LIPASE-CATALYSED CONDENSATION OF FATTY ACIDS WITH HYDROXYLAMINE

by

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Some time ago we reported preliminarily on two different types of enzymatic reactions leading to a condensation with hydroxylamine[1]. Acetate when incubated with adenosine triphosphate and hydroxylamine was found to yield acet-hydroxamic acid in fresh pigeon liver extracts. This reaction is specific for acetate, depends strictly on ATP, and occurs only in fresh liver extract of the pigeon but not of rat, rabbit or hog. The reaction is lost with aging but is regenerated on addition of coenzyme A and thus belongs in a class with the coenzyme A dependent acetyl transfer reaction. The characteristics of this type of hydroxamic acid acid formation will be reported on elsewhere in more detail.

The second reaction was of an entirely different type. It occurred only with higher concentrations of hydroxylamine and was fully independent of ATP. In the meantime we studied this reaction extensively and are reporting here the results obtained. It is found to occur only weakly with acetate but increasingly with the lengthening of the fatty acid chain, up to an optimum at octanoate. It is present in comparable strength in all liver extracts studied so far. It does not diminish appreciably on aging or dialysis. In contrast to the acetate reaction with ATP, it was strongly inhibited by fluoride. This and other observations eventually led to the conclusion that we were dealing here with a lipase-catalysed condensation of the fatty acid carboxyl with hydroxylamine.

METHODS AND ENZYME PREPARATIONS

Hydroxamic Acid determination. — The previously described method[2] was designed for a determination of acyl phosphate formed during enzymatic incubation. Hydroxylamine was added at the end of incubation to react non-enzymatically with pre-formed acyl phosphate at a p_H of slightly above 6. Subsequently, after deproteinization with trichloracetic acid, the color was developed with acid ferric chloride. In contrast to this earlier set-up, the hydroxylamine now is part of the reaction system and is present during incubation; the method is modified to determine the enzymatically formed hydroxamic acid. The experiment is generally terminated by addition of a mixture of trichloracetic acid, hydrochloric acid and additional hydroxylamine. Finally ferric chloride is added. The addition of hydroxylamine serves only to stabilize the color but does not participate in primary

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condensation. As previously described, the precipitate is eventually removed by filtration or centrifugation and the color determined in the supernatant.

Determination in 50% alcoholic solution.— When it appeared desirable to follow the hydroxamic acid formation with fatty acids of increasing chain length, it was observed that these hydroxamic acids became increasingly insoluble in water and on removing the protein precipitate, considerable amounts were lost. It was found, however, that these longer chain hydroxamic acids are easily soluble in 50% ethyl alcohol. Therefore in the experiments dealing with higher fatty acids, a revised procedure was used where, after incubation, the medium was brought to a concentration of approximately 50% in ethyl alcohol.

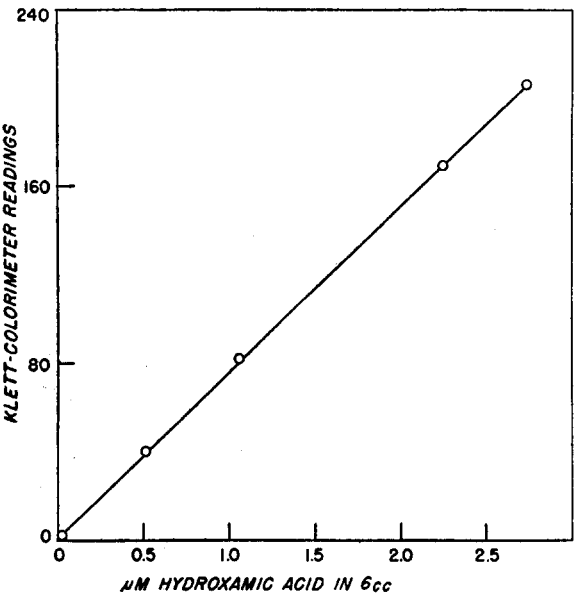

Fig. 1. Standard curve for hydroxamic acid determination in 50% ethanol. Lithium acetyl phosphate was used.

Procedure of Hydroxamic Acid Determination in Alcoholic Solution.— To 0.5 ml of enzyme-substrate-hydroxylamine mixture, 3 ml of 95% ethanol are added and well mixed. Then

1. 1.5 ml are added of a mixture of equal volumes of 28% hydroxylamine-HCl, 3.5 normal NaOH and a hydrochloric acid, obtained by dilution of concentrated HCl with 2 volumes of water,

2. 0.5 ml of 24% trichloracetic acid and finally,

3. 0.5 ml of 10% ferric chloride in 0.2 normal HCl are added. The precipitate is filtered or centrifuged off and the color measured in the supernatant. The main change of procedure is in the use of more highly concentrated solutions in order to keep the volume down and give space for the addition of ethanol.

Since the appearance of our original method, an interesting application of the hydroxamic acid-iron colour for colorimetry of fatty acid esters appeared[3]. Esters were found to react quantitatively with hydroxylamine in strongly alkaline solution and this reaction is used by HILL[3] for a determination of fatty acid esters. An extensive and very instructive discussion of the reaction between hydroxylamine and carboxyl derivatives may be found in the spot test analysis of FRITZ FEIGL[4].

ENZYME PREPARATIONS

Pigeon and rat liver homogenate were prepared as described previously[5] using 3 to 4 volumes of 1% potassium chloride and 0.02 M sodium bicarbonate solution.

Hog liver fractionation. — In this fractionation we followed roughly the procedure elaborated for the purification of liver lipase by KING and his collaborators[6–9]. Fresh hog liver was obtained from the slaughterhouse and 100 grams were homogenized in a Waring blender with 200 ml of 0.1 molar disodium hydrogen phosphate. The homogenate was frozen overnight and then centrifuged for half an hour after thawing.

Fraction L-1, obtained by removal of inactive protein by acidification. — 75 ml of the extract were further diluted with 2 volumes of 0.1 molar secondary phosphate and recentrifuged. To the supernatant 75 ml of water were added and the mixture was now acidified with 11.5 ml of normal acetic wherewith the p_H was brought to 4.8. A voluminous precipitate formed and was centrifuged off and discarded. 127 ml of strongly reddish, almost clear supernatant were collected. The extract was neutralized with 5 ml of normal ammonia to p_H 6.8. 10 ml were taken for analysis.

Fraction L-2, obtained by removal of inactive protein by half saturation with ammonium sulphate.— 122 ml of fraction L-1 were mixed with an equal volume of saturated ammonium sulphate solution. The mixture was shortly warmed to 30° and filtered. The filtrate was dialysed against distilled water.

Fraction L-3, 50% ammonium sulphate precipitate.— The precipitate on the filter was squeezed between filter paper layers and dried as far as possible. The precipitate was dissolved in about 10 ml of water and dialysed in cellophane against 4 liter of distilled water overnight in the cold room. Next morning the globulin precipitate formed on dialysis was centrifuged and once washed with water.

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The precipitate was dissolved with Krebs-Ringer containing 0.01 molar ammonium hydroxide in a 10 ml of Krebs-Ringer containing 0.01 ammonium hydroxide. Most of it went into solution and a little undissolved was discarded. This fraction L-3 was practically inactive.

Fraction L-4 obtained by full saturation with ammonium sulphate. — This is the most active fraction. To the half saturated ammonium sulphate solution (L-2) 37 grams per 100 ml of solid ammonium sulphate were added. The total volume of 250 ml obtained. This was warmed to 30–35° and filtered overnight in the cold room. The almost colourless filtrate was discarded. The precipitate was dissolved in 15 ml water; it dissolved very completely to a dark red fluid. It was dialysed against distilled water with agitation at room temperature for 3½ hours. The volume increased to 32 ml and very little precipitate was formed, which we centrifuged off and discarded. This is fraction L-4.

Pancreas Lipase

Pancreatine PARKE-DAVIS as obtainable on the market was used. Some fractionation of this product is described later on in the text.

RESULTS

In the first two tables, the lipase-catalysed hydroxamic acid formation is compared with the acetate + ATP reaction. In Table I, the inactivity of ATP with octanoate is contrasted with its action on acet-hydroxamic acid formation. It appears that the optimum concentration of hydroxylamine with ATP and acetate is 0.02 molar and that at 0.05 molar already an inhibition is observed. Table II shows the effect of increased concentrations of hydroxylamine on the condensation with octanoate. The strong dependence of this reaction on the high concentration of hydroxylamine will be noted as well as its independence on the presence of ATP. In the further study generally an 0.4–0.6 molar concentration of hydroxylamine was used.

TABLE I

HYDROXAMIC ACID FORMATION WITH ACETATE + ATP AT VARIOUS CONCENTRATIONS OF HYDROXYLAMINE

All tubes contained 0.5 ml of 10% fresh acetone pigeon liver extract in a total volume of 1.1 ml, pH 7.3, temperature 37°, 60 minutes incubation.

Octanoate M	Acetate M	ATP M	Hydroxylamine M	Hydroxamic Acid Formed μM
—	0.01	0.01	0.05	0.48
—	0.01	0.01	0.02	1.08
—	0.01	0.01	0.01	0.78
0.01	—	0.01	0.02	0.02

TABLE II

HYDROXAMIC ACID FORMATION FROM OCTANOATE AT HIGHER CONCENTRATION OF HYDROXYLAMINE

Each tube contained 0.5 ml rat liver homogenate (1:3 in 1% KCl, frozen for 4 days) in a total volume of 1.4 ml, adjusted to pH 7.3, 37°, 60 minute incubation in air.

Octanoate M	ATP M	Hydroxylamine M	Hydroxamic Acid Formed μM
0.014	0.001	0.43	1.99
0.014	—	0.43	1.90; 2.1*
0.014	0.001	0.14	0.54
0.014	—	0.14	0.48

Parallel experiment in a Warburg vessel with nitrogen in the gas phase.

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Table III shows the p_H optimum of the lipase reaction to be at 7.2. The measurements at the more acid range, however, do not give a true impression of the p_H dependence. A decrease of activity here is partly caused by the higher concentrations of free fatty acid which is rather strongly inhibitory[8].

TABLE III

THE p_H OPTIMUM OF HYDROXAMIC ACID FORMATION WITH PORK LIVER EXTRACT

Each tube contained 0.25 ml liver extract, 0.1 ml of 0.1 M octanoate, and 0.15 ml of 2 M hydroxylamine hydrochloride-NaOH buffer, 60 minute incubated at 37°. The buffer was prepared by neutralizing a 4 M hydroxylamine HCl solution with increasing amounts of NaOH and adjusting the volume with water

Hydroxylamine HCl NaOH	p_H	Hydroxamic Acid Formed μM
2:0.5	5.9	1.39
2:1	6.4	2.4
2:1.5	7.2	2.99
2:1.75	7.5	2.76
2:1.95	8	1.42

In Table IV, the activity of some lipase inhibitors is recorded. Like lipase the hydroxamic acid reaction is strongly inhibited by fluoride[10] and hexyl resorcinol[7]. The action of benzoate is of some interest. An inhibitory effect of benzoate on the oxidation of butyric but none or less of octanoic acid was observed by QUASTEL and his collaborators[11]. The hydroxamic acid reaction follows the same pattern of decreased inhibition with increasing chain length of the fatty acids. The inhibition of hydrolytic lipase action of this liver extract was checked manometrically with tributyrin in bicarbonate solution. It was found to a similar extent to be affected by fluoride and hexyl resorcinol; but benzoate showed only a small inhibition of about 10%.

TABLE IV

ACTION OF LIPASE INHIBITORS ON HYDROXAMIC ACID FORMATION WITH HOG LIVER EXTRACTS

Inhibitor	Concentration	Substrate	% Inhibition
Sodium fluoride	0.05 M	octanoate	71
	0.01 M	octanoate	51
	0.003 M	octanoate	35
Sodium benzoate	0.01 M	propionate	87
	0.01 M	butyrate	83
	0.01 M	hexanoate	20
	0.01 M	octanoate	15
Hexylresorcinol 0.25%		octanoate	40

In Table V, the lipase action and hydroxamic acid formation are compared with the various fractions, obtained as described above from hog liver extract. The parallel is rather striking. It may be noted that the absolute activity expressed in μM turnover

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is considerably smaller in the case of hydroxamic acid formation. The dependence of lipatic hydroxamic acid condensation on higher concentrations of hydroxylamine suggested a near equilibrium situation. Therefore, the influence of the concentration of

TABLE V

COMPARISON OF HYDROXAMIC ACID FORMATION AND TRIBUTYRIN HYDROLYSIS WITH VARIOUS HOG LIVER FRACTIONS

Hog Liver Fraction	Hydroxamic Acid μM/60′	Tributyrin Split μM/5′
L1	0.73	1.67
L2	0.75	1.6
L3	0.01	0.03
L4	1.2	3.25

For the hydroxamic acid determination 0.1 of the original fraction was used in a total volume of 0.5 ml, hydroxylamine 0.6 M, and octanoate 0.02 M, and incubated for 60 minutes at 37°. Tributyrin hydrolysis was measured manometrically with the manometer containing the fraction in appropriate dilutions, L1:1/12; L2:1/12; L3:none; L4:1/20. The vessels contained 0.1 ml of the diluted fraction, 0.6 ml of 0.1 M Na bicarbonate and 0.05 tributyrin was dipped in from the side arm. The gas room contained 5% CO_2 in N_2. To make the two series comparable the values recorded in the table for the manometric experiment were obtained by multiplication with the respective dilution factors.

the other reaction partner, the carboxyl ion, was likewise tested. In Fig. 2, two concentration levels, 0.02 and 0.2 molar are compared. The expected increase with carboxylate concentration is most evident at intermediate chain lengths. With longer chain lengths the often observed inhibition by free long-chain fatty acid overlaps. This also explains the change of the chain length optimum toward shorter chains at higher concentration, due to increasing hydrolysis of the salt at higher concentration levels. It is of special interest that the acetate ion starts to show appreciable activity at the 0.2 molar level.

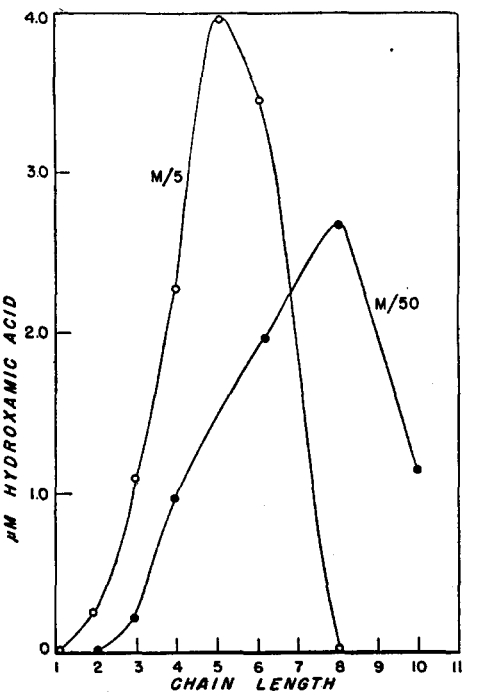

Fig. 2. Comparison of hydroxamic acid formation with 0.02 and 0.2 M octanoate, 0.6M hydroxylamine, and 0.1 ml enzyme solution in 0.5 ml total volume, 60 minutes incubabation at 37°.

In Fig. 3 the time curve of the reaction is traced. It appears that, with the reactants present in excess, the condensation occurs practically proportionally with time, indicating, as would be expected, an enzymatic reaction of the zero order.

Although in the experiment with carboxylate ion an intermediate formation of an ester was seemingly excluded, it appeared nevertheless of interest to explore the possibility of rapid enzymatic conversion of ester into hydroxamate. For this purpose, the enzyme was incubated with equivalent amounts of tributyrin and butyrate. As shown

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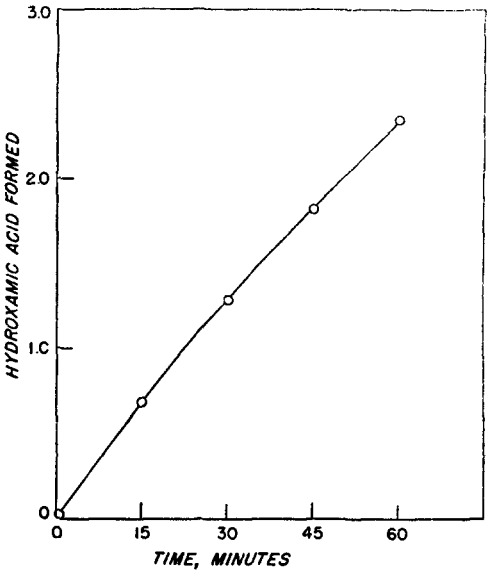

Fig. 3. Time curve of hydroxamic acid formation. Conditions as in Fig. 2, 0.02 M octanoate.

in an earlier table, the ester hydrolysis is much more rapid as the condensation reaction and very soon the tributyrin was split to completion. An appreciable exchange should, however, have been shown by a considerable increase of hydroxamate formation with the ester. The values found (Table VI) are practically identical, due to the presence of nearly equivalent amounts of butyrate during the major part of the incubation period. In the sample with tributyrin, the butyrate obviously originated from hydrolysis.

In similar experiments with equivalent amounts of ethyl and sodium butyrate, similar results were obtained. A slight increase of hydroxamate formation was observed in the earlier part of the incubation period, which evened out, however, with the progress of time. This may be due to a non-enzymatic reaction of the ester with hydroxylamine, recently observed under analogous conditions by CHANTRENNE[12] or to a slow enzymatic exchange reaction.

TABLE VI

COMPARISON OF EQUIVALENT AMOUNTS OF TRIBUTYRIN AND BUTYRATE

	Added μM	Hydroxamic Acid Formed μM
Tributyrin	3.4	0.76
	1.7	0.33
Butyrate	10.0	0.75

0.1 ml of hog liver extract in 0.5 ml total volume, 0.6 M hydroxylamine. The tributyrin was diluted with 9 volumes of 95% ethanol of which 0.01 ml was added. The same amount of ethanol was added to the butyrate sample to equalize conditions.

EXPERIMENTS WITH PANCREAS LIPASE PREPARATIONS

In order further to check the ability of lipase to condense carboxyl groups with hydroxylamine we turned to an exploration of the action of pancreas lipase on fatty acid and hydroxylamine. As source of the enzyme, the marketed pancreatine of PARKE-DAVIS was used. The condensation with hydroxylamine was easily observed likewise with pancreas enzyme, although somewhat less actively than with the liver enzyme. Significantly, the chain length optimum was shifted to the longer chains in accordance with the more truly lipatic nature of the pancreas enzyme.

By using an untreated suspension of pancreatine a rather large blank value was obtained. This could, however, be reduced considerably by washing with slightly acid fluid. Generally, not too much activity went into solution in this manner. The residue

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was used as a suspension. In Table VII, the hydroxamic acid formation with dodecanoate is described using various fractions. The results are analogous to those obtained with the liver enzyme.

TABLE VII

HYDROXAMIC ACID FORMED WITH PANCREATINE, PARKE-DAVIS

No.	Preparation	Dodecanoate M	Hydroxamic Acid μM
1	Orginal Suspension, 5%	—	1.39
		0.01	2.32
2	Supernatant	—	1.31
		0.01	1.63
3	Residue resuspended to volume	—	0.16
		0.01	0.74
4	Residue resuspended in $^1/_3$ original volume[c]	—	0.40
		0.01	1.94

0.5 g of pancreatine was suspended in 10 ml water, an aliquot was used in experiment 1. 20 drops of 0.02 molar acetic acid were added and the suspension shaken up. The suspension was centrifuged for half an hour in the cold room. The supernatant was neutralized and used for experiment 2. The residue was resuspended in 0.02 M ammonia buffer with final p_H of 8, and used for experiments 3 and 4.

Each tube contained 0.14 ml of 2 M hydroxylamine buffer of p_H 6.6, 0.25 ml enzyme solution and 0.1 ml of 0.1 M dodecanoate or 0.1 ml water. The dodecanoate solution had to be warmed up before addition. Incubation for 60 minutes at 37°; hydroxamic acid determination in alcoholic solution.

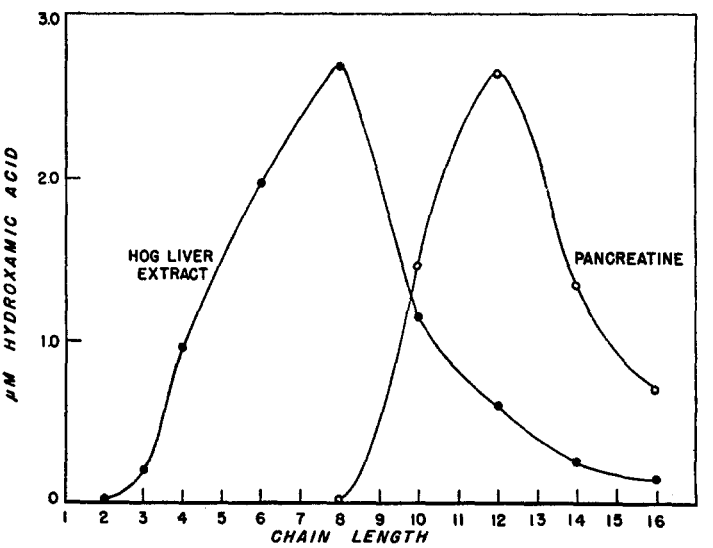

Fig. 4. Chain length optimum for liver and pancreas lipase. The conditions for liver extract were as described in Fig. 3, 60 minutes incubation time. Pancreatine, 5% suspension, 0.75 ml in 0.05 M secondary sodium phosphate, 0.45 ml 2 M hydroxylamine, p_H 7, 0.3 ml of 0.05 M fatty acid salts, 60 minutes incubation.

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A survey and comparison of results obtained with the liver and pancreas enzyme appear in Fig. 4. Particularly the difference in the chain length optimum may be noted, the optimum being found at octanoate for liver and at dodecanoate for pancreas lipase. The previously mentioned inhibitory effect of free long-chain fatty acids surely affects somewhat the situation of this optimum. In the experiments with solutions of the salts of higher members of the fatty acid series, the solution was prepared by warming the acid with equivalent amounts of sodium hydroxide. Such solutions jelled on cooling and had to be rewarmed for use in the experiment.

DISCUSSION

There are primarily two points that seem to deserve comment; *one*, the low energy requirement of the hydroxamic acid condensation and *two*, the apparent non-specificity of this reaction for an esterase. Although no attempts were made here to determine accurately the equilibrium point, it is quite obvious from the relatively low concentration of the reactants which are sufficient to support condensation on the catalyst that the change of free energy with this condensation cannot be more than a few hundred calories. It nevertheless is well known that spontaneous reaction between the free carboxyl group and hydroxylamine will not occur[4] and that therefore hydroxylamine remains to be regarded a trapping reagent for activated carboxyl groups. It is true that such activation need not mean the actual input of considerable energy by a creation of an energy-rich link. However, the acetate[1] or glutamate[13] activation by primary reaction with ATP, so easily measured by use of the hydroxamic acid reaction, bears evidence how valuable a tool hydroxylamine has become for a detection of this type of reaction. Nevertheless as rightly emphasized by CHANTRENNE[12], a judicious evaluation of the particular experimental conditions is required and the use of lower concentration of hydroxylamine may be recommended in cases where an activation of carboxyl by primary formation of an energy-rich linkage is suspected.

The "non-specificity" of the here described esterase activity appears of some significance. The link formed here by esterase action may be considered rather a peptidic link. It is thus tempting to look at this reaction as the reverse phenomenon to the esterase activity of chymotrypsin, uncovered recently by NEURATH and his group[15].

SUMMARY

A lipase-catalysed condensation of fatty acid and hydroxylamine is described. Reaction in liver extracts follows the inhibition pattern of liver lipase, hexyl resorcinol and fluoride acting as powerful inhibitors. On fractionation of hog liver extract, the esterase and condensation activities remain associated. An analogous reaction is found with pancreatine.

The condensation with hydroxylamine on lipase occurs only with relatively high concentrations of hydroxylamine and the reaction is further enhanced by increase of the fatty acid concentration. To obtain considerable hydroxamic acid formation, the concentration of 0.4 to 0.6 molar of hydroxylamine is required. With liver esterase, the chain length optimum is found with octanoate, while pancreas lipase reacts little with compounds containing below 8 carbons, and shows optimum activity with dodecanoate.

The observations indicate that a relatively small change of free energy occurs with condensation of fatty acids with hydroxylamine to form hydroxamic acid.

For the determination of the hydroxamic acid of long-chain fatty acids, a 50% alcoholic medium is required because of the water insolubility of this compound. The hydroxamic acid determination was modified for 50% ethanol-water.

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RÉSUMÉ

Les auteurs décrivent une condensation d'acide gras et d'hydroxylamine catalysée par une lipase. La réaction dans les extraits de foie suit le schéma d'inhibition de la lipase de foie, l'hexylresorcine et le fluorure agissant comme inhibiteurs puissants. Lors du fractionnement d'un extrait de foie de porc les activités d'esterase et de condensation restent associées. L'on trouve une reaction semblable pour la pancreatine.

La condensation avec l'hydroxylamine sous l'action de la lipase se produit seulement à des concentrations relativement élevées d'hydroxylamine et elle est accélérée par une augmentation de la concentration en acide gras. Pour obtenir une formation d'acide hydroxamique considerable, l'on doit avoir une concentration 0.4 à 0.6 molaire en hydroxylamine. Avec la lipase de foie l'optimum de longueur de chaine est atteint avec l'octanoate, tandis que la lipase de pancréas reagit peu avec les composés contenant moins de 8 atomes de carbone et montre une activité optima pour le dodécanoate.

Les observations que nous avons pu faire indiquent qu'un changement relativement faible d'énergie libre se produit lors de la condensation des acides gras avec l'hydroxylamine pour former les acides hydroxamiques correspondants.

Pour la détermination des acides hydroxamiques d'acides gras à longue chaîne, il faut employer un milieu contenant 50% d'alcool, parceque ces produits sont insolubles dans l'eau. La determination d'acide hydroxamique a été modifiée pour un milieu éthanol/eau à 50%.

ZUSAMMENFASSUNG

Eine durch Lipase katalysierte Kondensation der Fettsäuren mit Hydroxylamin wird beschrieben. Die Reaktion in Leberextrakten folgt dem Hemmungsschema der Leberlipase; Hexylresorcin und Fluorid wirken als starke Hemmstoffe. Bei der Fraktionierung eines Schweineleberextraktes bleiben die Esterase- und Kondensationsaktivitäten vereinigt. Eine analoge Reaktion wurde für Pankreatin gefunden.

Die Kondensation mit Hydroxylamin über Lipase findet nur bei verhältnismässig hohen Hydroxylaminkonzentrationen statt und wird durch Zunahme der Fettsäurekonzentration weiter gesteigert. Zur Erlangung einer erheblichen Hydroxamsäurebildung ist eine 0.4 bis 0.6 molare Hydroxylaminkonzentration erforderlich. Für Leberlipase ist die optimale Kettenlange mit dem Oktanoat erreicht, wahrend Pankreaslipase nur schwach mit Verbindungen reagiert, die weniger als 8 Kohlenstoffatome enthalten und für das Dodekanoat eine optimale Aktivitat zeigt.

Unsere Beobachtungen weisen darauf hin, dass bei der Kondensation von Fettsäuren mit Hydroxylamin unter Bildung von Hydroxamsäuren verhältnismässig geringe Änderungen der freien Energie stattfinden.

Zur Bestimmung der Hydroxamsäuren von Fettsauren mit langen Ketten muss, wegen der Unlöslichkeit dieser Verbindungen in Wasser, in 50% igem Alkohol gearbeitet werden. Die Hydroxamsäurebestimmung wurde für 50% iges Äthanol/Wasser angepasst.

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