blank (2)	1922
		1636
	HSA (5)	1088 ± 23 (S.E.M.)
2.	Reagent Blank (2)	3172
		2553
	HSA (3)	1106 ± 33 (S.E.M.)

Pyruvate

Experiment No.	Sample	Radioactivity of the Trapped CO_2 DPM
1.	Reagent Blank	4598
		4472
	HSA (4)	3027 ± 38 (S.E.M.)
2.	Reagent Blank	5451
		5333
	HSA (3)	3037 ± 34 (S.E.M.)

The number of determinations is shown in the parenthesis

Long chain fatty acids are highly insoluble at pH 7.4 and their presence in plasma is largely due to the binding properties of albumin. Ashbrook (1975) reported that 99.9% of the fatty acids in the circulation are bound to albumin. One or two fatty acid residues are bound per molecule of albumin by the insertion of the aliphatic chains into the hydrophobic clefts of the molecule (Peters 1975).

To investigate the effect of bound fatty acids on the acetoacetate decarboxylase activity of albumin, defatted Fraction V human serum albumin was assayed for its acetoacetate decarboxylase activity. The defatted Fraction V albumin (Miles Laboratories, Elkhart, Indiana, Code No. 82-323, Lot No. 13) was reported to be 98% pure as determined by cellulose acetate electrophoresis in 0.75 M barbital buffer, pH 8.6 and to contain less than 0.1 mole of fatty acid per mole of albumin. Ten mg samples of the defatted Fraction V human serum albumin were assayed for acetoacetate decarboxylase activity, as were 10 mg samples of Fraction V human serum albumin. Table 14 shows the percent increase in the acetoacetate decarboxylase activity of defatted Fraction V human serum albumin over that observed for Fraction V human serum albumin. This is not unexpected since the bound fatty acids most likely will interfere sterically with some of the epsilon amino groups of lysine residues. Once the fatty acids are removed from the hydrophobic clefts of albumin, more lysine residues are available for reaction with acetoacetate and hence, the acetoacetate decarboxylase activity of defatted albumin is greater.

The Effect of Guanidine-HCl on the Acetoacetate Decarboxylase Activity of Human Serum Albumin

Guanidine-HCl is known to cause disruptions in the secondary structure of proteins. Wallevik (1973) has shown by optical rotation

Table 14

Acetoacetate Decarboxylase Activity of Fraction V Human Serum Albumin and Defatted Fraction V Human Serum Albumin

Test Material	Number of Determinations	Acetoacetate* Decarboxylase Activity (Units)	Per Cent Increase
Fraction V Human Serum Albumin	5	0.95 ± 0.0	
Defatted Fraction V Serum Albumin	5	1.30 ± 0.1	36

* Mean ± Standard Error of the Mean

studies that minor changes occur in the secondary structure of human serum albumin at concentrations of less than 1 M.

Ten mg samples of human serum albumin were assayed in the presence of guanidine-HCl to study the effect that minor disruptions in the secondary structure of the molecule have on the acetoacetate decarboxylase activity. The concentration of guanidine-HCl used in the study ranged from 50 mM-1000 mM. Table 15 lists the mean percent of inhibition observed in the acetoacetate decarboxylase activity resulting from secondary structural disruption by guanidine-HCl. A graphical representation of the inhibition attained in the presence of various concentrations of guanidine-HCl is shown in Figure 10.

Very low concentrations of guanidine-HCl will cause a considerable inhibition of the acetoacetate decarboxylase activity of human serum albumin. Inhibition begins to level off somewhat at a guanidine-HCl concentration of 400 mM. Thus, the acetoacetate decarboxylase activity of albumin is extremely sensitive to minor changes in the secondary structure of the molecule.

The Effect of Various Monovalent Anions on the Acetoacetate Decarboxylase Activity of Human Serum Albumin

Studies of specific effects of anions on colloidal systems in aqueous systems date back to Hofmeister (1888) who measured the effects of various salts on the solubility of egg albumin. Series of anions arranged in order of ability to modify some observable function and similar in sequence to the series of anions found by Hofmeister are usually referred to as Hofmeister or Lyotropic Series (Fridovich, 1963). Acetoacetate decarboxylase of *Cl. acetobutylicum* has been shown to be strikingly sensitive to inhibition by anions (Fridovich, 1963). The anions react

Table 15

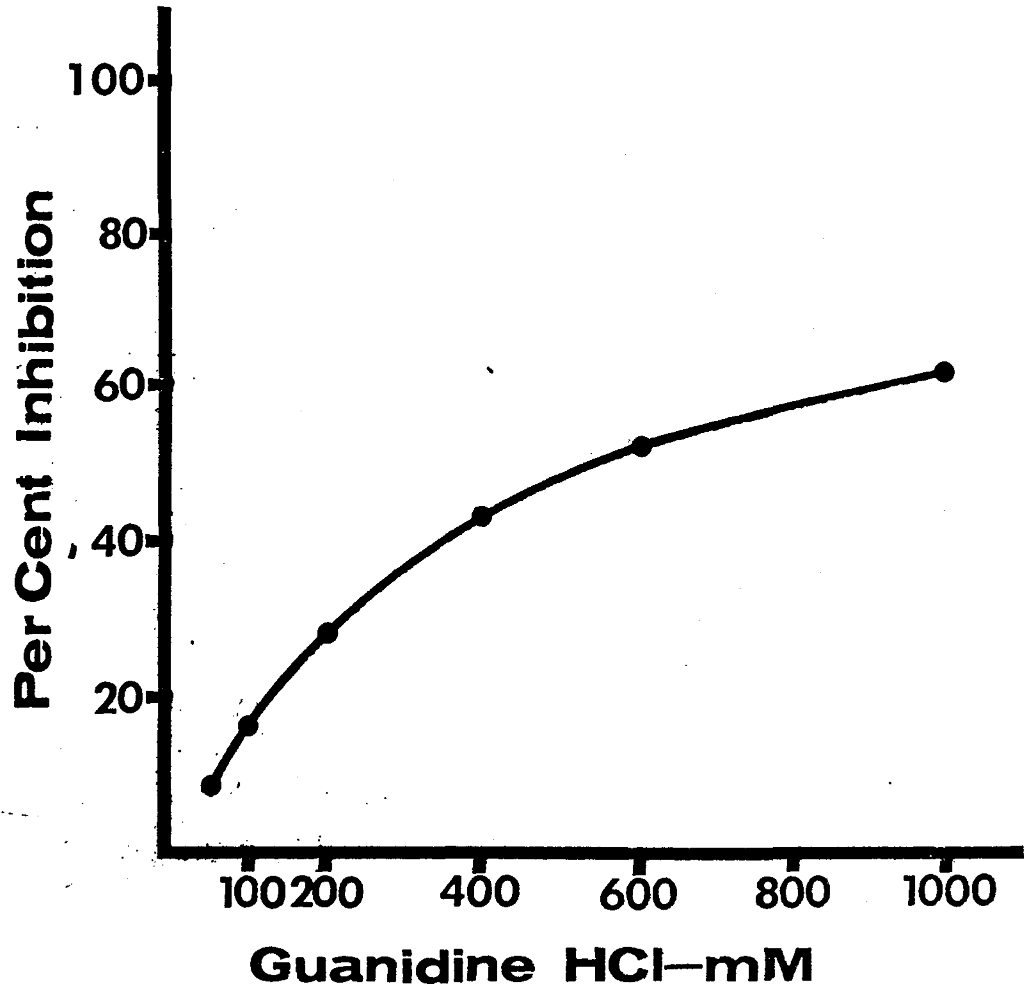
The Effect of Guanidine-HCl Upon the Acetoacetate Decarboxylase Activity of Human Serum Albumin

Concentration of Guanidine-HCl	Number of Determinations	Acetoacetate Decarboxylase Activity (Units)	Per Cent * Inhibition
Control	6	1.02 ± 0.01	
50 mM	5	0.95 ± 0.02	7.3 ± 2.2
100 mM	5	0.86 ± 0.02	15.7 ± 2.7
200 mM	5	0.74 ± 0.01	27.2 ± 1.3
400 mM	5	0.57 ± 0.01	43.8 ± 1.4
800 mM	5	0.49 ± 0.01	51.8 ± 0.5
1000 mM	5	0.40 ± 0.01	60.6 ± 0.5

* Mean ± Standard Error of the Mean

Figure 10. The effect of guanidine-HCl on the acetoacetate decarboxylase activity of Fraction V human serum albumin.

The acetoacetate decarboxylase activity was determined as previously described. The sample reaction mixture contained 2.6 ml of 50 m*M* Tris buffer, pH 7.4, 0.1 ml of a stock guanidine-HCl solution necessary to bring its concentration in the reaction mixture to the predetermined level, 0.1 ml of 0.45 *M* lithium acetoacetate and 10 mg of Fraction V human serum albumin in 0.2 ml of deionized water. For the blank reaction mixture 0.2 ml of deionized water was substituted for the test sample.


Per Cent Inhibition
100
80
60
40
20
100
200
400
600
800
1000
Guanidine HCl—mM

with the bacterial enzyme at a single sensitive site which seems to be cationic in nature having a pK of 5.8 or that consistent with an amino group (Fridovich, 1963). The ionic attraction of the anions to the sensitive site on the enzyme inhibits the substrate from gaining access to the active site. The ionic diameter of the anion seems to play an important role in the potency of the inhibitor (Fridovich, 1963). The series of anions listed in order of their potency as inhibitors nearly parallels the Hofmeister series in reverse (Fridovich and Westheimer, 1962; Westheimer, 1963).

The acetoacetate decarboxylase activity of human serum albumin is due to a sensitive lysine residue. It is not unexpected that anions may also inhibit the decarboxylase activity of albumin. To further characterize the acetoacetate decarboxylase activity of Fraction V human serum albumin, the effect of monovalent anions upon the decarboxylase activity was studied. Four anions from the Hofmeister series were investigated: bisulfite, thiocyanate, nitrate and chloride. These were chosen on the basis of their increasing effectiveness of inhibition of the bacterial enzyme of the following order: $HSO_3^- > SCN^- \gg NO_3^- \gg Cl^-$. For the assay, the test reaction mixtures contained 2.6 ml of 50 m$\underline{M}$ Tris buffer, pH 7.4, 0.1 ml of 0.45 $\underline{M}$ lithium acetoacetate and 0.2 ml of a solution of Fraction V human serum albumin (10 mg) and 0.1 ml of a solution of the sodium salt of the test anion. The control reaction mixtures were prepared similarly except that deionized water was used instead of the test anion solution. Various concentrations of the anion solutions were tried in setting up the protocol. Table 16 lists the concentration of each anion required for a comparable degree of inhibition of acetoacetate decarboxylase activity of Fraction V human serum albumin.

Table 16

The Effect of Monovalent Anions on the Acetoacetate Decarboxylase Activity of Albumin

Anion	Number of Determinations	Concentration	Per Cent Inhibition
SCN^-	5	10 m*M*	43.1% ± 2.8 (S.E.M.)
HSO_3^-	5	10 m*M*	41.8% ± 0.7 (S.E.M.)
NO_3^-	6	80 m*M*	40.8% ± 2.6 (S.E.M.)
Cl^-	6	200 m*M*	44.2% ± 1.0 (S.E.M.)

The degree of inhibition caused by the monovalent anions on the acetoacetate decarboxylase activity of human serum albumin parallels the order of effectiveness observed for the inhibition of the bacterial acetoacetate decarboxylase. The inhibition by monovalent anions further characterizes the acetoacetate decarboxylase activity of albumin and gives an indication that the sensitive site necessary for the decarboxylase activity is cationic in nature.

The Acetoacetate Decarboxylase Activity Associated with Crystalline Human Serum Albumin

Crystalline human serum albumin was assayed to obtain further evidence that acetoacetate decarboxylase activity is not due to artifactual contamination. The crystalline human serum albumin was obtained from Calbiochem-Behring Corp., La Jolla, California. It had the following specifications: catalog no. 126654; lot not. 801727; A grade; specially prepared under non-denaturing conditions by the method of Travis and Pannell (1973); composition as determined by electrophoresis, 97.6% albumin monomer, 2.4% albumin dimer, no alpha or beta globulins.

Nine 10 mg samples of the crystalline albumin were assayed in the usual manner. The mean acetoacetate decarboxylase activity was found to be 0.96 $\pm$ 0.01 (S.E.M.) units.

In practically all studies performed with Fraction V human serum albumin the activity was in the range of 0.90-1.0 unit. Thus, it appears that the acetoacetate decarboxylase activity of Fraction V human serum albumin is not artifactural. The active component is believed to be integral to the albumin molecule. The assay of crystalline albumin and previous experiments with Fraction V albumin indicate that the acetoacetate decarboxylase activity is due solely to the presence of the albumin.

CHAPTER IV

DISCUSSION

Most biochemistry textbooks cite the decarboxylation of acetoacetate to occur only spontaneously or nonenzymatically; a few state that the reaction is enzymatically controlled; others do not deal with the decarboxylation question at all. In 1972, Van Stekelenburg and Koorevaar presented evidence that human serum, and more specifically, the albumin fraction of human serum, contained acetoacetate decarboxylase activity. Their preliminary studies indicated that human serum contained active material whose properties were similar to those of the then known bacterial enzyme acetoacetate decarboxylase (EC 4.1.1.4 acetoacetate carboxylase) from Clostridium acetobutylicum. Among the properties of the postulated activator in human serum were the following: (a) the material is not ultrafiltrable, having a molecular weight of greater than 30,000, (b) the activator is albumin-like in nature, (c) the activity is not heat-stable, (d) the activity has enzyme-like substrate dependency, (e) the activity is inhibited by monoiodoacetate, urea, Hg^{2+} and is activated by lactoflavin. Also rat liver homogenates were reported to exhibit acetoacetate decarboxylase activity.

To follow the work of Van Stekelenburg, bovine liver homogenates were used in an attempt to partially purify the acetoacetate decarboxylase activity. The active component of the liver homogenates was found to have a molecular weight less than 30,000 as determined by ultrafiltration (Table 4) and an isoelectric point of approximately 4 (Figure 6).

Rat acetoacetate decarboxylase activity does not appear to be inducible during diabetes mellitus (Table 5). The non-inducibility of the catalytic activity indicates that the acetoacetate decarboxylase activity is constitutive.

Koorevaar and Van Stekelenburg (1976) presented further evidence of mammalian acetoacetate decarboxylase activity. They demonstrated that the degree of polymerization of albumin had no effect on the decarboxylase activity and that homogenates and subfractions of brain, liver and kidney had acetoacetate decarboxylase activity. Liver and kidney demonstrated relatively high specific activities throughout all their subfractions, while brain exhibited an extremely high specific activity in the nuclear fraction.

If albumin is responsible for the decarboxylation of acetoacetate in the circulation, how does it perform this function? The mechanism of action for bacterial acetoacetate decarboxylase occurs through a Schiff base intermediate which is formed between the epsilon amino group of a sensitive lysine residue and the beta carbonyl group of acetoacetate.

To investigate whether albumin has a similar mechanism of action on acetoacetate, albumin was acylated with dicarboxylic anhydrides. The acylation agents act on albumin by blocking the epsilon amino groups of lysine on the hydrophilic surface. Human serum albumin demonstrated drastic reductions in the decarboxylase activity after blockage of the epsilon amino groups. The epsilon amino groups of lysine in the albumin molecule play an integral part in mammalian acetoacetate decarboxylase activity. One noteworthy aspect of the inhibition by succinic anhydride is that extremely small amounts of succinate will inhibit markedly the decarboxylase activity (Figure 7). At a molar ratio of 0.25:1, succinate

to free amino groups, the activity is inhibited by 85%. The marked inhibition at low molar ratios of succinic anhydride to free amino group indicates that relatively few "active sites" are present on the hydrophilic surface of albumin for reaction with acetoacetate.

Similar results were obtained with a second acylating agent, citraconic anhydride (Tables 7 and 8). Low molar ratios of citraconic anhydride to free amino group markedly inhibit the decarboxylase activity. The citraconic anhydride has a distinct advantage over succinate as an acylating agent in that it can be readily removed from its complex with albumin by hydrolysis. After hydrolysis, the resultant albumin samples were found to demonstrate high levels of acetoacetate decarboxylase activities. Thus, the acetoacetate decarboxylase activity can be inhibited by small amounts of the acylating agent and that 68% and more of the decarboxylase activity can be recovered after removal of the blocking agent. These data strengthens the findings for the blockage of the lysine residues with succinate.

Behrens (1975) has determined the primary structure for human serum albumin. Human serum albumin contains 58 lysine residues per molecule; however, not all of these residues have the ability to react with acetoacetate. The acylation of human serum albumin with dicarboxylic anhydrides has shown that the acetoacetate decarboxylase activity is drastically inhibited by minor alterations in the free amino content. Relatively few lysine residues appear to play a role in the decarboxylation of acetoacetate.

A free epsilon amino group of lysine is necessary for the acetoacetate decarboxylase activity of albumin. The necessity of a free epsilon amino group for catalytic activity indicates that the mechanism of action occurs through a Schiff base intermediate. Schiff base intermediates

are susceptible to reduction by borohydride. In the bacterial mechanism, borohydride will reduce the ketimine-product intermediate (Tagaki et. al., 1968). The reduced ketimine-product is a stable complex which acts as a non-competitive inhibitor permanently tagging the enzyme with an intermediate in the mechanism and rendering it inactive. To investigate the possibility of a Schiff base mechanism for the action of albumin on acetoacetate, the borohydride reduction was conducted. Freund and Nuenke (1962) showed that borohydride will also reduce acetone. Thus, the assay used for the determination of acetoacetate decarboxylase by head space gas chromatographic analysis for acetone was not applicable. To measure the extent of the borohydride reduction, (3-^{14}C) acetoacetate was used as the substrate. After reduction occurred, the albumin was tagged radiochemically and the formation of the reduced complex was measured by liquid scintillation (Table 11).

The borohydride reducibility of the labeled albumin complex gives evidence that the mechanism of action for the decarboxylation of acetoacetate by albumin occurs through a Schiff base intermediate.

Beta diketones are known competitive inhibitors of the bacterial enzyme acetoacetate decarboxylase. The diketones react with a sensitive lysine at the active site of the enzyme and prevent acetoacetate from gaining access to the site. If albumin decarboxylates acetoacetate through a Schiff base mechanism, then beta diketones would be expected to inhibit this decarboxylation. Acetylpyruvate (Figure 8) and acetylacetone (Figure 9), two of the simplest diketones, were shown to inhibit the acetoacetate decarboxylase activity of albumin. This action of the beta diketones on the acetoacetate decarboxylase activity of albumin offers further evidence for the operation of a Schiff base mechanism.

Acetylpyruvate, a competitive inhibitor of the bacterial enzyme, forms a Schiff base with the active site of the enzyme rendering it inaccessible to acetoacetate. The Schiff base which is formed between the enzyme and acetylpyruvate is electron-rich in nature and borohydride will not reduce electron-rich Schiff bases. To gain further evidence for the proposed Schiff base mechanism of action for albumin on the decarboxylation of acetoacetate, the effect of acetylpyruvate was studied on the borohydride reduction of the albumin- (3-^{14}C) acetoacetate complex (Table 12). Acetylpyruvate was found to prevent the borohydride reduction of the labeled albumin complex. The inhibition of the borohydride reduction by acetylpyruvate strengthens the postulate that the decarboxylation of acetoacetate occurs through a Schiff base mechanism.

Monovalent anions have been shown to inhibit the bacterial enzyme by reacting with a sensitive cationic site. The sensitive cationic site is the epsilon amino terminal of a lysine residue in the active site of the bacterial enzyme. The list of anions which cause the inhibition follows the Hofmeister series in reverse as studied by Fridovich (1963). The anions possess a critical ionic volume which is necessary to inhibit the bacterial enzyme. The critical ionic volume necessary to inhibit the enzyme has been explained in terms of entropy changes. In order to approach the cationic site, the anions must alter the hydration sphere of the cationic site. The breaking of water bonds leads to a positive or favorable entropy change. Once the anion has approached the cationic site, the hydration sphere reforms. The reformation of the hydration sphere leads to a negative or unfavorable entropy change. However, the overall change in entropy is positive and the reaction is energetically favorable based upon the entropy change.

It was demonstrated that monovalent anions will inhibit the acetoacetate decarboxylase activity of human serum albumin. Four anions were used as representative of the Hofmeister series of anions studied by Fridovich (1963); bisulfite, thiocyante, nitrate and chloride. The concentration necessary to bring about a comparable degree of inhibition was found to be as follows: thiocyante = bisulfite < nitrate < chloride (Table 16). The order of inhibition by monovalent anions on the acetoacetate decarboxylase activity of albumin is thus similar to that reported for the bacterial enzyme. The binding of these anions most likely occurs at the sensitive lysine residues of albumin and this results in a competition between the anions and acetoacetate for the lysine sites.

Guanidine-HCl, a known denaturant of the secondary structure of proteins, was shown to inhibit the acetoacetate decarboxylase activity of albumin. Wallevik (1973) has shown by optical rotation that guanidine-HCl will produce minor changes in the secondary structure of albumin at concentrations less than one molar. The acetoacetate decarboxylase activity of albumin has been shown to be sensitive to the minor secondary structure disruptions produced by guanidine-HCl at concentrations below one molar (Figure 10). The inhibition of the decarboxylase activity is explained by the fact that albumin contains 58 lysine residues and minor changes in the secondary structure will make some of the lysines inaccessible to acetoacetate. However, some of the epsilon amino groups of lysine still remain near the surface of the albumin after denaturation unaffected and able to react with acetoacetate. Thus, the acetoacetate decarboxylase activity is not totally depleted by the denaturant.

The albumin molecule will bind one or two long chain fatty acids. The sites for binding the fatty acids are probably hydrophobic clefts

into which the aliphatic chains are inserted. Defatted Fraction V human serum albumin was assayed for acetoacetate decarboxylase activity and shown to contain 36% more activity than observed for Fraction V human serum albumin, $t_{0.05} = 13.0$ (Table 14). The long chain fatty acids of Fraction V human serum albumin appear to sterically interfere with the binding of acetoacetate and this results in a lower acetoacetate decarboxylase activity.

The bacterial enzyme acetoacetate decarboxylase is very specific for acetoacetate having no action on alpha or other beta keto acids. Fraction V human serum albumin is unable to decarboxylate the alpha keto acids pyruvate and alpha ketoglutarate (Table 13). These results are not unexpected for alpha keto acids will not form a Schiff base with primary amines (Westheimer 1963). The inability of albumin to decarboxylate pyruvate and alpha ketoglutarate presents evidence that the acetoacetate decarboxylase activity associated with human serum albumin occurs via the formation of a Schiff base intermediate.

The assay of crystalline human serum albumin demonstrates the same acetoacetate decarboxylase activity as that observed for Fraction V human serum albumin. This indicates that the activity associated with Fraction V albumin is not due to an artificial contamination of the Cohn fractionation. The assay of crystalline albumin for the decarboxylase activity indicates the activity is integral to the albumin molecule.

Koorevaar and Van Stekelenburg (1976) report a K_m for the acetoacetate decarboxylase activity of albumin to be around 0.1 <u>M</u>. Thus, the affinity of albumin for acetoacetate is rather low. The high binding constant is compatable with the fact that during ketosis, the oxybutyrates become a primary fuel source for cardiac and skeletal muscle and,

therefore, decarboxylation of acetoacetate would not be desirable. During severe ketosis there is an increase in the proton pool and this contributes to the lowering of the blood pH. The decarboxylation of acetoacetate removes a proton from the pool and helps combat the falling blood pH. The low affinity of albumin for acetoacetate insures that the oxybutyrates will reach the target tissue. The economy of the metabolic pathway dictates that albumin have a low affinity for acetoacetate.

Based upon the assumption that the acetoacetate decarboxylase activity of human serum albumin occurs through a Schiff base mechanism, one can rationalize some of the results reported by Van Stekelenburg and Koorevaar. For example, Van Stekelenburg and Koorevaar (1972) reported that human serum albumin is inhibited by iodoacetate. Iodoacetate reacts with free sulfhydryl groups; human serum albumin contains only one free half-cystine in the primary structure at position 34 (Peters, 1975). The closest lysine residue to this half-cystine is in position 41 and thus, it appears that the lysine-41 plays a major factor in the acetoacetate decarboxylase activity of human serum albumin.

Van Stekelenburg and Koorevaar (1972) also report a pH optimum for the decarboxylation reaction involving albumin. The pH optimum can be explained in terms of the microheterogeneity of albumin (Foster et. al., 1965); albumin undergoes an expansion and contraction with varying pH. The microheterogeneity of albumin is present in a single donor and is not a polymorphism within the population. The different species of albumin resulting from microheterogeneity can be separated by their different electrophoretic mobilities. As the pH of albumin is lowered, a species known as F for the fast moving electrophoretic component evolves. In the pH range of 2-4, ultracentrifugal, visocity and diffusion

measurements indicate that the albumin molecule expands, becoming longer and asymmetric without a change in molecular weight (Harrington et. al., 1956; Foster et. al., 1965). The helical content decreases and the molecule unfolds so that the interior parts become accessible (Benson et. al., 1964 and Wallevik, 1973). The unfolding makes more lysine residues available for reaction with acetoacetate. Van Stekelenburg and Koorevaar (1972) reported a pH optimum of 4.5. Thus, albumin is expanded and lysine residues are available to react with acetoacetate. As the pH is raised, another species evolves, the N or normal moving electrophoretic species of albumin. In the N species, the secondary structure of albumin contracts and sensitive lysine residues become unavailable for reaction with acetoacetate. The microheterogeneity of human serum albumin explains the pH optimum reported by Van Stekelenburg and Koorevaar.

Van Stekelenburg and Koorevaar (1972) described an experiment in which albumin was pretreated by heating at 80°C for various intervals of time before assay of acetoacetate decarboxylase activity. The heat-treated albumin showed a time-dependent response of inhibition. This thermal inactivation of albumin's acetoacetate decarboxylase activity is due to denaturation of the molecule. Albumin is quite resistant to thermal denaturation and this accounts for the slow inactivation of the enzymatic activity resulting from short periods of heating. The heating of albumin unwinds the normal secondary structure of the molecule. After short periods of time, albumin will renature in a manner similar to its normal structure, thereby loss of the acetoacetate decarboxylase activity is minimized. Longer periods of heating at 80°C will demonstrate a higher loss of activity because more intra-chain bonds are broken and renaturation occurs in a more random manner. After one hour

pretreatment of albumin at 80°C, the activity loss is 55% of its original activity. Of the 58 lysine residues present in albumin, some will become available for reaction with acetoacetate after renaturation and thus, albumin still demonstrates acetoacetate decarboxylase activity after thermal denaturation.

Van Stekelenburg and Koorevaar (1972) also report that mercuric ions will inhibit the acetoacetate decarboxylase activity of albumin. Hughes et. al., (1964) have shown that mercuric ions produce dimers of albumin by cross-linking the sulfhydryl groups. Human serum albumin contains one free sulfhydryl group, $CySH_{34}$. Blockage of this group will inhibit the lysine in position 41 in much the same way as was observed for the inhibition by iodoacetate.

In light of the results presented in the dissertation and those given by Van Stekelenburg and Koorevaar, it is difficult to rationalize the relatively high specific acetoacetate decarboxylase activities associated with rat brain homogenates. Albumin will not cross the blood brain barrier and it is not produced endogenously in the brain. The brain contains numerous compounds which could form a Schiff base with acetoacetate in the <u>in vitro</u> assay, eg., dopamine, epinephrine, norepinephrine, glutamine, serotonin and gamma amino butyric acid. All of these are primary amines that would catalyze the decarboxylation of acetoacetate. The brain also contains decarboxylase enzymes such as dopa and amino acid decarboxylase. It is possible that these could decompose acetoacetate. The pool of free amino acids in the brain extracts could also be involved in the decarboxylation of a acetoacetate by virtue of their free amino groups.

Albumin is the most abundant plasma protein. Albumin does not have a specific function but has numerous physiological roles. It is the principal agent responsible for the oncotic pressure of blood, transport of fatty acids and sequestration and transport of bilirubin. There are other less well defined functions; albumin transports various hormones, acts as a source of amino acids and transports tryptophan. Tryptophan is the only amino acid transported by albumin (McMenamy et. al., 1957; McMenamy and Oncley, 1958). This fact becomes very important when discussing the acetoacetate decarboxylase activity of albumin. Albumin will not absorb lysine or any other amino acid besides tryptophan. Thus, the decarboxylation must be due to lysine residues which are part of the primary structure of albumin.

Enzymatic activity associated with the albumin fraction is not restricted to acetoacetate decarboxylase activity. Some of the other enzymatic activities associated with albumin include: esterase (Tove, 1962, Casida and Augustinsson, 1959 and Popp et. al., 1966) and protease (Wilson and Foster, 1971), muclease (Anai et. al., 1972) and phospholipase (Elsbach and Pettis, 1973). Extensive purification of the albumin does not affect these enzymatic activities. This indicates that the enzymatic activities affiliated with the albumin are not artifactual but due to albumin itself.

Albumin is a very old protein in evolutionary terms, dating back between 2-3 millions years (Peters, 1975). It is interesting to note that albumin is the predominant circulatory protein in all species. However, albumin appears to have different functional roles in the various species. Van Stekelenburg and Koorevaar (1972) first indicated that albumin may function as a source of acetoacetate decarboxylase activity. The work

presented in the dissertation confirms their findings, characterizes the acetoacetate decarboxylase activity of albumin and presents evidence that the mechanism of action for the decarboxylation of acetoacetate by albumin occurs through a Schiff base intermediate.

CHAPTER V

SUMMARY AND CONCLUSIONS

Experiments performed by Van Stekelenburg and Koorevaar (Clin. Chem. Acta 39, 191, 1972) have demonstrated the presence of acetoacetate decarboxylase activity associated with human serum albumin and in rat liver homogenates. The purpose of this work was to investigate the acetoacetate decarboxylase activity of Fraction V human serum albumin, to characterize it further and to gain insight into the mechanism of decarboxylation of acetoacetate by albumin.

Experiments utilizing bovine liver as a source of acetoacetate decarboxylase activity have shown the active material to have a molecular weight of less than 30,000 and an isoelectric point of approximately 4.

Blockage of the epsilon amino groups of lysine in the albumin molecule with succinic anhydride has demonstrated a dose-dependent response of inhibition on the acetoacetate decarboxylase activity. At a molar ratio of 0.25:1, succinate to free amino group, the acetoacetate decarboxylase activity is inhibited by 85%. The marked inhibition at low molar ratios of succinic anhydride to free amino group indicates that relatively few "active sites" are present on the hydrophilic surface of albumin for reaction with acetoacetate.

A second acylating agent, citraconic anhydride, exhibited a similar effect on the acetoacetate decarboxylase activity of albumin. At a molar ratio of 0.10:1 and 0.25:1, citraconic anhydride to free amino group, the acetoacetate decarboxylase activity of albumin was inhibited by 62% and

88%, respectively. Hydrolysis and subsequent removal of the citraconyl group resulted in recovery of the decarboxylase activity; the deacylated albumin samples regained 92% and 68%, respectively, of the initial activities. This experiment indicates that the inhibition of acetoacetate decarboxylase activity resulting from the acylation is reversible.

Schiff base intermediates are susceptible to reduction by sodium borohydride. It has been shown that concentrations of 1 mM and 2 mM borohydride will reduce an albumin-(3-^{14}C) acetoacetate complex causing between 30% to 200% increase of label incorporated into albumin over control albumin samples not undergoing the reduction. The reduction of the complex by borohydride gives evidence that the mechanism of action utilized by albumin for the decarboxylation of acetoacetate occurs through a Schiff base intermediate which forms between the carbonyl group of acetoacetate and the epsilon amino group of lysine.

The acetoacetate decarboxylase activity associated with albumin is inhibited by the beta diketones acetopyruvate and acetylacetone, guanidine-HCl and various monovalent anions. At a concentration of 80 mM acetylacetone, the acetoacetate decarboxylase activity is inhibited by 78%. Acetopyruvate is a more potent inhibitor; the decarboxylase activity is inhibited on the order of 40% in the presence of only 2 mM acetopyruvate. Acetopyruvate also will inhibit the borohydride reduction of the albumin-(3-^{14}C) acetoacetate complex indicating that the diketone prevents the formation of a Schiff base. Guanidine-HCl at concentrations below 1 M cause minor disruptions in the secondary structure of albumin. These minor secondary structural changes in albumin cause marked inhibition in the decarboxylase activity. The presence of 0.8 M guanidine-HCl results in a 50% loss of the acetoacetate

decarboxylase activity of albumin. Monovalent anions inhibit the acetoacetate decarboxylase activity of albumin after they are attracted to the sensitive cationic site of the epsilon amino terminal of lysine and, thereby, prevent the binding of acetoacetate to that site. The concentration of anion necessary to bring about a comparable degree of inhibition was found to be as follows: bisulfite = thiocyanate < nitrate < chloride. The efficacy of inhibition by monovalent anions on the acetoacetate decarboxylase activity of albumin is similar to that reported for the bacterial enzyme isolated from *Clostridium acetobutylicum.*

Fraction V human serum albumin will not decarboxylate the alpha keto acids pyruvate and alpha ketoglutarate and this indicates some degree of specificity of the acetoacetate decarboxylase activity of albumin. The inability of albumin to decarboxylate the two alpha keto acids is supportive evidence for the formation of a Schiff base intermediate in the mechanism of action since primary amines will not form a Schiff base with an alpha keto acid.

The acetoacetate decarboxylase activity of defatted Fraction V human serum albumin was 36% greater than that for Fraction V human serum albumin. This indicates that bound fatty acids interfere with the binding of acetoacetate to some of the catalytic sites on the hydrophilic surface of albumin.

These data characterize the acetoacetate decarboxylase activity of albumin and give evidence that the mechanism of action occurs through a Schiff base intermediate.

BIBLIOGRAPHY

Anai, M., Haraguchi, H. and Tagaki, Y. (1972) An endonuclease associated with bovine plasma albumin fraction. J. Biol. Chem. 247, 193-203.

Ashbrook, D.J., Spector, A.A., Santos, E.C. and Fletcher, J.E. (1975) Long chain fatty acid binding to human plasma albumin. J. Biol. Chem. 250, 2333-2338.

Autor, A.P. and Fridovich, I. (1970a) The interactions of acetoacetate decarboxylase with carbonyl compounds, hydrogen cyanide and an organic mercurial. J. Biol. Chem. 245, 5214-5222.

Autor, A.P. and Fridovich, I. (1970b) The thermal inactivation of acetoacetate decarboxylase. J. Biol. Chem. 245, 5223-5227.

Behrens, P.Q., Spiekerman, A.M., and Brown, J.R. (1975) Structure of human serum albumin. Fed. Proc. 34, 591 (2106a).

Benson, E.S., Hallaway, B.E. and Lumry, R.W. (1964) Deuterium-hydrogen exchange analysis of pH-dependent transitions in bovine plasma albumin. J. Biol. Chem. 239, 122-129.

Bhagavan, N.V. (1974) Biochemistry, J.B. Lippincott Company, Philadelphia, page 683.

Bohinski, R.C. (1973) Modern Concepts in Biochemistry. Allyn and Bacon, Inc., Boston.

Bollman, D.H. and Mortinmore, D.M. (1972) Determination of carbon dioxide, hydrogen sulfide, sulfur dioxide, ethane and propane using a carbon molecular sieve column. J. Chrom. Sci. 10, 523-527.

Cantarow, A. and Trumper, H. (1975) Clinical Biochemistry. A.L. Latner, ed., W.B. Saunders Company, Philadelphia.

Casida, J.E. and Augustinsson, K. (1959) Reaction of plasma albumin with 1-naphthyl n-methyl-carbamate and certain other esters. Biochim. Biophys. Acta 36, 411-426.

Conn, E.E. and Stumpf, P.K. (1976) Outlines of Biochemistry. John Wiley and Sons, New York.

Davies, R. (1943) Studies on the acetone-butanol fermentation. Acetoacetic acid decarboxylase of Cl. acetobulylicum. Biochem. J. 37, 230-238.

Davis, R.E., Bromels, E. and Kibby, C.L. (1962) Boron hydrides. III. Hydrolysis of sodium borohydride in aqueous solution. J. Am. Chem. Soc. 84 (6), 885-892.

Dempsey, W.B. and Christensen, H.N. (1962) The specific binding of pyridoxal 5'-phosphate to bovine plasma albumin. 237, 1113-1120.

Elsbach, P. and Pettis, P. (1973) Phospholipase activity associated with serum albumin. Biochim. Biophys. Acta 296, 89-93.

Fischer, E.H., Kent, A.B., Snyder, E.R. and Krebs, E.G. (1958) The reaction of sodium borohydride with muscle phosphorylase. J. Am. Chem. Soc. 80, 2906-2907.

Foster, J.F., Sogami, M., Petersen, H.A. and Leonard, W.J. (1965) The microheterogeneity of plasma albumins. I. Critical evidence for and description of the microheterogeneity model. J. Biol. Chem. 240, 2495-2502.

Freund, T. and Nuenke, N. (1962) Hydrogen ion dependence of the rate of reduction of acetone by borohydride. J. Am. Chem. Soc. 84, 873-874.

Fridovich, I. (1963) Inhibition of acetoacetic decarboxylase by anions. J. Biol. Chem. 238, 592-598.

Fridovich, I. (1968) A study of the interaction of acetoacetic decarboxylase with several inhibitors. J. Biol. Chem. 243, 1043-1051.

Fridovich, I. (1972) Acetoacetate decarboxylase in the Enzymes, Vol VI, P.D. Boyer, ed., Academic Press, New York, pages 255-270.

Fridovich, I. and Westheimer, F.H. (1962) On the mechanism of the enzymatic decarboxylation of acetoacetate. J. Am. Chem. Soc. 84, 3208-3209.

Fridovich, I. and Westheimer, F.H. (1962a) Inhibition of acetoacetate decarboxylase by anions. Fed. Proc. 21, 232.

Grazi, E., Cheng, T. and Horecker, B.L. (1962) The formation of a stable aldolase-dihydroxyacetone phosphate complex. Biochem. Biophys. Res. Commun. 70, 250-253.

Guthrie, J.P. (1972) Acetopyruvate. Enamine formation with aminoacetonitrile. Models for acetoacetate decarboxylase. J. Am. Chem. Soc. 94, 7024-7029.

Hall, L. (1962) Preparation of crystalline lithium acetoacetate. Anal. Biochem. 3, 75-80.

Hamilton, G.A. and Westheimer, F.H. (1959a) A crystalline decarboxylase without biotin. J. Am. Chem. Soc. 81, 2277.

Hamilton, G.A. and Westheimer, F.H. (1959b) On the mechanism of the enzymatic decarboxylation of acetoacetate. J. Am. Chem. Soc. 81, 6332-6333.

Hamilton, P.B. and Van Slyke, D.D. (1943) The gasometric determination of free amino acids in blood filtrates by the ninhydrin-carbon dioxide method. J. Biol. Chem. 150, 231-250.

Harper, H.A., Rodwell, V.W. and Mayes, C.A. (1977) Physiological Chemistry. Lange Medical Publications, Los Altos, California, page 310.

Harrington, W.F., Johnson, P. and Ottewill, R.H. (1956) Bovine serum albumin and its behavior in acid solution. Biochem. J. 62, 569-582.

Hofmeister, F. (1888) quoted in Colloid Science. A.E. Alexander and P. Johnson, eds., Oxford University Press, London, 1949, page 16.

Horecker, B.L., Pontremoli, S., Ricci, C. and Cheng, T. (1961) On the nature of the transaldolase-dihydroxyacetone complex. Proc. Nat'l. Acad. Sci., USA, 47, 1949-1955.

Hughes, W.L. and Dintzis, H.M. (1964) Crystallization of the mercury dimers of human and bovine mercaptalbumin. J. Biol. Chem. 239, 845-849.

Johnson, M.J., Peterson, W.H. and Fred, E.B. (1933) Intermediary compounds in the acetone-butyl alcohol fermentation. J. Biol. Chem. 101, 145-157.

Jonas, A. and Weber, G. (1970) Partial modification of bovine serum albumin with dicarboxylic anhydride. Physical properties of the modified species. Biochemistry 9, 4729-4735.

Karlson, P. (1975) Introduction to modern biochemistry. Academic Press, New York, page 248.

Klotz, I.M. (1967) Succinylation in Methods in Enzymology, C.H.W. Hirs ed., Vol XI, Academic Press, Inc., New York, pages 576-580.

Koorevaar, G. and Van Stekelenburg, G. (1976) Mammalian acetoacetate decarboxylase activity. Its distribution in subfractions of human albumin and occurrence in various tissues of the rat. Clin. Chim. Acta 71, 173-183.

Kronman, M.J. and Foster, J.F. (1957) Sedimentation and optical rotation behavior of bovine plasma albumin at low pH in the presence of various anions. Effect of change on molecular expansion. Arch. Biochem. Biophys. 72, 205-218.

Laursen, R.A. and Westheimer, F.H. (1966) The active site of acetoacetate decarboxylase. J. Am. Chem. Soc. 88, 3426-3430.

Lederer, F., Coutts, S.M., Laursen, R.A. and Westheimer, F.H. (1966) Acetoacetate decarboxylase. Subunits and properties. Biochemistry 5, 823-833.

Leonard, W.J. and Foster, J.F. (1961) Changes in optical rotation in the acid transformations of plasma albumin. Evidence for the contribution of tertiary structure to rotatory behavior. J. Biol. Chem. 236, 2662-2669.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265-275.

Mahler, H.R. and Cordes, E.H. (1966) Biological chemistry. Harper and Row, New York.

Matthews, D.M., Muir, G.G. and Baron, D.N. (1964) Estimation of alpha nitrogen in plasma and urine by the colorimetric ninhydrin reaction. J. Clin. Pathol. 17, 150-153.

McGilvery, R.W. (1979) Biochemistry. W.B. Saunders Company, Philadelphia.

McMenamy, R.H. and Oncley, J.L. (1958) The specific binding of L-tryptophan to serum albumin. J. Biol. Chem. 233, 1436-1447.

McMenamy, R.H., Lund, C.C. and Oncley, J.L. (1957) Unbound amino acid concentrations in human blood plasmas. J. Clin. Invest. 36, 1672-1679.

Montgomery, R., Dryer, R.L., Conway, T.W. and Spector, A.A. (1977) Biochemistry. The C.V. Mosby Company, St. Louis, page 492.

Neece, M. and Fridovich, I. (1967) Acetoacetate decarboxylase: activation by heat. J. Biol. Chem. 242, 2939-2944.

Northrup, J.H., Ashe, H. and Senior, J.K. (1919) Biochemistry of Bacillus acetoethylicum with reference to the formation of acetone. J. Biol. Chem. 39, 1-21.

Orten, J.M. and Neuhaus, O.W. (1975) Human biochemistry. The C.V. Mosby Company, St. Louis.

Oyama, V.I. and Eagle, H. (1956) Measurement of cell growth in tissue culture with phenol reagent. Proc. Soc. Exp. Biol. Med. 91, 305-307.

Peters Jr., T. (1970) Serum albumin. Adv. Clin. Chem. 13, 37-111.

Peters Jr., T. (1975) Serum albumin in The Plasma Proteins, F.W. Putnam, ed., Vol I, Academic Press, Inc., New York, pages 133-181.

Petersen, H.A. and Foster, J.F. (1965) The microheterogeneity of plasma albumin. II. Preparation and solubility properties of subfractions. J. Biol. Chem. 240, 2502-2507.

Popp, R.A., Heddle, G., Canning, R.E. and Allen, R.C. (1966) Some physical and chemical properties of albumin esterase and albumin from mouse serum. Biochem. Biophys. Acta 115, 113-120.

Rafelson, M.E., Binkley, S.B. and Hayashi, J.A. (1971) Basic Biochemistry. The Mac Millan Company, New York, page 190.

Reilly, J., Hickenbottom, W.J., Henley, F.R. and Thaysen, A.C. (1920) The products of the "Acetone: n-butyl alcohol" fermentation of carbohydrate material with special reference to some of the intermediate substances produced. Biochem. J. 14, 229-251.

Schmidt, D.E. and Westheimer, F.H. (1971), pK of the lysine amino group at the active site of acetoacetate decarboxylase. Biochemistry 10, 1249-1253.

Seeley, H.W. (1955) Acetoacetate decarboxylase in Methods in Enzymology, S.P. Colowick and N.O. Kaplan, eds., Vol I, Academic Press, Inc., New York, pages 624-627.

Stryer, L. (1975) Biochemistry. W.H. Freeman and Company, San Francisco, page 417.

Tagaki, W. and Westheimer, F.H. (1968) Acetoacetate decarboxylase reassociation of subunits. Biochemistry 7, 891-894.

Tagaki, W. and Westheimer, F.H. (1968a) Acetoacetate decarboxylase. The molecular weight of the enzyme and subunits. Biochemistry 7, 895-900.

Tagaki, W. and Westheimer, F.H. (1968b) Acetoacetate decarboxylase catalysis of hydrogen-deuterium exchange in acetone. Biochemistry 7, 901-905.

Tagaki, W., Guthrie, J.P. and Westheimer, F.H. (1968c) Acetoacetic decarboxylase reaction with acetopyruvate. Biochemistry 7, 905-913.

Tove, S.B. (1962) The esterolytic activity of serum albumin. Biochem. Biophys. Acta 57, 230-235.

Travis, J. and Pannell, R. (1973) Selective removal of albumin from plasma by affinity chromatography. Clin. Chem. Acta 49, 49-52.

Van Stekelenburg, G.J. and DeBruyn, J.W. (1970) A simple gas chromatographic determination of acetone and β-ketobutyric acid in blood serum by means of head space gas sampling. Clin. Chem. Acta 28, 233-237.

Van Stekelenburg, G.J. and Koorevaar, G. (1971) Head space analysis. Clin. Chem. Acta 34, 305-310.

Van Stekelenburg, G. and Koorevaar, G. (1972) Evidence for the existence of mammalian acetoacetate decarboxylase with special reference to human blood serum. Clin Chem. Acta 39, 191-199.

Wallevik, K. (1973) Reverse denaturation of human serum albumin by pH, temperature, and guanidine hydrochloride followed by optical rotation. J. Biol. Chem. 248, 2650-2655.

Warren, S., Zerner, B. and Westheimer, F.H. (1966) Acetoacetate decarboxylase. Identification of lysine at the active site. Biochemistry 5, 817-823.

West, E.S., Todd, W.R., Mason, H.S. and Van Bruggen, J.T. (1966) Textbook of Biochemistry. The Mac Millan Company, New York, page 999.

Westheimer, F.H. (1963) The mechanism of the enzymic decarboxylation of acetoacetic acid. Proc. Chem. Soc., 253-261.

Westheimer, F.H. (1969) Acetoacetate decarboxylase from Clostridium acetobutylicum in Methods in Enzymol., J.M. Lowenstein, ed., Vol XIV, Academic Press, Inc., New York, pages 213-241.

White, A., Handler, P., Smith E.L., Hill, R.L. and Lehman, I.R. (1978) Principles of Biochemistry. McGraw-Hill, Inc., New York, page 603.

Wilson, D.W. and Foster, J.F. (1971) Conformation-dependent limited proteolysis of bovine plasma albumin by an enzyme present in commercial albumin preparations. Biochemistry 10, 1772-1780.

Wood, H.G., Brown, R.W. and Weikman, C.H. (1945) Mechanism of the butyl alcohol fermentation with heavy carbon acetic and butyric acids and acetone. Arch. Biochem. 6, 243-260.

Zerner, B., Coutts, S.M., Lederer, F., Waters, H.H. and Westheimer, F.H. (1966) Acetoacetate decarboxylase. Preparation of the enzyme. Biochemistry 5, 813-816.

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The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the Committee with reference to content and form.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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