THE RELATION OF METALS AND –SH GROUPS TO THE ACTIVITY OF PANCREATIC LIPASE

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SUMMARY

A study of the effect of metal chelating agents on purified pancreatic lipase has shown that the enzyme possesses an essential stabilising metal which is probably calcium. This may be replaced less effectively by some other metals such as Mg^{++} or Sr^{++}. The effect of several divalent metal ions on the rate of triglyceride hydrolysis by the enzyme has been investigated. These metals can be divided into three groups depending as to whether they (a) increase, (b) do not affect, or (c) inhibit, the rate of hydrolysis. The toxicity of certain heavy metals has been found to be reversed by addition of amino acids.

Lipase has been found to be inhibited by certain –SH reagents, but to be quite resistant to others. It is considered that the enzyme possesses –SH groups which are not part of the active centre.

INTRODUCTION

For many years it has been known that calcium salts have a significant effect on the activity of pancreatic lipase, but the precise mechanism of the activation still remains obscure.

The activation of lipase by calcium was observed by WILLSTATTER AND MEMMEN in 1924[1]. Subsequently BAMANN AND LAEVERENZ[2] confirmed that calcium stimulated the rate of lipase action and suggested that the main function of calcium was to remove as insoluble calcium soaps the fatty acids formed in the hydrolysis. This mechanism of the action of calcium was also supported by SCHONHEYDER AND VOLQUARTZ[3].

DESNUELLE, NAUDET AND CONSTANTIN[4] found, however, that the addition of Ca^{++} in increasing concentration could alter the end products of triolein hydrolysis by lipase. In the absence of Ca^{++}, diolein was the main end product, but the addition of Ca^{++} led to an increased production of monolein. These authors considered that Ca^{++} had a dual function, a direct activation of the enzyme and an activation effect resulting from precipitation of interfacial fatty acids, thus removing the products of hydrolysis.

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In contradiction to Ca^{++} activation, several heavy metals such as Cu^{++}, Hg^{++}, Co^{++} and Fe^{+++} have been found to be toxic to lipase[5].

The observation that lipase was inhibited by bromo- and iodo-acetate[5], *p*-chloromercuribenzoate and by certain arsenical drugs led to the suggestion[6] that lipase possessed –SH groups and SCOTT[7] proposed a theory of the mechanism of lipase action which was based on the assumption that sulfhydryl groups were present in the active centre.

In view of the fact that nearly all the experiments so far described, have been performed using relatively crude pancreatic extracts, it appeared desirable to investigate the role of calcium and –SH groups in the purified enzyme. This work has been undertaken using a purified lipase prepared by a method recently described by WILLS[8].

It has been found that an important function of Ca^{++} is to stabilise the active configuration of lipase and that although it is likely that –SH groups are contained in the enzyme they are unlikely to be components of the active centre.

A preliminary account of this work has already been described[9].

EXPERIMENTAL

Materials

Lipase was purified by magnesium sulphate precipitation and dialysis as previously described[8]. The resulting sodium chloride solution of the enzyme was diluted 1000–2000 times with water for use. For most experiments a solution containing 2γ protein was used.

Glycerides were obtained from Messrs. Kodak Ltd. and were used as purchased.

Methods

Five different methods were used for the determination of lipase activity and, whenever possible, results obtained with one method were checked using another: (a) The manometric method was used for most experiments with a sodium bicarbonate–carbon dioxide buffer system. The manometers were gassed either with N_2–CO_2 (95 : 5) or with 100 % CO_2, the concentration of bicarbonate being adjusted to give a resulting pH of 7.4. Details of this method have previously been described[10]. (b) A continuous titration method previously described[10]. (c) A continuous titration method in which the pH was measured by the glass electrode and kept constant by the continuous addition of 0.01 *N* NaOH. In a typical experiment 18 ml H_2O and 1 ml of tributyrin emulsion in gum arabic were brought to pH 7.5 and 1 ml enzyme solution added. The rate of addition of alkali was measured for a convenient time period. (d) The method of BALLS, MATLACK AND TUCKER was used in a slightly modified form as previously described[11]. (e) The method of FIORE AND NORD[12] was used for a few experiments.

The contents of the reaction flasks were shaken at 100 times/min in a water bath at 37° when methods (a), (d) and (e) were used, whilst the reaction mixtures used in methods (b) and (c) were stirred with a magnetic stirrer.

Tripropionin or tributyrin were the most commonly employed substrates, either either untreated or emulsified in 0.3 % gelatin or in 10 % gum arabic[13]. In addition, for comparison purposes, mono- and di-propionin, mono- and di-butyrin, trihexanoin, trioctanoin, trilaurin, tripalmitin and triolein were used in certain experiments.

RESULTS

The effect of metal complexing agents on lipase—the metal constituent of lipase

Dialysis of purified lipase against de-ionised water for 48 h did not cause any significant reduction in activity.

Lipase was, however, found to be strongly inhibited by the addition of a metal complexing agent such as Versene, which when used in a concentration of $2 \cdot 10^{-3}$ *M*, caused 70 % inhibition after contact with the enzyme for 15 min at 37°. The inhibiting effect of Versene was, however, found to be markedly dependent on the temperature of the mixture and the time that Versene had been in contact with the lipase. In order to investigate these factors more extensively, the enzyme was incubated in borate buffer at pH 8.0 with Versene ($2 \cdot 10^{-3}$ *M*) for various time periods and at various temperatures between 5° and 50°. At the end of the period the temperature was rapidly adjusted to 20° and an excess of Ca^{++} added to combine with all the added Versene. Enzyme determinations were carried out at 37°. At low temperatures, *e.g.* at 5° and with short contact periods Versene had no effect on lipase, but the enzyme was found to be much less stable to heat in the presence of Versene (Table I).

TABLE I

EFFECT OF VERSENE* ON THE HEAT STABILITY OF LIPASE

Temp.		*Enzyme activity (Activity of untreated enzyme = 100)*	
		After 10 min exposure	*After 14 h exposure*
5°	Control	100	100
	Versene	100	60
20°	Control	100	83
	Versene	100	40
35°	Control	91	57
	Versene	65	9
50°	Control	64	0
	Versene	14	0

* Concentration, $2 \cdot 10^{-3}$ *M*.

The addition of Ca^{++} (10^{-2} *M*) completely protected the lipase if added before addition of the Versene, but, once inactivation had occurred, neither Ca^{++} nor any other substance tested could restore the activity.

This lack of stability of the lipase to heat in presence of Versene appeared to be due to the removal of an essential stabilising metal. In order to ascertain which metals were able to function as stabilisers, lipase was treated in borate buffer pH 8.0, with Versene ($2 \cdot 10^{-5}$ *M*) for 15 min at 5°. This treatment removes the metal but allows the enzyme to retain full activity. A solution (10^{-2} *M*) of the metal ion under investigation was then added and left with the lipase for 5 min at 5°. The concentration of metal ion added was more than adequate to remove the Versene by chelation, so that the excess metal ion was available for combination with the lipase. After the elapse of the 5-min

period the lipase so treated was exposed at 50° for 10 min. It was then cooled to 37° and its enzymic activity compared (a) with the activity of the untreated enzyme exposed to 50° and (b) with the activity of the enzyme exposed in the presence of Versene at 50°. Any metal that enabled an activity similar to that of (a) to be obtained may be considered as a possible normal metallic constituent of lipase, whereas, if the enzymic activity was found to be similar to that of (b), then any such metal is unlikely to be a normal lipase constituent.

Calcium was found to be the most effective metal in stabilising lipase after Versene treatment but several other metals were partially effective (Table II).

TABLE II

A COMPARISON OF THE EFFECT OF DIVALENT METAL IONS ON THE RESTORATION OF LIPASE ACTIVITY AFTER TREATMENT OF THE ENZYME WITH VERSENE

After treatment with Versene ($2 \cdot 10^{-3}$ *M*), lipase was exposed to 50° for 10 min in presence of a 10^{-2} *M* solution of the metal ion. Enzymic activity was determined at 37°.

System	*Rate of tributyrin hydrolysis*
Control	100
Versene	20
Versene + Ca^{++}	88
Versene + Sr^{++}	83
Versene + Mg^{++}	80
Versene + Ba^{++}	62
Versene + Mn^{++}	56
Versene + Co^{++}	12

In the experiments so far described, the effect of Versene on lipase was examined at pH 8.0 in borate buffer. These experiments were extended to examine the effect of Versene when the pH was varied between 4.0 and 9.0. A buffer of the required pH was added to the lipase solution, followed by Versene ($2 \cdot 10^{-3}$ *M*). The mixture was exposed to 50° for 10 min, when it was rapidly cooled, brought to pH 7.4 and Ca^{++} (10^{-2} *M*) was added to combine with the Versene. Enzyme determinations were carried

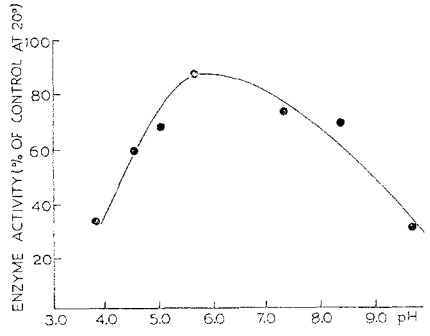

Fig. 1. The effect of pH on the heat stability of lipase. The enzyme was exposed to 50° for 10 min at the specified pH, cooled and adjusted to pH 7.4 for activity determinations. A comparison was then made with the activity of the enzyme at a comparable pH kept at 20°.

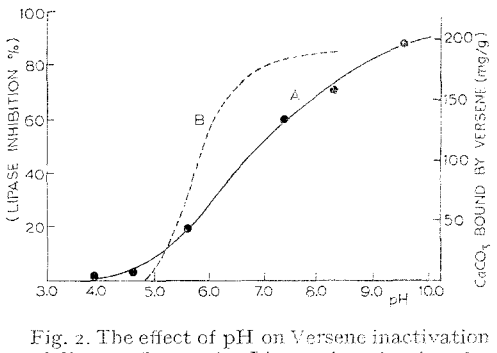

Fig. 2. The effect of pH on Versene inactivation of lipase. Curve A—Lipase inactivation by versene as a function of pH. Enzyme exposed to 50° for 10 min in presence of $2 \cdot 10^{-3}$ *M* versene at specified pH. Curve B—Binding of Ca^{++} by Versene as a function of pH (details from MARTELL AND CALVIN[31]).

out at pH 7.4. Lipase was found to be most stable to heat at about pH 5.6 (Fig. 1). The effect of Versene was markedly pH dependent, being very small when the solution was more acid than pH 5.0, but the inactivation effect increased with increasing pH. In Fig. 2 a comparison is made of the effect of pH on Versene inactivation of lipase with the effect of pH on the binding of Ca^{++} by Versene. A close similarity can be observed.

In addition to Versene several other metal complexing agents were tested on lipase. The procedure followed in each case was exactly similar to that using versene, the complexing agent under test being added to lipase in borate buffer at pH 8.0, the mixture exposed to 50° for 10 min, cooled and the enzyme activity measured. No substance tested was as effective as Versene, but the percentage reduction of enzyme activity was found to be closely paralleled by the increase of the stability constant for the calcium complex as shown in Table III.

TABLE III

COMPARISON OF THE EFFECTS OF SUCCINATE, ASPARTATE, OXALATE, CITRATE AND VERSENE ON THE HEAT STABILITY OF LIPASE

Enzyme exposed for 10 min at pH 8.0 to 50° after treatment with specified reagent.

Addition	*Log of the stability constant*[14] *for* Ca^{++}	*Concn.* *(M x 10²)*	*Enzyme activity*	*(Control = 100 at 20°)*
None	—	—	75	
Succinate	1.2	3.3	75	
Aspartate	1.6	3.3	75	
Oxalate	3.0	1.0	58	
Citrate	3.2	1.0	41	
Versene	10.77	0.2	14	

The effect of metal ions on lipase action

Although it is well known that the addition of Ca^{++} speeds up the rate of hydrolysis of glycerides of long chain fatty acids[1,2], it was found to have a much less, but still a significant effect on the rate of hydrolysis of the glycerides of short chain fatty acids such as tripropionin or tributyrin. Thus, using the method of BALLS, MATLACK AND TUCKER, the rate of hydrolysis of un-emulsified triolein was found to be increased 4.4 times by Ca^{++} (0.01 *M*), whereas under the same conditions the rate of tributyrin hydrolysis was stimulated only 1.73 × by the same concentration of Ca^{++}. Calcium stimulation of the hydrolysis rate was, however, dependent on the experimental conditions, being reduced from 1.73 × when using unemulsified tributyrin to 1.17 × when the tributyrin was emulsified in 10 % gum arabic. The stimulatory effect of Ca^{++} was also considerably reduced when strong (0.27 *M*), bicarbonate buffer solution was used in the manometric method, although the Ca^{++} remains in solution. Thus the rate of triolein hydrolysis was stimulated 10.5 times by Ca^{++} (0.01 *M*) in 0.015 *M* sodium bicarbonate solution, but was unaffected in 0.27 *M* sodium bicarbonate.

Solutions of Sr^{++} and Mg^{++} (0.01 *M*) stimulated the rate of hydrolysis of tributyrin less effectively than Ca^{++}, whilst Ba^{++}, Co^{++} or Mn^{++} (0.01 *M*) had no effect or slightly reduced the rate of hydrolysis.

On the other hand solutions of Zn^{++} and especially Cu^{++} and Hg^{++} were highly

toxic to lipase even in very dilute solutions. The effect of the metal, however, varied considerably with the experimental conditions and especially with the nature of the buffer employed. Using a continuous titration method in un-buffered solution, Cu^{++} ($2 \cdot 10^{-5}$ *M*) caused 90 % inhibition of the enzyme if unemulsified tributyrin was used as substrate, but only 27 % inhibition if the tributyrin was emulsified in gelatin. This reduction of the inhibitory effect is presumably caused by a competition of gelatin with the enzyme for the Cu^{++}. When the method of FIORE AND NORD[12] was used, the effect of Cu^{++} was considerably reduced, a $2 \cdot 10^{-4}$ *M* solution having no effect on the enzyme. This method utilises a buffer containing citrate, which is an effective chelating agent for Cu^{++}.

The effect of Cu^{++} on lipase was also reduced in the presence of bicarbonate buffer, although no precipitation of the metal occurred under the conditions used. When the experiment was carried out in 0.015 *M* $NaHCO_3$, Cu^{++} ($2 \cdot 10^{-4}$ *M*) caused 88 % inhibition of lipase, but there was only 52 % inhibition in 0.28 *M* $NaHCO_3$. In the stronger solution of bicarbonate lipase could be completely protected against inhibition by Cu^{++} ($2 \cdot 10^{-4}$ *M*) by the addition of Ca^{++} (10^{-2} *M*), less effectively by equivalent concentrations of Sr^{++} or Mg^{++}, but not at all by Ba^{++} or by any other metal tested. The enzyme could also be partially protected by Ca^{++} against inhibition by Hg^{++}. After short periods of contact, *e.g.* 10 min, Cu^{++} inhibition of lipase could be partially reversed by the addition of Ca^{++}. These protection and reversal effects could only be observed in $NaHCO_3$ buffers; under other conditions no protection or reversal occurred.

Glutathione or histidine ($4 \cdot 10^{-3}$ *M*) completely protected lipase against inhibition by Cu^{++} ($2 \cdot 10^{-4}$ *M*) and also reversed the inhibition completely after the Cu^{++} had been in contact with the enzyme for periods up to 45 min. Aspartic acid and glycine were also effective protective agents of lipase against Cu^{++} inhibition, but did not restore the activity of Cu^{++} inactivated lipase. Lipase was completely protected against inhibition by Hg^{++} ($5 \cdot 10^{-5}$ *M*) by the addition of glutathione ($4 \cdot 10^{-3}$ *M*), which also reversed the inhibition of Hg^{++} after a 5 min contact period. Histidine ($4 \cdot 10^{-3}$ *M*) was, however, less effective in this case, reducing the inhibition by Hg^{++} ($5 \cdot 10^{-5}$ *M*) from 95 % to 45 %.

Histidine and aspartic acid have been reported to activate lipase[15], but these findings could not be repeated by using the purified lipase, neither amino acid having any effect on the untreated enzyme.

Metal ions such as Cu^{++} and Hg^{++} may exert their inhibitory action on enzymes by the combination with –SH groups.

In order to examine this possibility for the case of lipase, the enzyme was treated with *p*-chloromercuribenzoate (10^{-3} *M*), which combines specifically with –SH groups. This treatment caused only about 50 % inhibition of lipase. The further addition of Cu^{++} or Hg^{++} in low concentrations (10^{-4} *M*), however, still produced 100 % inhibition of the lipase. Sites other than –SH groups must therefore be involved in the combination of lipase with these metals.

The effect of –SH reagents on lipase

WEINSTEIN AND WYNNE[5] found that pancreatic lipase was weakly (22 %) inhibited by a solution of bromacetic acid (10^{-2} *M*) and 32 % by iodoacetic acid. SINGER AND BARRON[6] found that iodoacetamide ($5 \cdot 10^{-3}$ *M*) had no effect on lipase

but that *p*-chloromercuribenzoate (10^{-3} *M*) caused 38 % inhibition and *p*-aminophenyldichlorarsine (10^{-3} *M*) caused 62 % inhibition of the enzyme. They concluded that pancreatic lipase was a typical –SH enzyme. All these experiments were carried out using relatively crude lipase preparations.

A more extensive study has now been made of the effect of a variety of typical –SH reagents on purified lipase and, although the enzyme was found to be inhibited by certain members of this group of –SH enzyme inhibitors, it was quite resistant to others (Table IV).

TABLE IV

THE EFFECT OF SULPHYDRYL REAGENTS ON LIPASE

Reagent	*Concentration (M)*	*Inhibition (%)*
Iodine	10^{-5}	100
Ferricyanide	$2 \cdot 10^{-3}$	0
Alloxan	10^{-3}	0
Iodosobenzoic acid	10^{-3}	0
Iodoacetate	$2 \cdot 10^{-3}$	0
Neoarsphenamine	10^{-3}	28
p-chloromercuribenzoate	10^{-3}	69

Most oxidising agents which convert –SH to –S–S had little or no effect, but the enzyme was very sensitive to iodine. Lipase could be protected against inhibition of iodine by glutathione, but, if iodine had had prior contact with the enzyme, no reversal was possible.

Using a purified lipase from wheat germ, SINGER[16] studied the effect of *p*-chloromercuribenzoate and *p*-aminophenylarsine oxide on the rate of hydrolysis of the triglycerides, triacetin, tripropionin and tributyrin.

The extent of inhibition of lipase by each compound was found to increase with increasing molecular weight of the substrate from triacetin to tributyrin. A similar variation of inhibition with molecular weight was observed when the mono- and the triglycerides of acetic, propionic or butyric acids were compared. In each case the inhibition of triglyceride hydrolysis was markedly greater than that of the monoglyceride hydrolysis.

The experiments described by SINGER[16] were repeated using the purified pancreatic lipase. Although the inhibition produced by *p*-chloromercuribenzoate (10^{-3}*M*) was found to be greater when using tributyrin than triacetin as substrate and to increase through the series mono-di-tri propionin as found by SINGER, the increase of inhibition with increase of molecular weight was not maintained through a series of triglycerides (Table V).

In order to explain the fact that *p*-chloromercuribenzoate inhibition of wheat lipase increases with the molecular weight of the substrate, SINGER[16] suggested that the –SH group of the wheat lipase may be located close to, but not within, the active centre. Combination of the –SH with a blocking reagent would be effective in preventing a large substrate molecule from combining with the active centre, but less effective in allowing a smaller substrate to reach the active site. Although this hypothesis may be true, an entirely different explanation of this phenomenon is,

TABLE V

INHIBITION OF LIPASE BY *p*-CHLOROMERCURIBENZOATE* IN THE PRESENCE OF DIFFERENT SUBSTRATES

Substrate	*Inhibition (%)*
Monopropionin	19
Dipropionin	35
Tripropionin	70
Triacetin	24
Tripropionin	70
Tributyrin	61
Trioctanoin	68
Trilaurin	46
Triolein	5

* Concentration, 10^{-3} *M*.

however, possible; namely, that the different inhibitions observed with different substrates are directly proportional to the quantity of *p*-chloromercuribenzoate absorbed at the glyceride water interface.

In order to examine the possibility that partition of the *p*-chloromercuribenzoate (*p*-CMB) between the substrate and aqueous phase was an important factor in determining the extent of inhibition of the lipase, the following experiment was carried out. Equal volumes of an emulsion of tributyrin in gum arabic and *p*-CMB (0.01 *M*) were vigorously shaken and then the tributyrin centrifuged down. The supernatant was discarded and the tributyrin was shaken with an equal volume of sodium bicarbonate solution (0.05 *M*) and recentrifuged. This procedure was repeated twice. Finally the tributyrin was suspended in water to give the original volume of the emulsion. A control emulsion was shaken with water instead of with *p*-CMB and subsequently treated in a similar manner.

When the tributyrin treated with *p*-CMB was used as substrate for lipase, it caused powerful inhibition of the enzyme, despite the washing with bicarbonate solution. The inhibition observed was only slightly less than that produced by the addition of an equivalent concentration of *p*-CMB directly to the lipase, thus proving the strong affinity of the glyceride for the *p*-CMB and the importance of partition into the fat as a factor determining the inhibition.

DISCUSSION

Lipase has been found to be much less stable after treatment with Versene and other metal complexing agents and therefore the enzyme may be assumed to possess a bound metal which helps to confer stability. The nature of the metal has not been definitely established, but several lines of indirect evidence indicate that it is likely to be calcium.

This evidence may be summarised as follows: (a) the metal displaced from lipase by Versene may be most effectively replaced by Ca^{++}, (b) the curve of pH/Versene inhibition of lipase is very similar to the curve of pH/Versene bound Ca^{++} (Fig. 2), and (c) the stability of lipase exposed to a series of chelating substances decreases

with increase of the chelating power of the substance for Ca^{++} through the series (Table III). Lipase does not, however, exert a specific affinity for Ca^{++} and this metal may be replaced less effectively by other metals such as Mg^{++} or Sr^{++}. This stabilising function of Ca^{++}, which does not appear to have been previously observed, is quite distinct from its activating effect on triglyceride hydrolysis.

There are, however, no reasons for believing that the originally proposed function of Ca^{++}, i.e. the removal of insoluble Ca^{++} salts of fatty acids, is not important when certain triglycerides which produce long chain fatty acids on hydrolysis are attacked by lipase. Such fatty acids are nearly insoluble in the aqueous phase and would tend to partition in the fat phase, thus slowing the rate of reaction. When short chain fatty acid glycerides are used, *e.g.* of butyric acid, Ca^{++} activation is much less than it is when long chain fatty acids are involved, but it is, nevertheless, still significant. In this case, the mechanism of Ca^{++} activation proposed above is inadequate, firstly, since the acid produced is freely water soluble and would diffuse into the aqueous phase, and secondly, since the Ca^{++} salts are soluble and ionised. A possible explanation of this phenomenon is that Ca^{++} has a special enzyme activating effect which it exerts by concentrating at the fat/water interface.

Calcium ions may therefore carry out three distinct roles in lipase action: (a) a removal of fatty acids as insoluble Ca^{++} salts in certain cases, (b) a direct enzyme activation resulting from concentration at the fat/water interface, and (c) a stabilising effect on the enzyme. The site of binding of the Ca^{++} in the stabilised enzyme is likely to be at the oxygen of the ionised carboxyl groups, since it is for these groups that Ca^{++} has the greatest affinity, having a much weaker affinity for nitrogen and sulphur.

Experiments using Cu^{++} demonstrate the impracticability of comparing results not obtained under identical experimental conditions.

In all experiments using this metal it appeared that a competition was set up between the chelating power of the buffer salt and vital enzyme centres for the Cu^{++}. The ability to remove Cu^{++} as a complex appeared to be especially powerful in the case of citrate and bicarbonate buffer and the inhibition was found to be correspondingly small.

Cu^{++} and Hg^{++} may be in part bound at –SH groups but when these groups are reacted with, and blocked by, *p*-chloromercuribenzoate, lipase is still powerfully inhibited by dilute solutions of these metals. The binding to the protein which causes the inhibition must be at other sites and the very effective reversal of inhibition observed when histidine is added suggests that a histidine residue might be an important site of metal binding.

It is possible that the activation effects observed with histidine and some other amino acids[15] may be explained on the basis of a removal of inhibitory metals such as Cu^{++}.

It has been previously suggested that lipase possesses –SH groups[5,6] and it has been proposed that they are essential constituents of the active centre[7]. The present experiments, however, have shown that the relatively pure lipase used is completely resistant to many –SH reagents and that inhibition by arsenicals and *p*-CMB is much weaker than would be expected if the –SH were actually part of the active centre. Most oxidising agents were completely without effect on the enzyme, but the strongly inhibitory effect of iodine appears to be anomalous. However, the fact that the iodine inhibition was not reversed by the addition of glutathione makes it appear probable

that oxidation of –SH groups to –S–S– is not the mechanism involved in iodine inhibition of lipase, since glutathione normally reduces –S–S– to –SH and should, therefore, have reactivated the enzyme. A more likely explanation of the powerful inhibition observed with iodine is that it is a result of iodination of tyrosine residues. A possible explanation of all these experiments is that lipase definitely possesses –SH groups, but that these groups are situated adjacent to, but not in, the active site. Combination with large molecules such as arsenicals or *p*-CMB may then inhibit sterically, whilst smaller molecules such as iodoacetate are without effect. It is also possible, however, that partition of the inhibitor between the glyceride and aqueous phase may be a more important factor determining the extent of the inhibition, as has been previously discussed for *p*-chloromercuribenzoate.

For two reasons it is considered unlikely that the suggestion of SINGER[16], that the effect of increased inhibition by –SH reagents observed with increased molecular weight of the substrate may be explained by a steric phenomenon, is true. Firstly, since the inhibition observed with *p*-chloromercuribenzoate only increases up to a certain molecular weight of substrate and then falls again (Table V) and secondly, the proof that the *p*-chloromercuribenzoate can partition in the glyceride to an extent sufficient to cause strong inhibition of the enzyme. The variation of lipase inhibition observed when using different substrates would then better be explained on the basis that this variation is really a measure of the different partitions of the *p*-chloromercuribenzoate between water and the insoluble substrate. This explanation is rendered the more likely in view of the recent clear demonstration of the action of lipase at the oil/water interface[17].

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