

THE PIGEON PANCREATIC LIPASE*

by

K. S. Scaria

Defence Science Laboratory, Delhi

ABSTRACT

Certain biochemical properties of lipase in aqueous extracts of an ether defatted dry powder of the pigeon pancreas were studied in a manometric system using tributyrin as substrate. No cation requirement for the enzymic activity could be demonstrated. This enzyme is activated by sodium taurocholate and is inhibited by Krebs cycle intermediates and lactic acid. This lipase is activated about 600% by mercuric chloride at very low concentrations of the salt and inhibited by PCMB and $HgCl_2$ at higher concentrations. The enzyme solution does not seem to contain —SH or even sulphur in its protein and still behaves as if it is a sulphhydryl enzyme in the presence of sulphhydryl reagents. The enzyme appears to be a metallo-protein or a protein requiring metal (probably iron) and reactive NH_2 groups for its activity.

Introduction

The occurrence of lipase in the breast muscle of pigeon and certain other vertebrate skeletal muscles has already been reported in an earlier paper by George and Scaria¹. In a subsequent paper it was shown that the vertebrate heart muscle also contains a lipase which appears to be similar to that of the pigeon breast muscle (George and Scaria)². The presence of this enzyme in these tissues was also demonstrated histochemically using "Tween 80" as substrate (George and Scaria^{3,4} and George, Susheela and Scaria⁵). The properties of the enzyme from these two different sources and from the pigeon pancreas were then studied under identical conditions to find out how far they are similar or otherwise. The properties and chemical nature of the pancreatic lipase of pigeon revealed by this study are so different from accounts on pancreatic lipase by previous workers that they merit special consideration.

Material and Methods

The material used for the enzyme was an ether defatted dry powder of the pancreas of the pigeon (*Columba livia*). It was observed that the lipolytic activity of the acetone powder of the pigeon breast muscle was nil and that when sections of the muscle and pancreas were treated with acetone, the lipase

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activity was completely destroyed (George and Scaria)³. Therefore, a dry powder of the material was made without treatment with acetone. Fiore and Nord⁶ used an ether defatted dry powder of *Fusarium lini* Bolly as the enzyme material for the study of this mould lipase. This method of preparing a powder was adopted and found highly satisfactory. The pancreas of decapitated pigeons were removed, cut into small pieces and dried at room temperature *in vacuo* in a desiccator over calcium chloride. Dehydration was quick and was complete within 12 to 24 hours. The dry tissue was then crushed into a fine powder in a mortar and the coarse parts removed by sieving through fine silk. The powder was then treated with a large quantity of cold ether for one hour. At the end of this period during which much of the fat was extracted, the ether was filtered off, the residue washed with more ether, spread on a filter paper and dried at room temperature, till all the ether was completely removed. An aqueous extract of this powder was used as the enzyme solution in all the experiments. The powder thus prepared keeps well in the refrigerator for months without much loss of the enzyme activity.

10 mg. of this powder was extracted in 5 ml. of water in cold for one hour. It was then centrifuged for 5 min. at about 2500 r.p.m. and the supernatant used as the enzyme solution. The protein content in 1 ml. of this solution was 0.6—0.7 mg. Protein was estimated according to the micro-Kjeldahl method by Hawk *et al.*⁷. The nitroprusside and the lead blackening test for sulphur gave negative results with this enzyme solution. A blue green precipitate was obtained when the enzyme solution was treated with excess BAL (British-Anti-Lewisite, 2:3-dimercaptopropanol). The precipitate formed with BAL, according to the descriptions of Webb and van Heyningen⁸ and Barron *et al.*⁹ is a BAL-iron compound. It was ashed and the ash taken up in 3N HCl was found to contain large quantities of iron.

Lipolytic activity of the extract was determined manometrically in a bicarbonate-CO₂ buffer system of pH 7.4 at 37°C (George, Vallyathan and Scaria).¹⁰ An emulsion of tributyrin (4% v/v) in 0.0148 M NaHCO₃ prepared by shaking in a conical flask with 1 drop of "Tween 80" (Atlas Powder Co. U.S.A.) was used as the substrate. Each reaction flask contained 1.5 ml. 0.025 M NaHCO₃, 0.5 ml. of the substance under test (the various chemicals used), in concentrations to give the final concentrations as noted below, and 0.5 ml. enzyme in the main chamber and 0.5 ml. substrate in the side arm in a total volume of 3 ml. This gives a final concentration of 0.0148 M NaHCO₃ with a pH of 7.4 (Umbreit *et al.*)¹¹. The test solutions were introduced into the reaction flask before the addition of the enzyme, except in cases where it is otherwise stated. The flasks and manometers were gassed for 3 min. with a mixture of 95% N₂ and 5% CO₂. After equilibration for 10 min. in the constant temperature water bath, the substrate was tipped in and again allowed to equilibrate for another 3 min. This period is sufficient to ensure complete mixing of the contents of the flask. The readings were taken at regular intervals of 1 hour.

For each experiment a control was run in which 0.5 ml. distilled water was added in place of the solutions under test. Auto-hydrolysis was found to be nil. The readings given are after correction for the thermo-barometer. The controls produced on the average 13 to 14 μ moles of CO₂ equivalent to the amount of butyric acid liberated.

Results

Effect of Various Substances:

Inorganic salts—(See Figs. 1 and 2) KCl , NH_4Cl , $CaCl_2$ and $MgCl_2$ were inhibitory. Activation or inhibition of the enzyme by $NaCl$ could not be demonstrated. Greatest inhibition was by NH_4Cl .

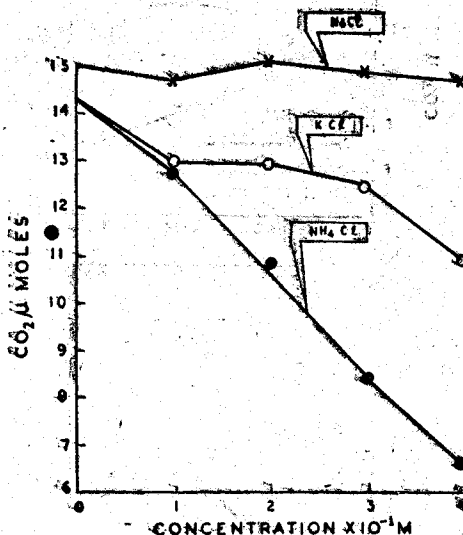


FIG. 1

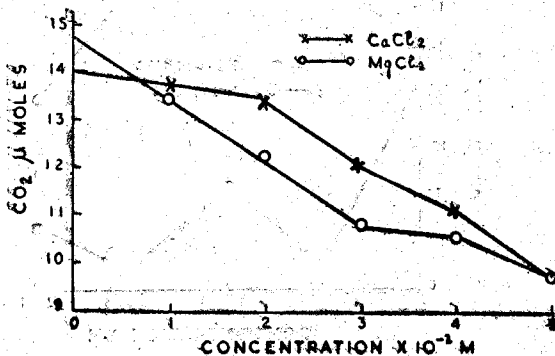


FIG. 2

Intermediary Metabolites—(See Fig. 3) All the intermediary metabolites tested had a marked inhibitory effect on this enzyme at concentrations $0.0025 M$ to $0.01 M$. Greatest inhibition was by citrate and the least by α -ketoglutarate.

Amino acids—(See Fig. 4) Of the two amino acids tested, histidine inhibited the enzyme whereas L-methionine activated it at low concentrations. At a concentration of $0.01 M$ the latter amino acid also was inhibitory.

ATP—(See Fig. 5) ATP was found to inhibit the enzyme. Inhibition was more than 50% at a concentration of $10^{-3} M$.

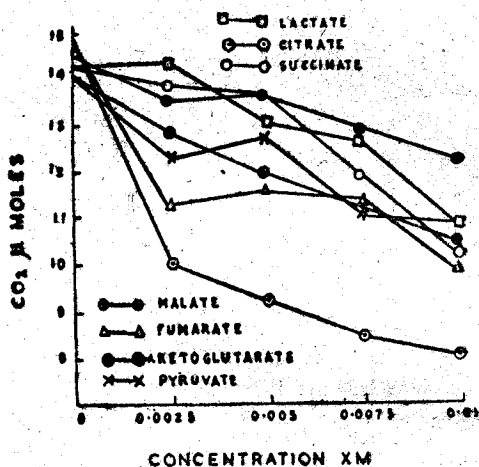


FIG. 3

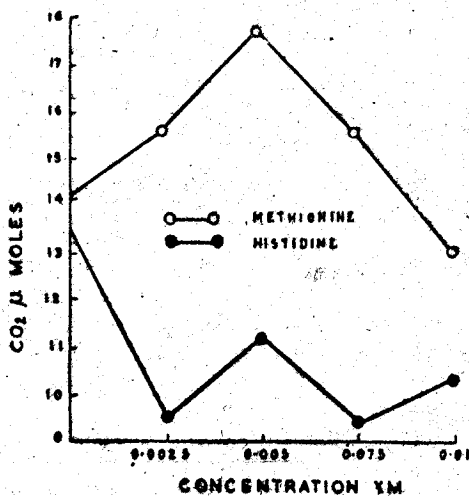


FIG. 4

Heparin—showed an activation of about 7% at a concentration of 10 $\mu\text{g}/\text{ml}$. Above this concentration the activity fell sharply and the inhibition was about 30% at a concentration of 40 $\mu\text{g}/\text{ml}$. (See Fig. 6).

Urethane—(See Fig. 7) The enzyme was slightly activated at a concentration of 0.2%. At higher concentrations the activity was considerably reduced, the inhibition being about 50% in a 1% solution of the substance.

Sodium taurocholate—was found to activate the enzyme about 200% at a concentration of 0.0025 M. Further activation at higher concentrations could not be demonstrated. (See Table 1).

TABLE I
Activation of pigeon pancreatic lipase by sodium taurocholate.

Addition	Activity, μ moles butyric acid produced				
	Concentration \times M				
	0	0.0025	0.005	0.0075	0.01
Sodium taurocholate	13.6	39.4	38.7	35.5	37.6

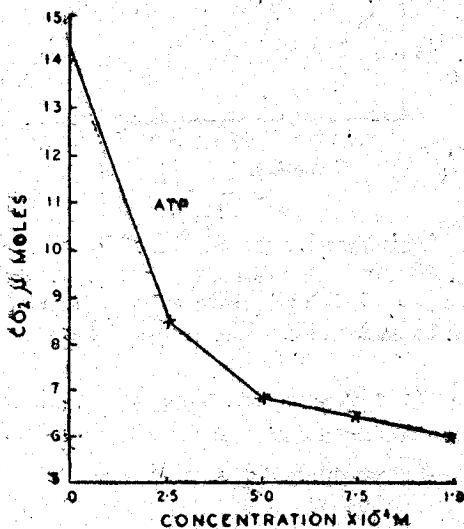


FIG. 5

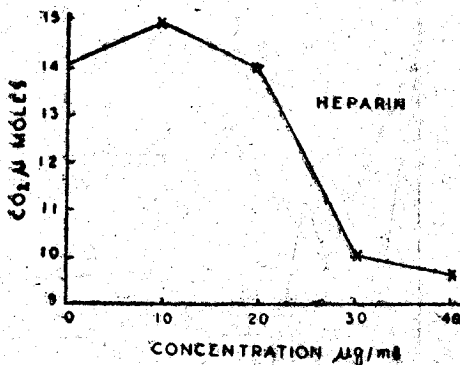


FIG. 6

Metal chelating agent—8-hydroxyquinoline is known to bind many metals in biological materials (Alber and Gledhill)¹². The solubility of this substance in water is extremely low. A saturated aqueous solution was prepared

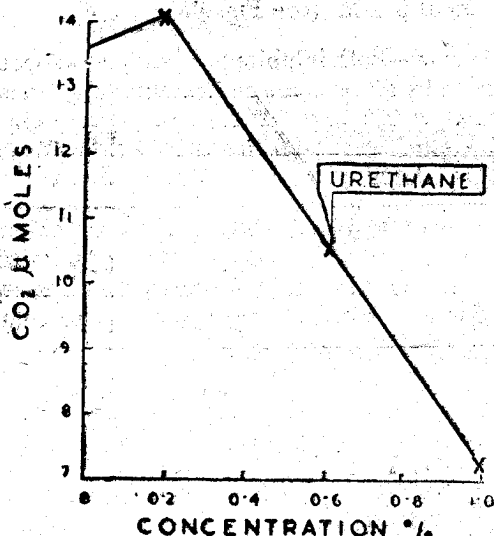


FIG. 7

(31°C) and 1.5 ml. of this solution was added to 1.5 ml. of the enzyme and incubated at 37°C for 30 min. A control was prepared by similarly incubating 1.5 ml. of the enzyme with 1.5 ml. water. 1 ml. each of these pre-incubated enzymes was added to each flask. The enzyme was inhibited about 84% by this chemical.

Thiol reagents: Alkylating agent—Iodoacetate was found to inhibit the enzyme about 35% at a concentration of 0.01 M. (See Fig. 8).

Oxidizing agent—Potassium ferricyanide was found to be a strong inhibitor. At a concentration of 0.004 M the activity of the enzyme was decreased by about 56% (See Fig. 8).

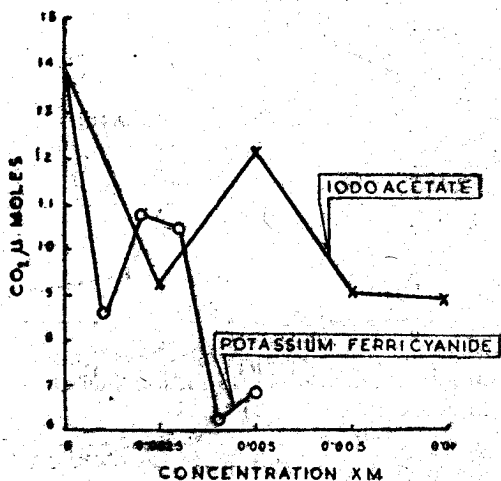


FIG. 8

Reducing agents—Monothiols, Thioglycollate inhibited the enzyme about 32% at a concentration of 0.1 M. (See Fig. 9).

Glutathione and Cysteine—Both inhibited the enzyme at concentrations 0.01 to 0.05 M, the inhibition by glutathione being more pronounced (See Fig. 10). At lower concentrations, 0.001 to 0.003 M, however, cysteine was still inhibitory but glutathione activated the enzyme about 32% at a concentration of 0.003 M. (See Fig. 11).

Dithiol—BAL at concentration 0.0002 M activated the enzyme by about 24%. Higher concentrations of the reagent had a marked inhibitory effect (See Fig. 12). When the enzyme was preincubated with excess BAL (1 drop in 3 ml.) for about 15 min. at 37°C, the supernatant after centrifugation was found to be completely inactive.

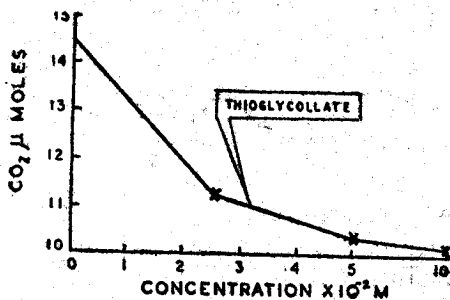


FIG. 9

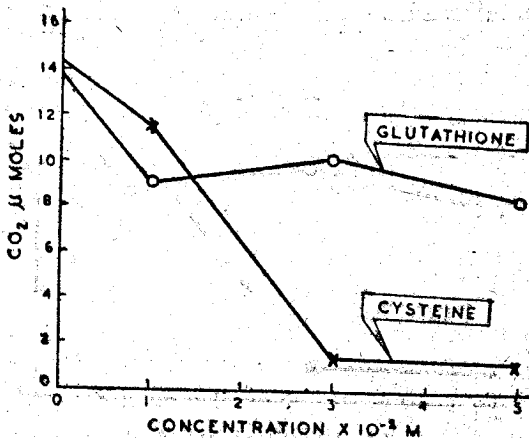


FIG. 10

Mercaptide forming Substances—PCMB (p-Chloromercuri-benzoate) at all concentrations was inhibitory. Complete inhibition was obtained at a concentration of 2×10^{-3} M in the flask.

Mercuric Chloride—was not so effective as an inhibitor at concentrations at which PCMB-inhibited the enzyme.

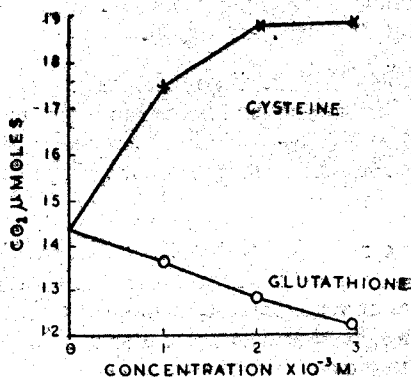


FIG. 11

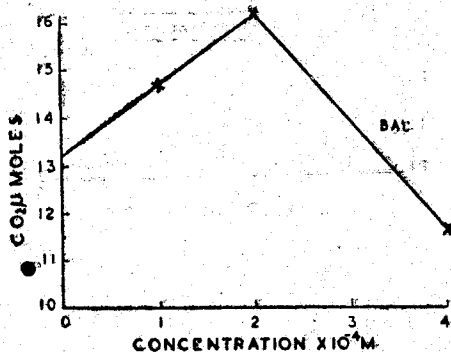


FIG. 12

At a concentration of 0.0025 M the inhibition by HgCl_2 was very slight. Even at a concentration of 10^{-2} M the inhibition by HgCl_2 was only about 66%. At a concentration of the order of micrograms of HgCl_2 , however, the enzyme was tremendously activated. The activation was about 600% at a concentration between 75 and 100 μg at which concentrations also PCMB behaved as an inhibitor (George and Scaria)¹³.

Effect of preincubating the enzyme first with HgCl_2 and then with BAL. To 1.5 ml. of the enzyme solution was added 0.5 ml. 2.4×10^{-4} M HgCl_2 and incubated for 15 min. at 37°C. A control was also similarly incubated side by side, with 0.5 ml. H_2O added to 1.5 ml. of the enzyme (tube 1). To 1 ml. of the former was added 1 ml. H_2O (tube 2) and to the remaining 1 ml. 6×10^{-4} M BAL. To tube 1 was added 2 ml. H_2O so as to make the concentration of the enzyme the same in all the three tubes. All the tubes were again incubated for 15 min. and 1 ml. each was added to the Warburg flasks in duplicate and the activity of the enzyme determined as usual. The results are given in table 2. Flask 2 had a final concentration of 10^{-5} M HgCl_2 and flask 3 contained 10^{-5} M. HgCl_2 and 10^{-4} M, BAL.

TABLE 2

Effect of preincubating pigeon pancreatic lipase with HgCl₂ and BAL
Activity, $\mu\text{l CO}_2$ liberated

Control, without addition	Enzyme + HgCl ₂ 10 ⁻⁵ M	Enzyme + HgCl ₂ , 10 ⁻⁶ M + BAL, 10 ⁻⁴ M
255	393	198

HgCl₂ was found to activate the enzyme about 54%. The activated enzyme was inhibited about 50% by the addition of BAL.

In another set of experiments in which the concentration of HgCl₂ and BAL was ten times that in the previous experiments, the following results were obtained (see Table 3).

TABLE 3

Effect of preincubating pigeon pancreatic lipase with HgCl₂ and BAL
Activity, $\mu\text{l CO}_2$ produced

Control, without addition (1)	Enzyme × HgCl ₂ 10 ⁻⁴ M (2)	Enzyme + HgCl ₂ 10 ⁻⁴ M + BAL, 10 ⁻³ M (3)
294	360	37

At this concentration the lipase was still activated 22% by HgCl₂ and the activated enzyme was inhibited about 90% by the addition of BAL.

Effect of preincubating the enzyme first with BAL and then with HgCl₂—1.5 ml. of the extract was incubated for 15 min. with 0.5 ml. 6 × 10⁻³ M BAL at 37°C. A similarly incubated enzyme to which 0.5 ml. H₂O was added was used as control (tube 1). To 1 ml. of the former was added 1 ml. H₂O (tube 2) and to the remaining 0.6 ml. 2.4 × 10⁻⁴ M Hg Cl₂ and 0.4 ml. H₂O (tube 3). 2 ml. H₂O was added to tube 1 and the tubes incubated for a further period of 15 min. 1 ml. each of these preparations was added to the flasks and the activity noted. The final concentration of BAL and HgCl₂ in the flasks was 2.5 × 10⁻⁴ M and 2.4 × 10⁻⁴ M respectively.

TABLE 4

Effect of preincubating pigeon pancreatic lipase with BAL and HgCl₂
Activity, CO₂ produced, μl

Control, without addition (1)	Enzyme + BAL 2.5 × 10 ⁻⁴ M (2)	Enzyme + BAL 2.5 × 10 ⁻⁴ M + HgCl ₂ 2.4 × 10 ⁻⁴ M (3)
403	350	373

The enzyme was inhibited about 12.5% by BAL. The inhibition was reversed by HgCl_2 to the extent of 50%.

Discussion

The extensive studies on lipases and esterases by the schools of Willstätter and Rona are well known. Ammon and Jaarma recently reviewed the literature upto 1950. It is generally agreed that the hydrolysis of tributyrin is effected by true lipase (Desnuelle)¹⁴. In the manometric system the pancreatic lipase hydrolyses tributyrin readily, but olive oil is not acted upon. This is in conformity with the observations of Martin and his colleagues (Martin and Peers)¹⁵. They observed that oat lipase does not hydrolyse olive oil in a manometric system and that tributyrin is readily hydrolysed. They explained the non-hydrolysis of olive oil in a manometric system as due to the predominance of water in the system. It is also realized that lipases from various sources differ from each other with regard to their solubility, specificity and kinetic properties (Martin and Peers)¹⁵. Each enzyme may be adapted for maximal activity in the physiological environment in which it acts. From the results presented above it is clear that sodium taurocholate highly activates the enzyme and all the krebs cycle intermediates and lactic acid inhibit the enzyme considerably. The reverse effect is observed in the case of the muscle lipase (George and Scaria)¹⁶. This can be appreciated when we consider that the activity of the muscle lipase is intra-cellular and the metabolic pool constitutes its physiological environment while it is not so with pancreatic lipase. This enzyme acts in an environment which is rich in bile salts which are known to enhance digestion. Evidently the activation of pancreatic lipase by sodium taurocholate is not due to emulsification of the substrate by the bile salt as is generally believed. Heparin and ATP are also likewise inhibitory. But a slight activation by heparin was observed at a concentration of 10 $\mu\text{g}/\text{ml}$. No cation requirement if any, could be demonstrated by adding these substances. It may be that the crude enzyme contains these substances in sufficient amounts, if required for maximum activity.

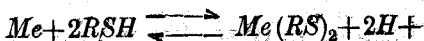
The behaviour of the pancreatic lipase in the presence of sulphhydryl reagents is highly interesting because this enzyme does not contain —SH or —S—S— groups as judged from the fact that the nitroprusside reaction for —SH groups and the lead-blackening test for sulphur are negative and still behaves as if it is a sulphhydryl enzyme, according to the literature by Barron¹⁷.

Potassium ferricyanide, an oxidizing agent inhibits the enzyme. Enzyme inhibition by ferricyanide is assumed to be due to the oxidation of sulphhydryl groups to the disulphide (Massart,¹⁸). It is evident that the inhibition of this enzyme by ferricyanide is not due to oxidation of sulphhydryl group. Iodoacetate, an alkylating agent, is also an inhibitor of the enzyme. According to Dickens¹⁹ iodoacetate reacts by substitution of the hydrogen of the sulphhydryl by the carboxymethyl group. Michaelis and Schubert²⁰ however, have noted that acid halides combine with amino groups. The inhibition of pancreatic lipase by iodoacetate may be due to the latter reaction.

Barron and Singer (Barron)¹⁷ have classified pancreatic lipase and esterases under sulphhydryl enzymes. Ions of heavy metals such as Hg are said

to inactivate such enzymes by combining with reactive —SH groups forming mercaptide compounds. Such inactivation can be reversed by the addition of BAL because of its greater affinity for the metal. This author could not confirm this, for it was found that small concentrations of the metal activates the enzyme to the extent of 600% and is inactivated by BAL. The activation of pancreatic lipase by mercury is difficult to explain. PCMB inhibits the enzyme at concentrations at which $HgCl_2$ activates it. The toxicity of PCMB may be due to the non-metal part of the compound. Hellerman²¹ used PCMB as an effective reagent for the detection of —SH groups in proteins. The inhibition of this enzyme by PCMB and $HgCl_2$ at high concentrations however, is not due to reaction with —SH groups. Barron¹⁷ has reported that all mercaptide forming substances can also react with the NH_2 groups of protein. This might perhaps explain the inactivation of lipase by PCMB and $HgCl_2$ at higher concentrations. It should be of interest to note here that Little and Cardwell²² have reported that free amino groups are essential for the activity of the pancreatic lipase. It appears from this study that these substances are not specific inhibitors of sulphhydryl enzymes and their usefulness in the detection of —SH groups in protein is limited.

Reducing substances such as thioglycollate, cysteine, glutathione and BAL considerably affect the activity of the enzyme. This cannot be explained as due to interference with the —SH or —S—S— groups of the enzyme protein. Other explanations have to be sought. It is well known that thiols can combine with metals forming mercaptides of varying degrees of reversibility (Barron *et al.*,⁹; Webb and van Heyningen,⁶).



The inhibitory effect of BAL on metal containing enzymes is explained as due to this reaction. The enzyme solution used in this study was found to contain large quantities of iron. It is possible that they contain other metals as well. Ions of metals can inhibit the activity of enzymes in a variety of ways depending upon the molecular structure of the enzyme protein. The activation of the pancreatic enzyme at very low concentration of BAL (0.0001 to 0.0003 M) may be due to the binding of some inhibitory metal ions present in the extract. The fact that pre-incubation of the enzyme with excess BAL completely inhibits the enzyme suggests that it may be an enzyme which requires metals for its activity, probably a metallo-protein. The essential metal appears to be iron because it is known that BAL forms an insoluble bluish green precipitate with iron and such a precipitate was obtained and found to contain iron. The inhibition of the $HgCl_2$ activated enzyme by BAL may be explained as due to two reasons: (1) the removal of the activating effect of mercury by combining with this metal, (2) inhibition of the enzyme by forming mercaptide with the metal required for the enzymic activity. On this basis it is easy to explain the reactivation by $HgCl_2$ of the enzyme inhibited by BAL. BAL leaves the enzyme metal and combines with mercury for which it has a greater affinity, bringing about a reversal of the inhibition. That the pancreatic enzyme may be a metal requiring enzyme is further indicated by the fact that 8-hydroxyquinoline which is a metal chelating agent (Albert and Gledhill)¹² inhibits its activity about 85%. The inhibition of this enzyme by monothiols like cysteine, glutathione and

thioglycollate also may be due to combination with the metal part of the enzyme. The oxidation of thiols by atmospheric oxygen is brought about by the catalytic action of metals (Warburg and Sakuma)²³ and it is known that cysteine forms a metal complex with iron previous to the oxidation of cysteine. Similar metal complexes may be formed with all thiols prior to oxidation. However, the activation of the pancreatic enzyme by glutathione at low concentration remains unexplained. Just as in the case of BAL this might be due to binding of some inhibitory metal. It is known that the efficiency of these substances in combining with different metals vary considerably (Barron)¹⁷. This author is fully aware that further studies on a purified enzyme are required before concluding that the lipase is a metallo-protein.

Acknowledgement

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