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EVIDENCE FOR THE EXISTENCE OF MAMMALIAN ACETOACETATE DECARBOXYLASE: WITH SPECIAL REFERENCE TO HUMAN BLOOD SERUM

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SUMMARY

In this article evidence is presented for the existence of mammalian acetoacetate decarboxylase (acetoacetate carboxy-lyase: E.C. 4.1.1.4). From experiments with human blood serum the presence of a non-ultrafiltrable activator, accelerating the decomposition of acetoacetate into acetone and carbon dioxide, is established. Kinetic studies about this activator are performed by means of repeated gas chromatographic determination of acetone in the head space vapour.

From the experiments with different, more or less purified, human protein fractions it can be concluded that the enzyme activity present in human blood serum is located in the albumin fraction and demonstrates: (1) an optimal activity at pH = 4.5, (2) a low substrate affinity ($K_m = 4 (\pm 1.5) \cdot 10^{-1}$ M), (3) a temperature coefficient (Q_{10}) of 2.5 in the range 20°–40°, (4) heat instability at 80°, (5) loss of activity by denaturation with $HgCl_2$, (6) inhibition of enzyme activity by iodoacetate and urea, (7) activation of enzyme activity by lactoflavin. No inhibition of the enzyme activity was found by addition of CN^- or pyruvic acid.

Also the presence of acetoacetate decarboxylase activity in rat liver tissue is mentioned.

INTRODUCTION

The enzyme acetoacetate decarboxylase has, so far, been demonstrated only in a few species of the genus *Clostridium*[1,2]. Except the studies of Warren *et al.*[3], using radioactive substrate, investigations of the properties of the bacterial enzyme were performed by measuring the velocity of the carbon dioxide production as a result of the decomposition of the substrate (acetoacetic acid). An alternative and very sensitive method of such investigation would be the determination of the acetone production rate by means of gas chromatography using a flame ionization detector. Recently, experimenting with the technique of head space analysis in order to determine the

concentration of acetone and acetoacetic acid in aqueous solutions[4], the existence of mammalian acetoacetate decarboxylase has been mentioned as a tentative conclusion from stability experiments of acetoacetic acid in human blood serum.

Although Seeley[5], referring to old publications, mentions a spontaneous, non-enzymatic decarboxylation of acetoacetic acid by blood serum, organ extracts and by solutions of peptones, and several amino acids[6], we believe that the enzyme acetoacetate decarboxylase is also present in mammals. It is the purpose of this acticle to present sufficient evidence for the existence of this enzyme in solutions containing human proteins (blood, serum or plasma).

METHODS

The enzyme kinetic studies were performed by gas chromatographic determination of the increase of the acetone concentration in the head space vapour. The instrumental and analytical–technical details of the gas chromatographic determination of acetone (and acetoacetic acid) in aqueous solutions, by determining the acetone concentration in the head space vapour, have already been described in detail[4,7]. However, as the increase of the acetone concentration in the head space vapour is to be related to the enzyme activity of acetoacetate decarboxylase in an aqueous solution, some special analytical aspects have to be taken into consideration.

(1) The chemical instability of the substrate: acetoacetic acid makes it necessary to measure in each experiment the increase of the acetone concentration, due to the spontaneous decomposition of the substrate, under identical experimental conditions (blank experiments).

(2) To maintain conditions of equilibrium between the acetone concentration in the aqueous and in the vapour phase a shaking thermostatic waterbath, of $37^\circ \pm 0.1$, is used.

(3) As the acetone concentration in the vapour phase does not only depend upon the acetone concentration in the aqueous phase but also upon the osmolality of the aqueous solution[4], this variable must also be considered in every experiment.

(4) In general, the duration of every kinetic experiment is at least 4 h, during which the acetone concentration in the vapour phase is determined at seven different times of incubation. The total change of the acetone concentration in the vapour phase, expressed in mm peak height per unit of time, minus the change of the acetone concentration in the vapour phase of the corresponding blank experiment is a measure for the enzyme activity.

For computation of the regression coefficient (the acetone peak height in relation to the time of incubation), the ordinate intercept including the corresponding standard deviation (S_{yx}), and the correlation coefficient, we used the Olivetti P 101 computer, program code 2.19 (Williams[8]).

MATERIALS

(1) *Preparation of acetoacetic acid*

The substrate is prepared according to the method of Procos[9], with some minor modifications to increase the yield.

Ten ml ethyl acetoacetate (Noury-Baker N.V., Holland) are added to 80 ml

N sodium hydroxide solution. The mixture is incubated at 37° for 2 h, cooled and shaken four times with 50 ml ether in order to remove any unchanged ethyl acetoacetate. The aqueous solution is made acid to pH 2.5–3 with fuming hydrochloric acid and again shaken four times with 100, 75, 50 and 50 ml ethyl ether respectively.

The combined ether extracts are dried over sodium sulfate at 4° overnight. The ethereal extract is filtered and evaporated under reduced pressure on a waterbath at 30°–40°. The colourless syrup obtained is stored under vacuum for 24 h at 4° to remove rests of ethyl ether and ethyl alcohol. The yield of acetoactic acid is 2.85 g (method of Procos[9] under similar consitions 0.95 g) and stored at −80°.

(2) Solutions

(a) For all experiments, unless otherwise stated, we used buffer solutions with an ionic strength of 0.1; in the acid range: acetate buffers, and in the neutral range: tris buffers.

(b) Several purified human protein fractions, dissolved in physiological saline, were investigated as to their ability to accelerate the decomposition of acetoacetic acid into acetone and carbon dioxide (see Table I, section RESULTS).

RESULTS

A. Properties of the proteinous activator

(1) Ultrafiltration experiment with human blood serum. Fig. 1 demonstrates the increase of the acetone concentration in the head space vapour, when acetoacetic acid is present in: physiological saline (blank experiment), the ultrafiltrate of a human serum specimen, the untreated serum specimen, and a solution of proteins in physiological saline, respectively; the latter containing an identical protein composition as the original serum specimen. The pH of all solution is kept at 7.0. The ultrafiltrate is prepared by means of a collodion tube (Sartorius Membranfilter, G.m.b.H.), retaining any substances with a M.W. of over 30000. The process of ultrafiltration is accelerated by means of centrifugal force[10]. The protein solution is prepared from the

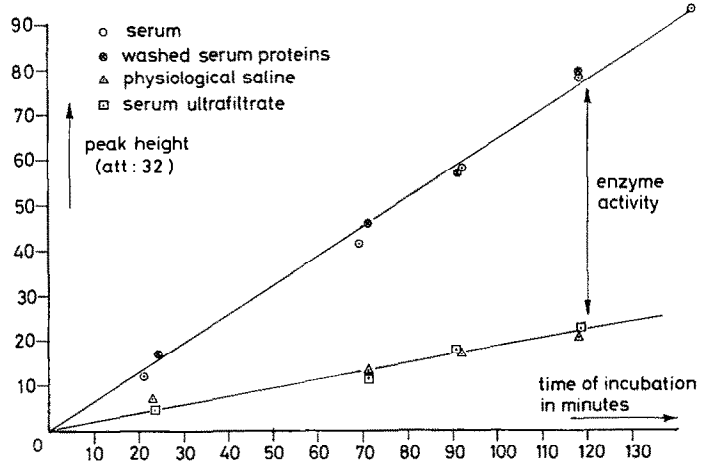


Fig. 1. Increase of the acetone concentration (in mm peak height; att = attenuation gas chromatograph) when different aqueous solutions are incubated with acetoacetate.

TABLE I

ACETOACETATE DECARBOXYLASE ACTIVITIES OF MORE OR LESS PURIFIED HUMAN PROTEIN FRACTIONS expressed in (1) μmole·min^{-1}·g^{-1} (corrected and uncorrected values) and (2) in relative activities to albumin (= 100%); the latter being calculated from the corrected values.

Protein fraction	*Manufacturer* (cat. No.)*	*Activity in μmoles·min^{-1}·g^{-1}*		*Relative activity in %*
		(a) original	*(b) corrected***	*(Albumin = 100%)*
Albumin	1	1.54	—	100
Albumin	2 (1702)	1.51	—	98
Mercapt-albumin	2 (5017)	1.46	—	95
Azo-albumin	2 (5355)	0.30	—	19
Globulins:				
α-	2 (157)	0.38	0.11	7
α-	2 (7591)	0.27	0	0
α_2-	2 (5127)	0.30	0.19	12
β-	2 (7183)	0.22	0.22	14
γ-	1 —	0.14	0.14	9
γ-	2 (457)	0.16	0.16	10

* 1: Red Cross Laboratories, Amsterdam, The Netherlands; 2: Mann Research Laboratories, U.S.A.

** By subtraction of the activity caused by the presence of albumin.

original serum sample by means of repeated ultrafiltration and subsequent washing with physiological saline (5 times). The original protein content, present before ultrafiltration, is re-established with physiological saline. From Fig. 1 it can be seen that the ultrafiltrate exhibitsdentical activity in comparison with the blank experiment (physiological saline). It can also be seen that the activity of the mixture of proteins in the serum sample is not altered by repeated washing of the proteins. From the figure it may be concluded that the catalyst, present in the human serum sample, is probably a protein or a small molecule firmly attached to the protein(s).

(2) *Activity of different human serum protein fractions.* Ten different, more or less purified protein fractions were tested for their catalytic activity. The experiments were performed by incubation at 37° of a mixture of 0.2 ml protein solution ($\pm$ 50 mg/ml), 2.5 ml tris–HCl (pH = 7.40) and 0.1 ml acetoacetic acid (80 mg/ml).

The activities of the different protein fractions are expressed in μmoles·min^{-1} per gram protein. In Table I the activities of the human protein fractions are presented.

To understand the significance of the observed activities in Table I, we calculated, from 14 determinations of the activity of an albumin solution, a standard deviation of $\pm$ 0.09 μmole·min^{-1} per gram albumin.

From Table I it can be seen that for the greatest part, the activity is located in the albumin fraction. Observing the values of the different globulin fractions the activities of these fractions can be explained either by (1) "impurity" of these fractions by the albumin-like catalyst, or (2) by a small (non-specific?) activity of the globulins themselves. Therefore the albumin contents of the different globulin fractions were determined immunochemically, by means of the Ouchterlony plate technique (purchased from Behringwerke A.G., Germany).

Assuming that the catalyst and albumin are substantially the same, the activities of the globulin fractions can be corrected for albumin impurities present. From these corrected values (see Table I) it may be concluded that the globulins do not exhibit significant acetoacetate decarboxylase activity.

Because of differing amounts of free fatty acids (FFA) bound to albumin (1) and albumin (2) (see Table I), *viz.* 1.38 and 0.28 mequiv FFA per mmole albumin respectively, it can be concluded that FFA does not affect the activity of albumin.

The observation that albumin and mercapt-albumin (the latter a dimer of albumin) exhibit identical activities, suggests that SH groups of the albumin are not involved in the reaction. As azo-albumin exhibits a much lower activity than albumin, this observation leads to the suggestion that, according to Olcott *et al.*[11], either the imidazole-, or the phenol-, or the indole-group may be involved in the reaction.

(3) Activities of random serum samples from patients. 0.2 ml of serum was incubated with 0.1 ml of acetoacetic acid (20 mg/ml) and 2.5 ml Tris–HCl buffer (pH = 7.40). Activities from a total of 56 serum specimens were determined. Fig. 2 represents the frequency distribution of the results as a histogram.

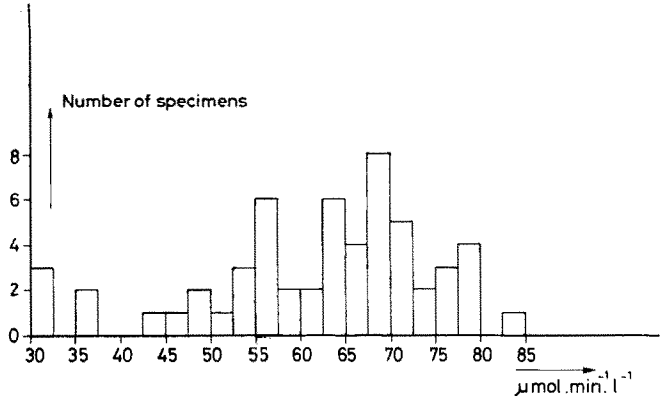


Fig. 2. Frequency distribution of the specific activities of 56 serum specimens (chosen at random) expressed in μmole·min^{-1}·l^{-1}. The mean value corresponds to a activity of about 62 μmole·min^{-1}·l^{-1}.

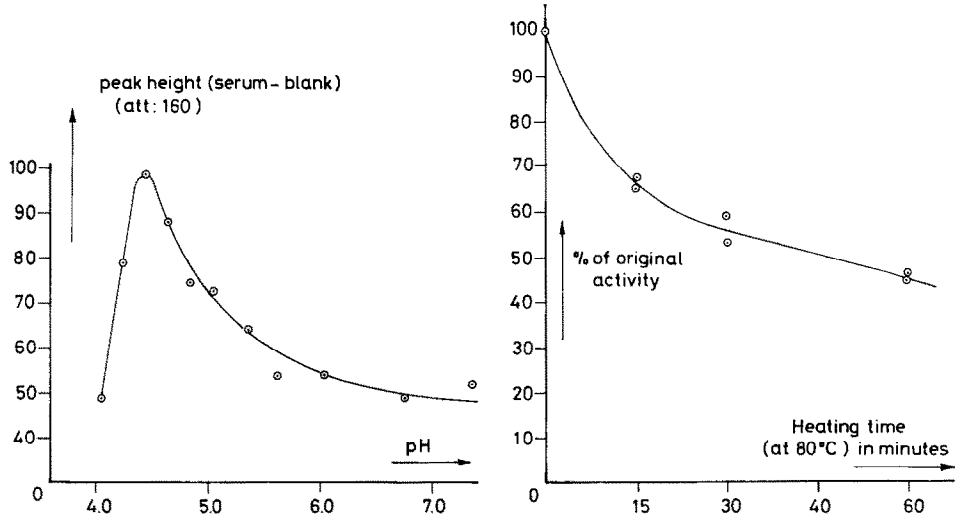


Fig. 3. Influence of the pH of the incubation medium upon the specific activity of albumin.

Fig. 4. Activity remaining after heating albumin at 80° for different lengths of time, expressed in percent of the original activity (= 100%).

We calculated a mean activity of 62.2 ± 12.9 (S.D.) μmoles·min^{-1}·l^{-1}. We realize that a different value may be found if optimal experimental conditions (pH, substrate conc., *etc.*) are used.

Comparing the mean activity found in the serum specimens having a mean albumin concentration* of 42 g·l^{-1}, with the activity found in the purified albumin (see Table I), excellent agreement is observed.

(4) Influence of the pH upon the activity of albumin. Fig. 3 demonstrates the influence of the pH upon the rate of decomposition of the substrate, acetoacetic acid, when albumin is present. The presented curve is a mean of 4 experiments covering the pH range demonstrated. From this curve it can be seen that: (1) optimum activity is found at pH 4.5; (2) at pH 7.4 considerable activity is still present.

In the foregoing experiments the buffer pH was kept at 7.4 because at acid pH the blank activity increases considerably.

(5) Influence of the substrate concentration. The Michaelis constant (K_m) was determined at pH 5.0 and pH 7.4. For these experiments the buffer used had an ionic strength of 0.2.

K_m was evaluated by studying the effect of the substrate concentration (s) on the velocity (v), the latter being the increase of the acetone peak height in our incubation experiments. By means of computer program 2.19 (see section METHODS (4)) the slope of the Lineweaver–Burke curve ($1/v$ against $1/s$) is given by the regression coefficient, the latter being equal to K_m/V (V = maximum velocity). K_m can now be calculated by dividing the slope of the line by the ordinate intercept (= $1/V$).

Table II gives details of the K_m-values calculated from the results of our experiments. The correlation coefficients presented in this table are a measure for the probability that the relationship between $1/v$ (in our case $1/\text{tg}\ \alpha$, where tg α represents the specific increase of the acetone peak height, due to the presence of albumin, in relation to the time of incubation) and $1/s$ is strictly linear. From Table II it can be concluded that the affinity of the protein for its substrate is very low and probably not affected by the pH of the incubation medium.

(6) Effect of temperature. The temperature coefficients (Q_{10}) were determined at pH 7.4 and 5.0, for the range 20°–30° and 30°–40°. Because of the deviations in the

TABLE II

RESULTS OF EXPERIMENTS WITH PURIFIED ALBUMIN FOR THE DETERMINATION OF THE MICHAELIS CONSTANT (K_m)

The correlation coefficients (value between 0 and 1.00) refer to the strict linearity between $1/v$ and $1/s$ (Lineweaver–Burke plot).

Experimental code	*Number of concentrations used*	*pH buffer*	K_m *(M)*	*Correlation coefficient*
1	10	7.4	$26 \cdot 10^{-2}$	0.990
2	5	7.4	$49 \cdot 10^{-2}$	0.995
3	7	7.4	$50 \cdot 10^{-2}$	0.970
4	9	7.4	$21 \cdot 10^{-2}$	0.959
5	10	7.4	$65 \cdot 10^{-2}$	0.994
6	9	5.0	$38 \cdot 10^{-2}$	0.998
7	11	5.0	$52 \cdot 10^{-2}$	1.000

Mean: 4 (± 1.5)·10^{-1}.

* Determination by means of cellulose acetate electrophoresis, staining (with Ponceau S) and subsequent elution of the different fractions.

results we calculated an approximate value for Q_{10} of 2.5 with a standard deviation of 0.47, while for the Q_{10} of the spontaneous decomposition of the substrate, under identical experimental conditions, a value of about 5 was found.

Also the influence of heating of the protein solution upon the activity was examined. 0.5 ml serum, diluted with 1.0 ml of physiological saline, was heated at 80° for 15, 30, and 60 min respectively. The activity was determined at pH 7.4 (tris/HCl: $I = 0.05$). Fig. 4 demonstrates the loss of activity in % of the original activity (= 100%). Identical results were obtained when, instead of serum, purified albumin was used.

Autor and Fridovich[12] investigated the thermal inactivation of bacterial acetoacetate decarboxylase in the range 74–82°. Heating for 30 min at 80°, they found an activity loss of 50% (our study: 46%).

B. Inhibition and activation of the protein activator

(1) Influence of Hg^{2+}. According to Davies[1] a 10^{-5} M concentration of $HgCl_2$ gives a total loss of activity of the bacterial enzyme. Investigating $HgCl_2$ as a possible inhibitor in our case, experimenting with human purified albumin (Buffer: tris–HCl, pH 7.2, $I = 0.10$), we did not observe a loss of activity when the $HgCl_2$ concentration was 10^{-4} or 10^{-3} M.

When, however, the concentration was 10^{-2} M a decrease of 76% of the original activity was found. Also from the start of this experiment, a turbidity, indicating denaturation of the protein, was observed.

(2) Influence of CN^-. Neither serum nor purified albumin demonstrated a loss of activity with KCN concentrations of 10^{-4}, 10^{-3}, and 10^{-2} M respectively.

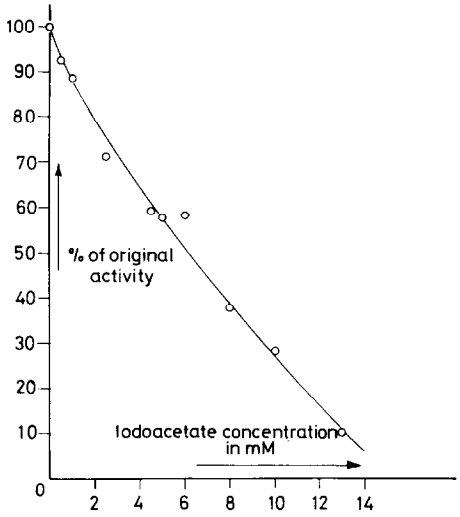
(3) Influence of iodoacetate. Fig. 5 demonstrates the influence of monoiodoacetate upon the activity of purified albumin. Experiments were performed with Tris–HCl buffer (pH 7.40, $I = 0.20$). It can be seen, that at a concentration of the inhibitor of 10^{-2} M, 74% inhibition is found (Davies[1] found 63% inhibition for the bacterial enzyme).

(4) Influence of urea. Fig. 6 demonstrates the influence of increasing concentrations of urea upon the activity of purified albumin. Although no 100% inhibition is obtained, the decrease of the activity by urea is significant.

(5) Influence of EDTA. The activity of purified albumin, dissolved in a tris–HCl buffer (pH 7.4, $I = 0.20$), was determined with increasing concentrations of EDTA (Fig. 7). In Fig. 7 it can be seen that, after an initial rise of the activity by increasing the EDTA concentration, a maximum is reached at 1.4 mM, whereafter the activity decreases with a further increase of the EDTA concentration. The mechanism of the influence of EDTA, however, needs further investigation.

(6) Influence of pyruvic acid. In contrast to the findings of Davies[1], who observed 64% inhibition of the bacterial enzyme activity when pyruvic acid was present in a concentration of $5 \cdot 10^{-2}$ M, we did not observe a loss of activity in the concentration range of 10^{-3}—$6 \cdot 10^{-2}$ M with purified human albumin (pH 7.4, $I = 0.20$).

(7) Influence of vitamin B_2 (lactoflavin). Fig. 8 demonstrates the influence of lactoflavin upon the activity of purified albumin. From this figure it can be seen that at about 1 μg lactoflavin/ml this vitamin acts as an activator, whereas at 5 μg lactoflavin/ml inhibition is observed. From the blank experiments it was found that the spontaneous decarboxylation of acetoacetic acid is not influenced by lactoflavin.



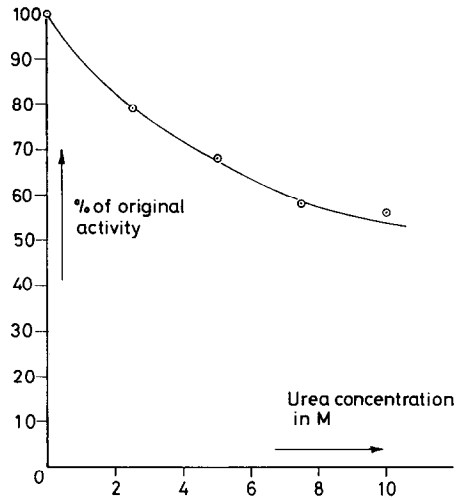
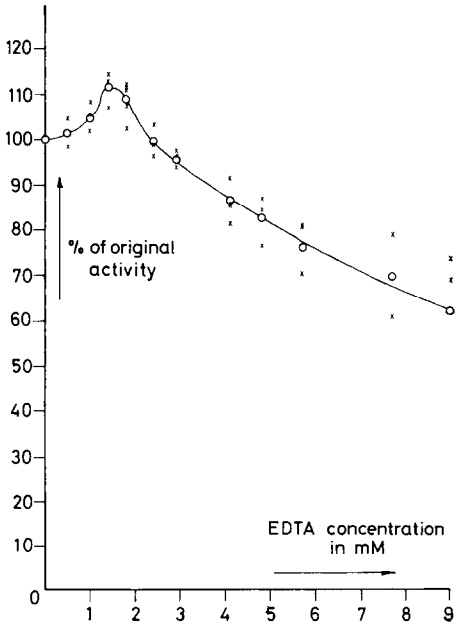


Fig. 5. Influence of different concentrations of mono-iodoacetate upon the specific activity of albumin expressed in percent of the original activity (= 100%).
Fig. 6. Influence of different urea concentrations upon the specific activity of albumin expressed in percent of the original activity (= 100%).



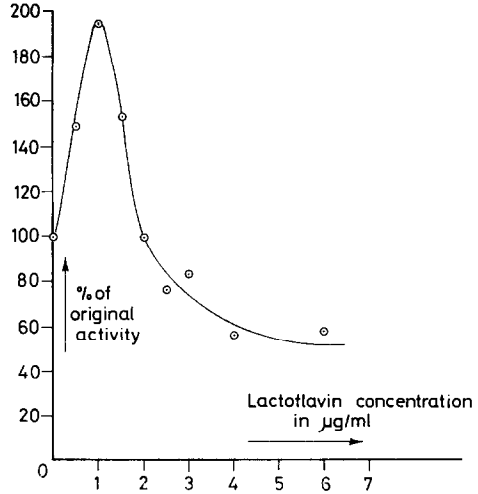


Fig. 7. Influence of different concentrations of EDTA (ethylene diamine-tetra-acetate) upon the specific activity of albumin expressed in percent of the original activity (= 100%).
Fig. 8. Influence of lactoflavin upon the specific activity of albumin expressed in percent of the original activity (= 100%).

DISCUSSION

According to Dixon and Webb[13] the most satisfactory definition of an enzyme is: "a protein with catalytic properties, due to its power of specific activation".

The question arises how many properties and kinetic aspects of an activator must be investigated before this activator may be called an enzyme. Summarizing all the aspects of the catalyst: (a) not ultrafiltrable, (b) probably of albumin-like nature, (c) not heat-stable, (d) has a pH optimum, (e) has an enzyme-like substrate dependency (Michaelis constant), (f) is inhibited by mono-iodoacetate, urea, Hg^{2+} (in relatively high concentrations) and activated by lactoflavin, we believe that sufficient information is presented to justify our conclusion that the enzyme acetoacetate decarboxylase, is present in human serum.

Up to now the experiments, mentioned in this article, have been performed with purified human protein fractions or with blood serum samples.

Investigating acetoacetate decarboxylase activity in homogenates of rat liver tissue about half the activity, compared with human blood serum, per gram protein was found. This activity was, for about 2/3, located in the $20000 \times g$ supernatant. In a further study the presence of acetoacetate decarboxylase will be investigated in different tissues, including the quantitative distribution of this enzyme in the different parts of the tissue cell. Also the clinical significance of a possible elevated, or lowered, enzyme activity in blood serum will be investigated.

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