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Edison J. Geromel for the degree of Doctor of Philosophy

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Title: RELEASE OF LIPASE FROM LYSOSOMES OF RAINBOW TROUT (SALMO GAIRDNERI) MUSCLE SUBJECTED TO LOW TEMPERATURES

Abstract approved: Dr. M.W. Montgomery

Homogenates of dark lateral line muscle of rainbow trout (Salmo gairdneri) were prepared in a solution containing 0.25 M sucrose, 0.175 M potassium chloride and 0.001 M disodium (ethylenedinitrilo) tetraacetate (EDTA) soon after the animals were sacrificed. The homogenates were submitted to successive centrifugations at 1,000xG for 10 min and 27,000xG for 20 min. Each of the pellets were resuspended in the solution indicated above and were called the sediment and lysosomal fractions, respectively. The supernatant was used with no further treatment.

Under the assay conditions employed, lipase activity in the lysosomal fraction with tridecanoylglycerol as substrate was found to be optimal at pH 4.2-4.5 and was greatly enhanced by Triton X-100 (2% w/v). Fasting the fish for 24 or 72 hr before sacrifice resulted in

an increase of the lipase activity.

On a protein basis, relative proportions of 61, 21 and 18% of the acid lipase activity were found to be associated with the lysosomal, sediment and supernatant fractions, respectively.

Low temperature treatments commonly encountered in the fish processing industry were studied to determine if these treatments would promote in situ release of lipase from the lysosomes of the dark lateral line muscle of rainbow trout. A treatment was considered to have an effect on the release of lipase if a significant decrease of lipase activity in the lysosomal fraction accompanied by a significant increase of the activity in the supernatant and/or sediment fractions resulted from the treatment.

Storage of fillets for 7 days on ice caused no detectable release of the lipase from the lysosomes. On the other hand, temperature fluctuations of fillets in the range of -10° to -35° during 4 days was found to promote the release of the enzyme.

Considerably less lipase liberation from the lysosomes was observed when the fillets were frozen either at a fast or at an intermediate rate of freezing as compared to those frozen slowly. No significant difference in release of lysosomal lipase was noted between the fast and intermediate freezing rates. Rates of freezing studied were 6 (fast), 56 (intermediate) and 193 (slow) min for the internal temperature of the fillets to be reduced from -1° to -5°.

When fillets were stored at -11°, -23° or -40° for 30 or 120 days, a significantly larger lipase liberation from the lysosomes was noticed at the higher temperatures. Extending the storage period from 30 to 120 days caused no additional release of the enzyme.

Release of Lipase from Lysosomes of Rainbow Trout (Salmo gairdneri) Muscle Subjected to Low Temperatures

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Head of Department of Food Science and Technology

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Typed by Christine Dewey for Edison Jose Geromel

dedicated to

Alba and Edo

and to my parents and brothers

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INTRODUCTION

In recent years, as fishing boats have increased in size and the time that the boats remain at sea has increased, on board freezing of the fish to preserve quality has become a common practice. In addition, freezing is an effective method of preserving fish after processing. Fish tend to decrease in quality during frozen storage due to texture alterations. Liberation of fatty acids by muscle lipases appears to be related to these texture alterations.

Enzymic lipolysis in fish flesh is well documented and has been shown to proceed at a faster rate in the dark muscle than in the white muscle of fish during frozen storage.

Lysosomes are subcellular particles surrounded by a membrane that contain hydrolytic enzymes exhibiting acid pH optima. These hydrolases are inactive on external substrates as long as the lysosomal membrane is intact. During handling and processing of foods, lysosomes may be disrupted and the hydrolases released.

Tappel (1966a) stated that:

> "Because of their high latency and content of hydrolytic enzymes of broad specificity, the lysosomes appear to be the most important cell structure involved in freezing damage. ...For freezing and thawing of animal cells and tissues with

> minimum damage it is apparent that attention should be given to conditions for minimum damage to lysosomes."

The presence of a lipase among the enzymes that compose the lysosomal hydrolytic apparatus has been demonstrated in several tissues of different animals. In fish, an acid lipase of the dark lateral line muscle has been characterized as being lysosomal (Bilinski et al., 1971).

It has been suggested that the promotion of enzymic lipolysis in frozen fish is due to disruption of the lysosomes by freezing (Olley et al., 1962). Demonstration that lipolytic enzymes are in fact released from the lysosomes upon freezing would support this hypothesis.

The present work was undertaken to determine if low temperature treatments, such as those commonly found in the fish processing industry, would have an effect on in situ release of lipase from lysosomes of the dark lateral line muscle of rainbow trout (Salmo gairdneri).

LITERATURE REVIEW

The Texture Problem of Frozen Fish

During frozen storage of fish adverse changes have been reported to take place that resulted in a decline of product quality and have been reputed to be of great commercial importance (Mills, 1975). Some of these changes were apparent in the frozen product while others were manifested only when the product was consumed. Among the reported changes in appearance were: a) loss of moisture from the surface causing "freezer-burns", b) discoloration of the flesh due to lipid oxidation ("rust"), and c) loss of liquid ("drip") that flows spontaneously from the fish on thawing (Mills, 1975; Dyer and Dingle, 1961). Deterioration that have been evident upon consumption were reflected by: a) changes in the texture, b) development of tastelessness, and c) appearance of off-flavors and off-odors due to oxidative rancidity (Cutting and Spencer, 1968; Dyer, 1951).

Texture deterioration has been found to be an important change in frozen lean fish (Ackman, 1967; Dyer, 1967), that is, in those species where the fat content of the flesh was lower than 1%. Depending on the frozen storage conditions (especially time and temperature), adverse texture changes were also reported to be a problem in the more fatty species (Dyer and Morton, 1956; Dyer *et al.*, 1956).

The nature of the texture changes has been related to a loss of tenderness that gradually led to increased toughening (Dyer, 1967). Development of toughness in frozen fish was reported to be very undesirable (Partmann, 1977) and an important factor in the storage life of the product (King et al., 1962). Adverse texture changes have also been described in terms of firmness, dryness, and fibrousness (Connell, 1975; Cutting and Spencer, 1968; Dyer and Dingle, 1961).

As opposed to fish, which were inedible as a result of texture deterioration in a relatively short period of time, other types of muscle foods such as beef, lamb and poultry were very stable during frozen storage (Connell, 1975; Bramsnaes, 1969). According to King et al. (1962), this may have been due to the reduced stability of the fish myosins as compared to those of warm-blooded animals (Connell, 1961).

Development of adverse changes in the texture of frozen fish has been associated with alterations in the protein fraction (Cutting and Spencer, 1968; Connell, 1964; Moorjani et al., 1960; Dyer, 1951). Among the proteins, it has been established that only the myolibrillar group was involved (Connell, 1975; Dyer, 1967; Dyer and Dingle, 1961; Sawant and Magar, 1961). The correlation between protein alterations and toughness development appeared to be dependent on the frozen storage temperature. In general, both variables correlated fairly well during storage above -29° (Partmann, 1977). In some cases, however, the lower temperature limit was reported to extend to approximately

-20° (Cowie and Little, 1966; Luijpen, 1957).

Factors Causing Protein Alterations in Frozen Fish

The subject of protein changes in frozen foods in general (Partmann, 1977) and in frozen fish in particular (Sikorski et al., 1976) has received attention in recently published reviews.

Changes have been found to be manifested by a considerable reduction in the solubility of the proteins. However, Sikorski et al. (1976) and Connell (1962) have proposed that the term denaturation may not fully describe the phenomenon. In addition to what would normally be considered denaturation, such as changes in the native conformation caused by alterations in the secondary and tertiary structures of the proteins, solute-mediated aggregation of the molecules while still in their native, unaltered state were found to occur. This equally resulted in reduced solubility.

Factors related to the freezing of the water out of the solution were reported to bring about alterations of the proteins (Sikorski et al., 1976; Connell, 1968) with consequent texture deterioration (Partmann, 1977). Sikorski et al. (1976) suggested that one such factor was the disorganization of the structural water surrounding the protein molecules. When these structures collapsed, support for the native conformation of the proteins was lost due to disruption of water-mediated hydrophobic-hydrophilic interactions. Another factor was related to the increased

localized concentrations of protein molecules with resulting aggregation.

While often negligible in model systems, solute-mediated aggregation of the proteins may be important in more complex systems such as fish muscle. In other muscle foods, protein alterations during frozen storage were also observed but frequently not as a result of aggregation (Partmann, 1977). The solutes involved were of two origins (Sikorski et al., 1976): those present in the muscle (which did not exert their action until their concentration was altered by the freezing of the water) and those formed in the muscle as a result of biochemical reactions. In the first category are the inorganic salts, while the latter category includes lipid oxidation products, formaldehyde (in some species) and free fatty acids.

Inorganic Salts

Inorganic salts were suggested to act by a combination of effects, namely depression of the freezing point of the tissue fluids, dehydration, changes in the interfacial tension and ionic interactions with charged side groups of the protein molecules (Sikorski et al., 1976). Changes in the solubility of the proteins of frozen stored fish were attributed by Love (1958a) to be caused by inorganic salts present in the unfrozen liquid portion. Protein-salt interactions in frozen foods have also been mentioned by van den Berg (1968).

Lipid Oxidation Products

Although no relationship between fat oxidation and protein alterations in frozen fish was apparent to Dyer and Dingle (1961), the results of later research, mainly with model systems containing fish myofibrillar proteins, have shown that lipid autoxidation caused protein changes during frozen storage. To Braddock and Dugan (1973), evidence was strong that some of the products of lipid autoxidation caused at least part of the deterioration of muscle proteins in frozen fish and other meats. Even fish containing less than 1% unsaturated lipids in the muscle was found to develop a tough texture due to interaction of products of lipid oxidation with proteins during frozen storage (Castell, 1971). Both the initiation products and the secondary scission products of oxidation were shown to be involved in the deterioration of frozen fish proteins. Among the former, free radicals (Sikorski et al., 1976) and hydroperoxides (Jarenbäck and Liljemark, 1975) have been implicated. Among the secondary products, malonaldehyde has received the greatest attention. This compound was able to react with several amino acids in the protein molecules under frozen conditions. The rate of reaction of the ε amino group of lysine residue of isolated trout myosin with malonaldehyde at -20° was found to be greater than at 0° and almost as great at 20° (Buttkus, 1967).

Formaldehyde

Formaldehyde also has been linked with protein alterations in frozen fish (Babbitt et al., 1972; Castell, 1971). This subject has been reviewed by Sikorski et al. (1976). Only a few species have been shown to produce formaldehyde in the flesh during storage, which is probably because most species lack the required enzyme (Partmann, 1975). Sikorski et al. (1976) reported that only fish of the family Gadidae (such as cod, cusk, haddock, hake, pollock) and some members of the orders Scopeliformes and Macruriformes have been documented to contain the capacity to degrade trimethylamine oxide to form formaldehyde during storage. These authors showed that the reactivity of formaldehyde was so high that its action overshadowed other factors potentially capable of causing protein alterations in the frozen fish. Formaldehyde was reported to act by promoting inter- as well as intra-molecular cross-links in protein molecules. In frozen Alaska pollock, formaldehyde formation was maximum at -10°. Trimethylamine oxide was broken down to trimethylamine to a great extent during storage of the fish in ice. Therefore, reduced formaldehyde formation and consequently lower damage to the proteins were observed during frozen storage of fish that had been stored in ice for long periods before freezing (Sikorski et al., 1976).

Free Fatty Acids

The most studied factor contributing to solute-induced damage to proteins in frozen fish is undoubtedly that related to the action of free fatty acids. Several aspects of this subject have been covered in reviews dealing with the effects of freezing and frozen storage on fish proteins (Partmann, 1975, 1977; Sikorski et al., 1976; Powrie, 1973; Connell, 1968; Dyer, 1967; Connell, 1964; Dyer and Dingle, 1961). It was initially suggested that the decrease in protein extractability in salt solutions, which was paralleled by increased toughness, could have been a result of changes in the lipid fraction of frozen American plaice fillets (Dyer and Morton, 1956). A better correlation was observed between decrease in protein extractability and fatty acid liberation than with lipid oxidation in frozen rosefish fillets (Dyer et al., 1956). Supporting evidence for the effect of free fatty acids was later reported for additional species (Dyer and Fraser, 1959). Aggregation of isolated fish myosin upon addition of fatty acid was observed even under conditions preventing oxidation of the fatty acid (Menzel and Olcott, 1964). In fact, Castell et al. (1966) attributed the observed lack of lipid oxidation during frozen storage of cod to the liberation of free fatty acids in the muscle.

According to Anderson et al. (1965) protein changes induced by frozen storage was associated with lipid hydrolysis. Ultracentrifugal

studies revealed that protein-free fatty acid complex formation was favored by freezing and frozen storage and that the reaction rate was greater at -29° than at non freezing, low temperatures (Anderson and Ravesi, 1969). Based on experimental observations, the suggestion was made that "free fatty acids may exert an *in situ* effect" on the texture of frozen fish by promoting an increased interaction between myofibrills (Jarenbäck and Liljemark, 1975). Awad *et al.* (1969) claimed to have obtained a straight line relationship between free fatty acid liberation and actomyosin insolubilization in frozen stored fish muscle. Maximum instrument-measured toughness in frozen fish muscle was found to occur at -1.5°. In contrast, in the muscle supercooled to the same temperature (no ice formation), no toughness development was observed (Love, 1962a).

An interesting result emerged from the work of Love (1962b) when the decrease in actomyosin extractability, the increase in instrument-measured toughness and the liberation of fatty acids from phospholipids were compared. It was discovered that, during frozen storage of cod, the Arrhenius activation energies calculated for the three variables was impressively similar. Olley and Lovern (1960) also observed a similar course of phospholipid hydrolysis and protein insolubility in frozen cod but cautioned that this should not be taken as evidence of a cause-and-effect relationship. Further observations confirmed the results for cod but not for three other species (Olley *et al.*, 1962).

In those studies, the variable compared with the amount of protein insolubilization was fatty acid liberated as percentage of total lipids. The suggestion was made that probably a better correlation could have been obtained if the rates of fatty acid liberation rather than absolute amounts had been used in the comparison (Olley et al., 1969). Even though the relationship was found to hold for a number of species it did not for others and Lovern (1962) recommended that generalizations should not be made regardless of species.

In vitro studies by King et al. (1962) proved that small concentrations of free fatty acids caused a reduction in the solubility of fish actomyosin. The extent of the insolubilization was found to be a function of the nature of the free fatty acid, its concentration and the time of reaction. Based on those results, the authors suggested that some of the failures in the attempt to correlate fatty acid liberation with protein insolubilization could be related to the fatty acid composition of the fish muscle lipids. Indeed, although no obvious trends were observed, species with higher content of C_{22} hexenes in the fatty acids liberated during frozen storage seemed to show higher protein insolubilization (Olley and Duncan, 1965).

In addition, the lack of correlation observed in some studies could probably be explained on the basis of data obtained by Anderson and Steinberg (1964). These investigators observed that a different critical concentration of free fatty acid was required to trigger the process of

protein insolubilization for each of the species studied. Furthermore, the critical concentration was found to correlate positively with the lipid content of the fish muscle for most of the species studied.

This may provide an explanation for the findings of Dyer et al. (1956) who observed a difference in the susceptibility to protein alterations among species of fish having different muscle lipid contents. According to Dyer's theory (Dyer, 1967), neutral lipids somehow exert a protective effect against free fatty acid-protein interaction in the frozen fish muscle. A similar conclusion was also reached in the review prepared by Sikorski et al. (1976).

The extent of the interaction also appears to be a function of the storage temperature. Thus, while a good correlation was found during storage at -12°, almost no change in protein extractability and sensory panels scores was found at -23° even though an increase in free fatty acid concentration was observed (Dyer and Fraser, 1959). Another factor influencing the effect of free fatty acids was the presence of other solutes capable of inducing alterations in the proteins. Childs (1974) observed that formaldehyde concentrations as low as 0.04% enhanced the effect of free fatty acids by about three times.

The mechanisms by which free fatty acids induce alterations in the proteins is not well understood. To Anderson and Ravesi (1970), the non polar groups of amino acids in the myofibrillar proteins would interact hydrophobically in situ with the hydrocarbon chain of the liberated

fatty acids. To Sikorski et al. (1976), hydrophobic interactions also appeared to predominate under the conditions encountered in the environment of frozen fish muscle. In the study of Jarenbäck and Liljemark (1975), electron microscopic observations showed micelles of free fatty acids adhering to the actomyosin filaments. Non specific adsorption of the micelles was thought to be the cause of aggregation of the filaments. In the same study, the fatty acid was found to be ten times less effective than its correspondent hydroperoxide in inducing the protein changes. The mechanism of action of the fatty acid and the hydroperoxide was found to be entirely different from each other. Electrostatic bonds between the protein and the charged heads of the fatty acid molecules was also suggested as a possible mechanism (Powrie, 1973). Indeed, Tanford (1972) has demonstrated for another class of proteins, the serum albumins, that the binding of fatty acids does not occur only due to hydrophobic interactions but also through hydrophilic affinity for the fatty acid head groups.

The subject of free fatty acid-protein interaction does not appear to be settled. While some investigators defend the idea that this type of association plays a role at least in a number of species and under certain conditions, others reject this possibility. After attacking many of the evidences claimed for the role of free fatty acids, Connell (1968) concluded that:

"Numbers of chemical and enzymic changes occur simultaneously

> and exhibit similar time course during frozen storage of fish; fatty acid production is merely one change which happens to coincide in some species with the decline in extractability."

On the other hand, Ackman (1967) suggested that:

> "The development of free fatty acids in stored frozen fish has been recognized as occurring concurrently with protein denaturation, possibly prior to the latter process and undoubtedly related to it."

Dyer (1967) stated that:

> "The original suggestions, first, the sensitization of protein to denaturation by liberated free fatty acids and, secondly, possible stabilization of protein by unhydrolyzed lipid or a lipoprotein complex are supported by the experimental findings that dispersed actomyosin was insolubilized by added fatty acid, that the amount of fatty acid salts necessary to reduce protein extractability was related to the total lipid present in muscle from a series of seven species, and that neutral lipid protected against free fatty acid."

Sikorski et al. (1976) concluded that:

> "The result of lipid hydrolysis is detrimental to quality in lean fish, while at higher concentrations of neutral lipids dispersed in the muscle tissue in the form of droplets their competition for hydrophobic binding sites may reduce the participation of polypeptide side chains in hydrophobic adherences with fatty acid hydrocarbon residues. ...Simultaneous production of free fatty acids and formaldehyde both contribute to the effects of freezing changes on proteins."

From the evidence presented in this section, protein alterations in frozen fish appear to be caused by the interactions of inorganic salts, products of lipid oxidation, formaldehyde and free fatty acids. Increase in toughness of the fish muscle during frozen storage most likely is

due to the interactions of these substances with muscle protein.

Lipid Hydrolysis in Frozen and Ice Stored Fish

Increases in free fatty acid concentration as a result of lipid hydrolysis in the flesh are well documented both for fish stored in ice (Stodolnik et al., 1974; Lovern, 1962; Lovern et al., 1959) as well as during frozen storage (Shaw and Botta, 1977; Botta et al., 1973a,b; Ackman and Ke, 1969; Bligh and Scott, 1966; Dyer and Fraser, 1959). The opinion has been expressed that the driving force for this hydrolysis was the interaction of the liberated fatty acids with muscle proteins (Anderson et al., 1965).

Lovern et al. (1959) presumed that lipid hydrolysis could affect, besides the texture, also the flavor of stored fish. Indeed, a significant correlation between fatty acid liberation and the decrease in taste panel scores was observed in refrozen cod stored at -18° for 8 months (Peters et al., 1968). However, no correlation was found either in cod stored at -23° for 11-13 months (Dyer and Fraser, 1959) or in chinook salmon and Pacific halibut stored at -30° for up to 81 weeks (Botta et al., 1973a,b).

Lipid Classes Hydrolyzed

According to Lovern (1962),the nature of the lipid class hydrolyzed may vary depending on the species. Therefore, generalizations should

be made only with a great deal of caution. For instance, some species of fish such as cod and haddock were reported by Lovern (1962) to have very little triacylglycerols in the flesh. Consequently, the free fatty acids that became available during storage (Dyer, 1951) were derived almost entirely from phospholipids. Moreover, the elasmobranchs skate and nursehound,which had no triacylglycerols in the flesh, did not show evidence of fatty acid liberation from any lipid class (Olley et al., 1962). Absence of phospholipid hydrolysis appears to be a characteristic of the elasmobranchs since another member, the dogfish, also showed no phospholipid hydrolysis during storage. However, because the dogfish had a rather high content of neutral lipid in the flesh, fatty acid liberation in the frozen fish did occur (Lovern, 1962).

The situation was different with most teleostean fishes. Teleostean species that deposit almost no fat in the muscle, of which cod is a typical example, has already been mentioned above. For the members that do accumulate neutral lipids in the flesh, phospholipid hydrolysis may or may not have been accompanied by triacylglycerol hydrolysis depending on the species (Lovern, 1962). This is in contrast with the idea of Ackman (1967), which was later rectified by Ackman and Ke (1969), that only phospholipids were hydrolyzed in cold stored teleostean fishes. Nevertheless, at least in one fatty species (the fresh water whitefish) fatty acid liberation was observed to originate only from phospholipids during storage at -10° (Awad et al., 1969).

However, in some other fatty species such as rosefish, very little fatty acid liberation was observed during storage at -12° or -23° for 42 weeks regardless of the lipid class examined (Dyer et al., 1956). Herring, another fatty species, was thought to exhibit no post-mortem triacylglycerol hydrolysis (Lovern and Olley, 1962) but evidence is now available that both phospholipids and triacylglycerols are hydrolyzed in frozen (Bosund and Ganrot, 1969) as well as in ice stored herring (Addison et al., 1969).

A most interesting result that illustrates the influence of the storage conditions on the predominance of hydrolysis of one lipid class or another can be found in the work reported by Stodolnik and colleagues. When cod, herring and sprat were stored at -18°, phospholipids accounted for 95% of the fatty acid liberated in the cod and triacylglycerols accounted for 70 and 85%, respectively, in herring and sprat (Stodolnik and Podeszewski, 1975). On the other hand, during storage in ice phospholipid hydrolysis predominated in herring and sprat whereas triacylglycerol hydrolysis was found to be more important in cod (Stodolnik et al., 1974).

Studies have shown that the only phospholipids hydrolyzed during frozen storage of cod were phosphatidyl choline and phosphatidyl ethanolamine (Bligh and Scott, 1966; Lovern and Olley, 1962). In frozen herring, phosphatidyl choline was observed to be hydrolyzed at a faster rate than phosphatidyl ethanolamine and these were claimed

to be the only phospholipids hydrolyzed (Bosund and Ganrot, 1969).

Free fatty acids and water-soluble phosphorus derivatives have been shown to be the only products of phospholipid hydrolysis in cod stored at various temperatures either above or below freezing (Olley and Lovern, 1960). Lysophosphatides did not accumulate to any extent and the presence of diacylglycerols or phosphatidic acid was not detectable (Lovern, 1962). The relative concentrations of fatty acids hydrolyzed from phospholipids in frozen herring have been reported (Bosund and Ganrot, 1969).

As opposed to the nature of the lipid class hydrolyzed, the rate of fatty acid liberation was observed to be very similar at least among eleven teleostean species, regardless of the fat content in the flesh and of the storage temperatures studied (Olley et al., 1962). However, in the studies of Wood and Hagg (1962) a clear distinction in the hydrolysis rates was observed between the two species examined. Disimilar results were also obtained by Olley et al. (1969) for two additional species. In addition, different hydrolysis rates were observed in the experiments reported by Castell (1971) when five gadoid species were compared. Curiously, it was noted that those species showing the higher rates of fatty acid liberation were the ones presenting the lower rates of dimethylamine formation. It should be noted that dimethylamine was produced concomitantly with formaldehyde which, similarly to free fatty acids, is another compound known to cause protein alterations

during frozen storage of fish.

Lipid Hydrolysis in Different Locations of Fish Muscle

Some differences in the amount and type of lipid hydrolysis have also been observed within the same specimen depending from what location the sample was taken. The work of Olley et al. (1962) illustrates this fact. Depending on the species examined, the amount of lipid hydrolysis in the skin plus subcutaneous layer was found to be equal or greater than that of the flesh. The fat content of the skin plus subcutaneous layer was also observed to be greater but the higher hydrolysis noticed in this location was not proportional to the lipid content.

As far as the type of lipid class hydrolyzed is concerned, Olley and Watson (1969) observed that in iced gutted herring approximately half of the free fatty acids originated from triacylglycerols in the skin plus subcutaneous layer whereas phospholipids were found to be the sole contributors in the muscle proper.

A distinction has also been made between lipid hydrolysis in the ordinary or white muscle and the dark, heavily pigmented lateral line muscle. Despite the higher fat content in the dark muscle (Stansby, 1973; Ackman, 1967; Bosund and Ganrot, 1969), the lipid composition was very similar to that of the white muscle (Bligh and Scott, 1966; Bosund and Ganrot, 1969).

A much higher concentration of free fatty acids was reported in the dark muscle than in the white muscle of "commercially handled fish" and this difference was attributed to the greater metabolic activity of the dark muscle (Ackman, 1967). In frozen stored fish, a higher concentration of total free fatty acids (Botta et al., 1973a) as well as of numerous individual free fatty acids (Botta et al., 1973b) was found to be higher in the outside muscle (which includes the dark portion) than in the inside (white) muscle.

A higher rate of lipid hydrolysis was found in the dark muscle than in the white muscle of frozen herring (Bosund and Ganrot, 1969). Despite the similar lipid composition of both types of muscle, triacylglycerol hydrolysis was found to contribute 55% of the fatty acids liberated in the dark muscle as compared to 25% in the white muscle. Phospholipid hydrolysis was responsible for the balance of fatty acids liberated in both muscles. A similar pattern was observed during frozen storage of skipjack except that in the dark muscle hydrolysis of triacylglycerols was much more pronounced than that of phospholipids (Tsukuda, 1976).

Influence of Storage Conditions on Lipid Hydrolysis

In the initial stages of storage at low non freezing temperatures, lipid hydrolysis in the flesh of fish was characterized by very low rates of fatty acid liberation followed by an increased rate of hydrolysis.

This lag period was reported to extend for approximately 10 days in cod stored at 0^{o} (Olley and Lovern, 1960; Lovern and Olley, 1962), 2 weeks in cod stored in ice (Lovern et al., 1959) and 10 days in herring stored in ice (Olley and Watson, 1969). In lingcod, no phospholipid hydrolysis was detectable in 3 weeks of storage at 0^{o} (Tomlinson et al., 1960). At -1.4^{o}, where approximately 35% of the water is frozen, a lag period of 5 days was observed in stored cod (Lovern, 1962).

Olley and Lovern (1960) reported that after the lag period, the rate of fatty acid liberation became rather high but declined at latter stages in cod stored at 0^{o}. Unlike at 0^{o}, these authors observed no lag phase at $+20^{o}$, -7^{o}, -14^{o} or -20^{o}.

The effect of the freezing rate on the lag period of fish frozen, immediately thawed and subsequently held in ice, was studied by Lovern and Olley (1962). It was found that the lag phase was eliminated by the use of fast or slow freezing rates but not by intermediate rates of freezing.

Freezing followed by thawing without intermediate storage was observed to cause fatty acid liberation (Olley et al., 1962; Lovern, 1962). It was also found that the rate at which the fish was thawed did not seem to affect the amount of fatty acids liberated. Freezing followed by frozen storage of fish that had been previously frozen and thawed was noticed to lead to a sharp rise in the free fatty acid concentration

(Peters et al., 1968).

The method by which fish was frozen (brine freezing vs. plate freezing) was observed to have a significant effect on the free fatty acid concentration during subsequent frozen storage (Botta et al., 1973a). Longer frozen storage at -30° resulted in higher concentrations of free fatty acid although the increase was not uniform with time. A large rise in the first part of the storage was followed by a slower rate at later stages (Botta et al., 1973a).

Not only the rates of fatty acid liberation changed during frozen storage but also the nature of the lipid class hydrolyzed. Thus, Tsukuda (1976) noticed that while triacylglycerol hydrolysis predominated at the early stages of storage in both dark muscle and white muscle, phospholipid hydrolysis became predominant in the white muscle, whereas triacylglycerol hydrolysis was still more significant in the dark muscle.

The effect of the temperature of frozen storage on lipid hydrolysis of fish has been examined in many studies. While a fast rate of fatty acid liberation was observed during storage at -12°, the reaction proceeded very slowly at -29° (Dyer and Fraser, 1959). However, Olley and Lovern (1960) found the rate of the reaction to be only a little higher at 0° than at -14° and at +20° the rate of fatty acid liberation tripled that at 0°. Therefore, as with most reactions, the rate of lipid hydrolysis in frozen fish decreased as the storage temperature was

reduced. However, this relationship was not valid in the upper range of freezing temperatures (Lovern and Olley, 1962). It has been shown that the rate actually increased as the temperature was reduced just below the freezing point and was maximum at approximately -4°. Even at -7° fatty liberation was higher than at 0° (Lovern and Olley, 1962).

Anderson et al. (1965) predicted that maximum protein-free fatty acid combination would take place at a storage temperature where maximum lipid hydrolysis occurs. Development of maximum toughness was observed by Love (1962a) to occur during storage at -1.5°. In contrast, if the fish was only supercooled to -1.5° (no ice formed), no toughness development was observed. It should be noted that -1.5° was close to the temperature of maximum lipid hydrolysis (-4°) observed by Lovern and Olley (1962).

Factors Responsible for Lipid Hydrolysis

Dyer et al. (1956) suggest that the increase in free fatty acid concentration in frozen fish was due to enzymes and that probably phospholipids, triacylglycerols and other lipid constituents were the substrates for such enzymes (Dyer and Fraser, 1959). The latter investigators went further in suggesting that bacterial enzymes were probably not involved. This was concluded from the fact that ice stored samples that differed markedly in the degree of spoilage did not show a significant difference in the rate of free fatty acid development during

subsequent frozen storage.

Olley and Lovern (1960) compared the development of free fatty acid among normally handled fish with sterile and cooked samples stored in ice and were able to demonstrate that lipid hydrolysis was due to enzymes actually present in the flesh. Microbial and non enzymic lipolysis were found to be negligible. Lovern and Olley (1962) have shown that absence of microbial lipolysis was not a consequence of limited invasion of the flesh by the microrganisms due to the presence of skin. Skinned fillets exhibited the same rate of fatty acid liberation as whole fish. Also, reduced surface area for microbial attack was ruled out by comparing the lipolysis of minced and intact fillets stored in ice.

Investigators have been intrigued by the fact that the enzyme responsible for lipid hydrolysis a) did not act during the early stages of storage in ice and b) showed increased activity as the temperature was reduced just below the freezing point. According to Fennema (1975), the responsible factor leading to increased rates in some enzyme catalyzed reactions during the early stages of freezing of cellular systems appeared to be the damage caused by freezing to membranes of cellular structures with the consequent decompartmentation of enzymes, substrates and activators. The hypothesis set forth by Lovern and Olley (1962) to explain the delay in the start of lipolysis in iced fish required cell damage before the enzyme could act since, in

their concept, the enzyme was in some way kept apart from the substrate in the intact cell. Only after cellular disorganization, for instance as a result of autolysis, enzyme and substrate could come together and lipolysis take place.

Lovern and Olley (1962) also studied the effects of different rates of freezing on the pattern of lipolysis of fish subsequently stored in ice. Fish were frozen at different rates of freezing, immediately thawed, stored in ice and the rate of lipolysis followed. The usual lag period observed with unfrozen fish could not be detected in the fish frozen at high or low rates of freezing. Intermediate rates were found to cause no change in the pattern of the lag phase. Coupled with the findings of Love (1958b) that damage to the cellular structures of fish was less at intermediate rates of freezing, these results appeared to support the hypothesis that cellular disorganization was necessary for the lipolytic mechanism to have been activated.

Activation of the lipolytic enzyme system during freezing and frozen storage of fish has been reported (Olley and Lovern, 1960). Peters et al. (1968) attributed the increase of the lipolysis rate observed after thawing, processing and refreezing fish fillets to "an enzyme activation, or, more likely, to a disruption of membranes or other tissue structure." In line with these observations are the suggestions of Olley et al. (1962) and Lovern (1962) that the enzyme involved would be contained in subcellular structures such as the lysosomes. These organelles have

been described as resembling bags of hydrolytic enzymes (Wattiaux and de Duve, 1956). The hydrolases are inert on external substrates as long as the lysosomes are intact. Under certain conditions, freezing has been shown to disrupt the lysosomes with a resulting release of the enzymes in their fully active form (de Duve et al., 1955).

From the above discussion, it is evident that fatty acids are liberated during freezing and frozen storage of fish. At least part of this lipid hydrolysis is probably catalyzed by a particle bound lipolytic enzyme which is liberated during freezing and subsequent frozen storage.

Lipases and Their Significance in Foods

The Commission on Biochemical Nomenclature of the International Union of Pure and Applied Chemistry and International Union of Biochemistry (Florkin and Stotz, 1973) recommended the name triacylglycerol lipase (systematic name triacylglycerol acyl-hydrolase, code number 3.1.1.3) for the enzyme catalyzing the reaction:

triacylglycerol + H_2O = diacylglycerol + a fatty acid anion

Desnuelle (1972) indicated, however, that the hydrolysis of triacylglycerols by most, though not all, lipases goes beyond the diacylglycerol stage shown in the equation above. In this case, substantial amounts of monoacylglycerols and sometimes free glycerol

would also be formed.

Acting exclusively on carboxyl ester bonds, lipases were classified as carboxylesterases. In practice, however, a distinction is made between lipases and ordinary carboxylesterases. While monoesters or acylglycerols of long chain fatty acids are the ideal substrates for lipases, ordinary carboxylesterases show optimum activity against single esters of short chain carboxylic acids. However, this distinction is not absolute since some lipases are known to hydrolyze tributyrin and some ordinary carboxylesterases are able to hydrolyze naphtyl laurate (Nachlas and Blackburn, 1958).

The most suitable compound for differentiation of ordinary carboxylesterases from lipases was claimed to be sodium taurocholate. This compound inhibited the esterases while having an accelerating effect on lipases (Nachlas and Blackburn, 1958). However, the physical state of the substrate was believed to be a better distinctive criterion than the length of the fatty acid chain in the substrate or the effect of inhibitors (Desnuelle, 1972). While ordinary carboxylesterases were able to act on substrates that were completely soluble in water, lipases required a substrate with a certain degree of molecular aggregation such as that encountered in micelles or in emulsions. Accordingly, Jensen (1971) defined lipases as "enzymes hydrolyzing the esters from emulsified glycerides at an oil-water interface". A review on the specificity of lipases has been published (Desnuelle and Savary, 1963).

Lipases are widely distributed in nature. Microbial lipases were reported to be produced in significant amounts by a few species (Alford et al., 1964). Desnuelle (1972) found most of the microbial lipases to be exocellular and to exhibit specificity for the primary ester bonds in the triacylglycerol molecule although a few exceptions were reported that show no positional specificity. The lipase of the mold Geotrichum candidum was found to be unique in its specificity for oleic acid regardless of the position of this fatty acid in the substrate molecule (Alford et al., 1964).

In plants bearing oil seeds, lipase activity was reported to be observed during germination (Desnuelle, 1972). In castor bean, an additional lipase was reported by Desnuelle (1972) to be active in the dormant seed. While the lipolytic activity that appeared after germination exhibited an optimum pH near neutrality, the dormant castor bean seed enzyme showed an optimum at 4.2 (Ory, 1969). The acid castor bean lipase hydrolyzed triacylglycerols to completion (Ory et al., 1960) and was reported to be probably the best characterized plant enzyme (Desnuelle, 1972). Lipases have also been detected in cereal grains and fruits (Desnuelle, 1972).

The presence of lipase activity has been detected in the muscle of invertebrates (Stevenson, 1969). Invertebrate lipases did not exhibit rigid positional or fatty acid specificity (Berner and Hammond, 1970). Fish lipases have been reviewed by Bilinski (1974).

Among animal lipases, those of mammals are probably the best studied. Desnuelle (1972) has reviewed those mammalian lipases occurring in milk, tissues and gastrointestinal tract. At least two lipases were reported to be found in milk. One was found to be specific for the primary ester bonds of triacylglycerol molecules (Jensen, 1971) and in this respect resembled pancreatic lipase and most microbial lipases. However, unlike those enzymes, the milk lipase did not exhibit faster rates of hydrolysis with short chain fatty acids than with longer ones when both types of chains occupied similar positions in the triacylglycerol molecule (Jensen et al., 1962). Forster et al. (1961) suggested that the B-esterase of milk was a component of the milk lipase system. Some properties of milk lipase have been reviewed by Shahani (1975). The other lipase present in milk is a lipoprotein lipase believed to originate from serum leakage (Brockerhoff, 1974).

Desnuelle (1972) has discussed mammalian tissue lipases under three categories: lipoprotein lipase, hormone-sensitive lipase and liver lipases. Lipoprotein lipase was found in the plasma as well as in a number of tissues. Stimulated by heparin, this lipase was reported to act on the triacylglycerol components of chylomicra transported by the blood (Desnuelle, 1972).

Hormone-sensitive lipase was so called because of the marked stimulation exerted on the activity by hormones such as glucagon, thyroid-stimulating hormone, adrenocorticotropic hormone and

cathecolamines (Desnuelle, 1972). Epinephrine, norepinephrine and serotonin were other hormones reported to stimulate the enzyme (Jensen, 1971). Hormone-sensitive lipase was reported to be found in the adipose tissue where it mobilized depot fat (Brockerhoff, 1974).

The presence of three distinct lipases in rat liver was demonstrated by Guder et al. (1969). One of the enzymes was shown to be microsomal and exhibited maximum activity at pH 8.5. Another alkaline lipase was found to require Ca^{+2} ions and was stimulated by heparin. The third lipase exhibited optimum pH 5.0 and was demonstrated to be bound in the lysosomes. Lysosomal lipases exhibiting low pH optimum have also been demonstrated in tissues other than liver in mammals (Mahadevan and Tappel, 1968; Weglicki et al., 1976) and fish (Bilinski et al., 1971).

Rat skeletal muscle has been shown to contain four lipases (Wallach, 1968). Other mammalian organs and tissues known to exhibit lipase activity include arteries, brain, heart, kidney and serum (Desnuelle, 1972).

The main digestive mammalian lipases were reported to be intestinal lipase, gastric lipase and pancreatic lipase (Desnuelle, 1972). The existence of a pregastric or oral lipase was mentioned by Beck and Scott (1974). The intestinal lipase was found in the mucosa and exhibited a high degree of preference for monoacylglycerols (Pope et al., 1966). The presence of a lipolytic enzyme distinct from pancreatic

lipase has been demonstrated in the gastric juice (Cohen et al., 1968) but little is known about this enzyme.

Pancreatic lipase, probably the best known of all lipases, has been shown to be synthesized by the acinar cells of the pancreas and discharged into the intestinal tract where it converts dietary triacylglycerol mostly to 2-monoacylglycerols and free fatty acids (Desnuelle, 1972). Unlike the triacylglycerols, these products of hydrolysis are able to penetrate the epithelial membrane of the mucosa cells when combined with bile salts (Hoffmann and Borgstrom, 1962). This penetration is an essential step in the absorption of fats by the body.

Pancreatic lipase has been reported to be highly specific for the primary ester bonds in triacylglycerol molecules (Desnuelle, 1972) and to require a protein cofactor (colipase) for optimal activity (Julien et al., 1972). Borgstrom (1972) found that colipase counteracts inhibitory effects of certain detergents on pancreatic lipase. Certain effectors have been shown to be important in the hydrolysis of triacylglycerols containing long chain fatty acids by pancreatic lipase: sodium chloride (Benzonana and Desnuelle, 1968), bile salts (Brockerhoff, 1971) and Ca^{+2} ions (Benzonana, 1968).

A mechanism to explain the unique ability of lipolytic enzymes to hydrolyze water insoluble substrates at oil-water interfaces has been proposed by Brockerhoff (1974). In this mechanism the substrate would be located at the surface of a matrix or of a membrane that would be

much larger than the enzyme molecule. Matrix and membrane were collectively called supersubstrate. The substrate would be imbedded in the supersubstrate and would only be allowed to move laterally but not to leave the structure. The lipase was envisaged as having a non polar head which permitted the enzyme to bind hydrophobically to the supersubstrate. The binding site would be distinct from the active center but would probably be located close to the latter. Binding of enzyme and supersubstrate would result in the correct positioning between the lipase active center and the substrate. The polar tail of the enzyme molecule would contribute additional stability and orientation for the binding of the enzyme to the supersubstrate.

Effect of Lipase on Food Flavors

According to Richardson (1976), fatty acids formed in foods as a result of endogenous lipase activity would impart off flavors to the product.

Most of the work reported in the literature that related lipases with food applications dealt with milk and dairy products. In milk, a distinction has been made between the lipases naturally present, the lipases of contaminating or innoculated microorganisms and lipase preparations that were added to milk in the manufacture of various products.

Milk lipases have been reported to be responsible for off flavor

development when milk was unduly agitated, for instance during transport through pipelines (Brockerhoff, 1974). A high correlation between lipase activity and milk taint was observed by Hemingway et al. (1970). Psychrotrophic microorganisms that produce lipolytic and proteolytic enzymes have become increasingly important in milk spoilage in recent years (Brunner, 1976).

In the production of certain cheeses, lipolysis of milk fat by lipases of innoculated microrganisms have been reported to play an important role in the development of characteristic flavors (Beck and Scott, 1974).

The flavors produced by lipase preparations added to milk in the manufacture of certain dairy products are directly related to the flavors of the fatty acids liberated from the milk fat. Thus, preparations made from the gullet of calves have been reported to produce higher ratio of short chain fatty acids whereas pancreatic lipase produced long chain fatty acids which exhibit soapy flavors (Wieland, 1972). A pre-gastric lipase preparation obtained from the stomach of kids or lambs was reported to be responsible for the characteristic piquant flavor of Italian cheeses (Richardson, 1975). In butter, lipolysis was reported to generate rancid flavors which may be desirable or undesirable depending on individual preferences (Beck and Scott, 1974).

The liberation of fatty acids in stored grains or in flour milled from such grains has been reported to have a detrimental effect in

bread baking. The enzymes responsible for such lipolysis were lipases naturally present in the grain, in the flour or produced by contaminating molds (Barrett, 1975).

Lipases present in oil seeds were reported to be active during industrial extraction of the oil. Such activity was reported to be undesirable and to lead to additional refining steps (Brockerhoff, 1974).

Other detrimental aspects resulting from the action of lipases in foods were listed by Beck and Scott (1974). Hydrolysis of fat in cocoa powder imparted off flavors to milk chocolate products. In egg white powder, lipolysis produced off flavors that were noticeable in products manufactured from such raw material. Such lipolysis was due to pancreatin added to remove traces of yolk lipids. Potthast et al. (1975) observed triacylglycerol hydrolysis in freeze dried beef even at the low relative humidity of 10%.

Effect of Low Temperatures on Lipase Activity

The activity of microbial lipases at temperatures below freezing has been examined in several studies. Psychrotrophic microrganisms have been reported to be enzymically active even near -10° although, at least for some species, the optimum production of lipase occurs at 20° (Stokes, 1968). Alford and Pierce (1961) examined the lipase activity of several microrganisms at low temperatures. Considerable

activity was observed within 2 to 4 days at -7° and within a week at -10°. Close examination of the results shows that a yeast, Candida lipolytica, produced almost the same amounts of free fatty acids in corn oil emulsion during six weeks at -29° as during 45 min at 35°.

Lipase activity at low temperatures has been studied in a variety of model and food systems. As early as 1938 it was realized that enzymic hydrolysis of fats in frozen foods "probably has an important bearing on the quality of the stored product" (Balls and Tucker, 1938). Using a model system, those authors were able to detect enzymic lipolysis even below -30°. The rate of lipid hydrolysis decreased with temperature but did not show any abrupt change corresponding to the change in physical state as the temperature was reduced below 0°. In contrast, Sizer and Josephson (1942) did observe a sharp break at 0° which led these investigators to conclude that the kinetics of lipase activity below and above 0° were different. Appreciable activity was noticed even at -25°. The enzyme was not inactivated during storage at -70°.

Beesley et al. (1969) observed that sodium chloride inhibits or stimulates lipase activity in frozen systems depending on the concentration of this salt in the unfrozen liquid. At -10°, maximum activity was found close to the saturation point of sodium chloride.

Working with a system containing pancreatic lipase and olive oil emulsion or milk as substrate, unusual results were observed by

Mullenax and Lopez (1975). Higher activity was found at -23° than at -14.4° or -19.4° and the difference was noticed to be even greater at higher enzyme concentrations. Those investigators found no logical explanation for such behavior.

Parducci and Fennema (1978) examined the effect of the freezing rate and the final freezing temperature (temperature nadir) on the lipase activity in a model system. Rapid freezing to -80° resulted in a much slower initial rate of lipase activity at -8° than either rapid freezing to -20° or slow freezing regardless of the temperature nadir.

Lipolysis was observed during frozen storage of unblanched frozen peas by Bengtson and Bosund (1966). Development of off flavors due to lipolysis was observed in unblanched frozen peas by Lee et al. (1956) and in unblanched corn, peas and snap beans by Lee et al. (1955).

Very slow autolytic degradation of the lipids was observed by Rhode and Lea (1961) during the first few days when lamb livers were held at 15°. After this lag period, rapid hydrolysis was observed. Freeze-thawing was found to eliminate the lag period and precipitate rapid hydrolysis in the livers subsequently held at 15°. Triacylglycerols were hydrolyzed at a faster rate than phospholipids. To explain these findings, it was suggested that the lipolytic enzymes in lamb liver were particle bound and could only express their activity after being released (Rhodes, 1961; Rhodes and Lea, 1961).

Fish Flesh Lipases

Lipids appear to constitute a more important energy reserve than carbohydrates in fish (Cowey and Sargent, 1977; Drummond and Black, 1960). In mammals, lipids are stored mainly in the adipose tissue whereas liver and skeletal muscle are the chief storage sites for lipids in fish (Bilinski, 1969). According to this author, liver is the most important storage site for the depot lipids in bottom-dwelling sluggish fish such as cod. On the other hand, in more active species including rainbow trout, lipids are stored mainly in the skeletal muscle.

George (1962) compared two types of fish skeletal muscle with respect to their ability to utilize fat as a source of energy for muscular activity. The dark lateral line muscle was observed to be better adapted for such task than the white muscle. While in the dark muscle energy was generated mainly through aerobic metabolism of fats, the white muscle was found to derive energy requirements mostly through anaerobic metabolism of glycogen. However, under continuous and prolonged physical activity, even the white muscle utilized fat as fuel.

The presence of a lipase able to hydrolyze triacylglycerols containing short chain fatty acids was demonstrated by George (1962) both in the white muscle and in the dark lateral line muscle of mackerel. The activity was determined at pH 7.4 and observed to be four times greater in the dark than in the white muscle. Histochemical observations

of the dark lateral line muscle of an unspecified fish species revealed the presence in the mitochondria of a lipase able to hydrolyze Tween 85 (Bokdawala and George, 1964).

A lipase having an optimum pH of 8.2 has been isolated and partially purified from lingcod muscle (Wood, 1959a). The rate of hydrolysis of monoacylglycerols was observed to increase with the chain length of the fatty acids but the same did not apply to triacylglycerols. Olive oil emulsion, fish liver oils and trioctanoin were not hydrolyzed. Short chain triacylglycerols were found to be hydrolyzed at a faster rate than monoacylglycerols regardless of the chain length of the latter. Further studies showed that this enzyme was not uniformly distributed in the fish muscle (Wood, 1959b). A gradual increase in activity was observed from the tail section to the head section of the fillets. Very little loss of activity was found after storage of the fillets at -20° for several weeks.

Searching for lypolytic activity against long chain triacylglycerols in the dark lateral line muscle of rainbow trout, Bilinski and Lau (1969) found that the demonstration of such activity was highly dependent on the method used to emulsify the substrate. No significant hydrolysis occurred when emulsification was carried out with a number of compounds or procedures. High activity was accomplished only when the substrate was dispersed in the phospholipid fraction isolated from the tissue. The lipase activity was found to be non uniformly distributed

along the lateral line muscle. Optimum pH was determined to be 7.3 in the range studied (6.0 to 8.5). Tripalmitin was hydrolyzed at a faster rate than triolein. Tristearin was slowly attacked.

Another lipase distinct from the above by exhibiting a low pH optimum (4.0 to 4.5) has also been demonstrated in the dark lateral line muscle of rainbow trout (Bilinski et al., 1971). This enzyme, which was shown to be associated with the lysosomes, will be discussed below under Lysosomal Lipases. Lipolytic enzymes of fish skeletal muscle have been reviewed by Bilinski (1969, 1974).

Lysosomal Lipases

Studies with rabbit polymorphonuclear leucocytes provided suggesting evidence (Elsbach and Rizack, 1963), reinforced by later work (Elsbach and Kayden, 1965), that the lysosomes may contain an acid lipase. No lipase activity was detected in lysosomes of rat liver (Shibko and Tappel, 1964a) but the cause of failure was later attributed to the assay conditions used (Mahadevan and Tappel, 1968). Further studies with rat liver lysosomes indicated considerable acid lipase activity in these structures (Mellors et al., 1967).

Mahadevan and Tappel (1968) reported what they considered to be the first clear demonstration of a lipase in the lysosomes. The rat liver enzyme exhibited an optimum pH of 4.2 and showed a marked requirement for Triton X-100 in the reaction mixture. A high concentra-

tion (5.3%) of this detergent was required for maximum activity. Of the several triacylglycerols tested as substrate, maximum hydrolysis was accomplished with tridecanoylglycerol.

The existence of a lysosomal lipase in rat liver was later confirmed by other investigators (Guder et al., 1969). Fowler and de Duve (1969) observed not only a requirement for a high concentration (5%) of Triton X-100 for maximum activity but also that triacylglycerol hydrolysis would not take place in the absence of the detergent. In addition, those authors showed that diacylglycerols were also hydrolyzed and suggested that the same enzyme was responsible for both activities since Triton X-100 strongly stimulated diacylglycerol hydrolysis. On the other hand, the presence of Triton X-100 inhibited almost completely the activity on monoacylglycerols while its absence strongly stimulated such hydrolysis.

Additional work with rat liver lysosomes showed some disagreement with the early studies with respect to the effect of Triton X-100 on the acid lipase activity. Approximately 80-90% inhibition of tridecanoylglycerol hydrolysis was observed in the presence of 2% Triton X-100 as compared to a similar system in which the detergent was omitted (Hayase and Tappel, 1970). The pH optimum also shifted from 5.2 in the system containing no Triton X-100 to 4.2 in the presence of the detergent. No explanation was offered for such apparent discrepancies with the early studies reported by Mahadevan and Tappel (1968) and

Fowler and de Duve (1969).

The lysosomal lipase of rat liver has been purified to a great extent and some of the properties studied (Teng and Kaplan, 1974). The pH optimum was found to be approximately 4.0 and the activity was observed to be greatly enhanced by added phospholipids, especially cardiolipin. Unlike the lysosomal phospholipase of the same tissue (Mellors and Tappel, 1967), the lipase was found to be tightly bound to the lysosomal membrane.

Lysosomal lipases from sources other than rat liver have also been studied. Evidence for the presence of a lysosomal lipase in pig liver has been presented by Muller and Alaupovic (1970). Temperature-induced release of a lipase and phospholipase from isolated lysosomes of canine cardiac tissue was investigated by Weglicki et al. (1976).

Studies on lysosomal lipase from skeletal muscle are scarse. Bilinski et al. (1971) demonstrated the presence of such enzyme in homogenates of dark lateral line muscle of rainbow trout. The effect of Triton X-100 on the activity was found to be striking. In the absence of the detergent, no activity could be detected at low pH values (4.0 - 5.0) where lysosomal enzymes usually exhibit maximum activity. An increase in Triton X-100 concentration corresponded to an increase in activity in that pH range until a concentration was reached (2%) above which no further increase in the enzyme activity was observed. At the 2% level, the pH optimum was found to be 4.0 - 4.5. The main

products of tripalmitoylglycerol hydrolysis were determined to be palmitic acid and dipalmitoylglycerol. Fractionation studies permitted to locate the main activity in the light mitochondrial fraction where most of the lysosome particles sediment.

The acid lipase of castor bean (pH optimum 4.2) might be considered a lysosomal enzyme according to the concept of plant lysosomes set forth by Matile (1969). To that author, lysosomes occur under several types of structures in plant tissues. Castor bean acid lipase has been shown to be associated with one of such structures, the spherosomes (Ory, 1969).

Electron microscopy and biochemical analysis have been used in the establishment of a microbial lipase, that of the yeast Candida utilis, as a lysosomal enzyme (Biriuzova and Lobyreva, 1975).

Inhibitor studies have shown (Mahadevan and Tappel, 1968) that the liver lysosomal lipase has different properties from a number of other liver lipases, namely those found in the plasma membrane, microsomes, soluble fraction and the lipoprotein lipase. Adipose tissue lipase was also found to be different from the hepatic lysosomal lipase.

The physiological role played by lysosomal lipase has been speculated in several communications. To Vignais et al. (1976), lysosomal lipase would be important in the energy metabolism of animal cells, especially in the adipocytes and hepatocytes. A

similar function has been attributed by Bilinski et al. (1971). Mahadevan and Tappel (1968) suggested that the lysosomal lipase would be involved in intralysosomal digestion of triaclyglycerols that enter lysosomes in the process of autophagy.

Lysosomal lipase has been implicated in Wolman's disease. Extensive deposition of neutral fat and cholesterol as well as widespread occurrence of foamy lipid-laden cells were observed in individuals afflicted by the disease. Lysosomal lipase activity was observed to be entirely absent from the affected tissues of such individuals (Patrick and Lake, 1969).

Brief accounts of lysosomal lipase occurrence and properties have been published (Vignais et al., 1976; Barrett, 1969, 1972; Tappel, 1969).

Lysosomes

Studies on intracellular localization of enzymes led de Duve et al. (1955) to propose the existence of a new group of cytoplasmic particles, the lysosomes. The original lysosome concept set forth by de Duve and co-workers has been summarized by Pitt (1975). The concept was founded on the observations that five hydrolases, all exhibiting optimum pH around 5.0, were found to be sedimentable after differential centrifugation of rat liver homogenates. The hydrolases differed widely in substrate specificity and were observed to display maximum activity

only after the particulate fraction in which they were located had been submitted to treatments known to disrupt cellular membranes. Under such conditions, all of those hydrolases were released concomitantly. This suggested that the five enzymes were associated with the same structure in the cell.

Later it was found that those and many other acid hydrolases occurred in the cell separated from the cytoplasmic substrates by a membrane what helped to explain the observed latency of the enzymes. It should be noted, however, that all acid hydrolases in the cell do not occur in the lysosomes (Pitt, 1975).

The reduced ability of intact lysosomes to hydrolyze external substrates in vitro has been confirmed in many reports (de Duve, 1963; Sawant et al., 1964a,c). The gradual increase in hydrolytic activity of lysosomal enzymes on external substrates in vitro was found to be due to disrupture of the lysosomal membrane rather than increased permeability of the membrane to the substrates. The lysosomal membrane was found to be virtually impermeable to all substrates tested (Baccino and Zuretti, 1975).

The single lipoprotein membrane was reported to be rather fragile and to impose a series of precautions during isolation of intact lysosomes (Pitt, 1975). De Duve (1963) found the need for homogenization of the tissue to be performed under gentle and carefully controlled conditions. Factors reported by de Duve (1963) to favor disruption of

the lysosomal membrane *in vitro* included inadequate osmotic protection, certain ranges of pH values, temperature a few degrees above 0^{o}, freezing and thawing and the presence of detergents. According to Weglicki *et al.* (1976), the stability of the lysosomal membrane was related to the integrity of the constituent lipids.

A number of compounds were shown to affect the stability of lysosomes *in vivo* (de Duve *et al.*, 1962; Weisman, 1964). Labilizing compounds included etiocholanone, endotoxins, streptolysin S and vitamins A, D_2, E and K_1. Among stabilizing compounds were cholesterol, cortisone, hydrocortisone and chloroquine. Recent studies on toxicity of food additives revealed *in vivo* damage to rat liver lysosomal membrane by butylated hydroxyanisol (BHA) when this compound was used in concentrations sufficient to inhibit peroxide formation in foods (Sgaragli *et al.*, 1975).

Osmotic protection to lysosomes during isolation has generally been provided by including sucrose in the homogenizing and fractionation medium. In the pioneer work of de Duve *et al.* (1955) with rat liver lysosomes, an unbuffered solution containing 0.25 M sucrose was used for this purpose. With or without modifications, this solution as well as the fractionation scheme developed by these researchers has been used in studies reported by many workers.

Shibko and Tappel (1965) have examined the effect of osmolarity of the suspension medium on the stability of isolated rat kidney

lysosomes. No change was observed in the osmolarity range of 0.6 to 0.25. A combination of decreased osmolarity and increased temperature was found by Tappel et al. (1963) to result in increased lysosomal disruption.

Isolated rat kidney lysosomes were found to be unstable at pH values both below 5.0 and between 8.0 and 9.0. Maximum stability was observed when the pH of the suspension medium was between 6.0 and 7.0 (Shibko and Tappel, 1965).

In studies involving the assessment of lysosomal integrity it is customary to distinguish between two types of lysosomal enzyme activity. Soluble or free activity is determined under conditions that prevent the disruption of the lysosomes during the assay. On the other hand, the determination of total activity requires the release of the enzymes before the activity can be measured (de Duve, 1963). A low ratio between free and total activity indicates that a high degree of integrity was accomplished during the isolation of the lysosomes.

The two most common procedures employed to accomplish in vitro release of lysosomal enzymes involve on one hand repeated freezing and thawing the lysosomes suspension (e.g., in Sawant et al., 1964b) and on the other hand addition of the non-ionic detergent Triton X-100 to the suspension (Wattiaux and de Duve, 1956).

Fennema (1975) indicated that while mitochondria were only moderately affected by freezing, lysosomes were highly susceptible to freezing

damage. Repeated freezing and thawing was found to release in a parallel fashion β-galactosidase, cathepsin and β-glucuronidase from beef skeletal muscle lysosomes (Ono, 1970). During beef aging (4°) however, β-glucuronidase and cathepsin D were observed to exhibit different patterns of release from the lysosomes (Valin, 1970). While β-glucuronidase showed a latent period of about two days followed by high activity, cathepsin D activity was high at the early stages of the aging period but levelled off later.

The availability of lysosomal enzymes to substrates after repeated freezing and thawing was studied by Sawant et al. (1964c). It was observed that arylsulphatase and β-glucuronidase were completely solubilized after freezing and thawing the lysosomes suspension ten times whereas ribonuclease and acid phosphatase were only partially solubilized by the same treatment (73% and 55%, respectively). Differential release of individual lysosomal enzymes upon treatment of lysosomes suspensions with Triton X-100 has also been observed (Romeo et al., 1966).

Sawant et al. (1964a) have produced evidence that the individual enzymes in the lysosomes were bound to the membrane to differing degrees. The bound enzymes did not appear to be constituents of the membrane structure. The possibility exists, however, that at least some of the enzymes occur free in the soluble phase within the lysosomes (Pitt, 1975).

Release of enzymes from the lysosomes was found to follow a two-stage process (Shibko and Tappel, 1965; Shibko et al., 1965). In a first step, the enzymes became available to the substrates while still bound to the lysosomes (sedimentable). Later, the soluble activity increased and approached the total activity.

Over 50 distinct types of hydrolases have been shown to occur in lysosomes (Barrett, 1972). The spectrum of activities was wide and included substrates such as lipids, phosphates, the oligosaccharide portion of glycoproteins and glycolipids, proteins, peptides, polysaccharides, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) (Tappel, 1969).

Lysosomal hydrolases have been shown to exhibit pH optima in the range of 3.5 to 6.4. Many of the lysosomal enzymes have been characterized as glycoproteins (Desnick et al., 1976). Characteristics of the individual enzymes known to be of lysosomal origin have been reviewed by Barrett (1969, 1972) and Tappel (1969).

Differences in individual enzyme activities between tissues of the same specimen have been reported (Shibko et al., 1963; Shibko and Tappel, 1964b). As suggested by Shibko and Tappel (1965), in addition to a common group of hydrolases, the lysosomes of each tissue may contain enzymes that are specific for the metabolism of that particular tissue. The suggestion has been made that there may be a functional differentiation among lysosomes in the various tissues (Hayashi, 1967).

Even populations of lysosomes isolated from the same tissue may be enzymically heterogeneous (de Duve, 1963; Pitt, 1975).

The individual lysosomes do not only differ with respect to their enzyme content and activities but also in shape and size of the particles (de Duve, 1963). Several functional types of lysosomes have been proposed (Pitt, 1975; Desnick et al., 1976; Holtzman, 1976). Primary lysosomes were classified as storage structures for acid hydrolases but would not be involved in the digestive events in the cell. The hydrolases would probably be present in an inactive state in these structures. The fusion of primary lysosomes with certain types of vacuoles would result in the digestive units which were called secondary lysosomes. The vacuoles would be mostly phagosomes, secretory vesicles and autophagosomes in which the molecules to be hydrolyzed had been sequestered. It was presumed that the inactive hydrolases present in the primary lysosomes would become activated in the fusion process. Secondary lysosomes were classified in heterophagic or autophagic. Heterophagic vacuoles were defined as the types of lysosomes in which acid hydrolases were present along with material to be digested that was of extracellular origin. In contrast, autophagic vacuoles contained acid hydrolases along with material originated from the own cytoplasm of the cell. After repeated use, the secondary lysosomes would lose their hydrolytic capacity and would become residual bodies. A detailed account of enzymic and non-enzymic

components of lysosomes has been published (Tappel, 1969).

Most of the work conducted with lysosomes has employed liver, kidney or spleen of mammals as sources. Evidence has been presented for the presence of lysosomes in skeletal muscle of mammals (Buchanan and Schwartz, 1967; Stagni and de Bernard, 1968) as well as fish (Milanesi and Bird, 1972). According to Bird et al. (1969), the reason for fewer studies with skeletal muscle lysosomes is related to the difficulty in fractionation and maintenance of integrity of muscle lysosomes. In addition, skeletal muscle has a low content of lysosomes (Reddi et al., 1972). Related difficulties in studies with fish muscle lysosomes have been reported (Warrier et al., 1972).

The effect of decreasing osmolarity of lysosome suspensions has been studied with both chicken skeletal muscle and chicken liver lysosomes. While the decrease from 1.0 to 0.25 M caused no change in the availability of lysosomal enzymes from skeletal muscle, increased availability of lysosomal enzymes from liver was observed (Harikumar et al., 1974).

In contrast with organs such as liver, kidney and spleen which are known to be good sources of lysosomes, a low yield of these organelles was observed in skeletal muscle (Harikumar et al., 1974). Low activity of lysosomal enzymes in skeletal muscle as compared to organ tissues has been reported by many workers (Shibko et al., 1963; Tappel, 1966b; Buchanan and Schwartz, 1967).

Stagni and de Bernard (1968) observed that acid hydrolases were liberated from Triton X-100 treated lysosomes more homogeneously from rat than beef skeletal muscle.

For the isolation of lysosomes, Bird et al. (1969) reported that fish skeletal muscle was more amenable to fractionation than was rat skeletal muscle.

Two distinct populations of lysosomes were detected in rat skeletal muscle (Canonico and Bird, 1970). One type was suggested to be composed of muscle cell lysosomes whereas the other was thought to originate from macrophages and connective tissue cells. A marked difference in the activity of certain individual enzymes was noticed between the two populations.

Lysosomes have not only been found in animal tissues but also in plants and microrganisms. Comprehensive reviews on plant lysosomes have been published (Matile, 1969, 1975; Gahan, 1973).

The main physiological role of lysosomes was reported to involve intracellular digestion of cell components (Pitt, 1975). This activity might take place under normal physiological conditions or it might arise as a response to metabolic disturbances of the cell by pathological agents. Intracellular digestion was reported by Pitt (1975) to be an important step in the recycling (turnover) of cell components. Some pathological conditions involving extensive cellular breakdown by lysosomal hydrolases were mentioned by Sawant et al. (1964b) and

included liver necrosis, hypervitaminosis A, muscular distrophies and bruises. Lysosomes involvement in arthritis and a number of other diseases was discussed by Holtzman (1976).

Books covering many aspects of the accumulated knowledge on lysosomes include those published by Dingle and Fell (1969), Dingle (1972, 1973), Pitt (1975) and Holtzman (1976).

MATERIAL AND METHODS

Preparation of the Crude Enzyme

Enzyme Source

Rainbow trout (Salmo gairdneri) Mount Shasta strain were used in this research. The fish were obtained from the stock of the Food Toxicology and Nutrition Laboratory, Department of Food Science and Technology, Oregon State University, and were raised under the conditions described by Sinnhuber et al. (1977a,b). Fish of both sexes were used indistinctly and ranged in age from 18 to 24 months.

Preparation of the Tissue for Homogenization

The fish were caught in a net, given a blow on the head and immediately packed in ice. All of the following work leading to the enzyme preparations was conducted in a cold room at 4° or in an ice bath.

Fish were allowed to chill in ice for 30 min while being transported to the laboratory and were weighed, decapitated and eviscerated. The red muscle along the lateral line was excised by making cuts at about 2-3 cm both dorsal and ventral to the lateral line. The cuts extended all the way through approximately the posterior part of the caudal peduncle. A strip of flesh with the skin attached having a thickness

of about 1-1.5 cm was removed from the fish. White muscle, fat and connective tissue were removed from the strip of flesh as much as possible, leaving the dark lateral line muscle attached to the skin. The dark muscle was removed from the skin, care being taken that no skin material was excised along with the muscle. Due to the structural organization of the dark muscle, complete removal of contaminant tissues was not feasible. This difficulty was also reported by Bilinski et al. (1971). The slices of the dark muscle were cut with scissors into pieces roughly 0.5-1.0 cm^2. Usually about 4 g of dark muscle were obtained per each side of the fish. The pieces were mixed for randomization purposes since Bilinski and Lau (1969) have reported that the activity of an alkaline lipase was not uniformly distributed along the lateral line muscle of rainbow trout.

Homogenization

The extracting solution had a composition of 0.25 M sucrose, 0.175 M potassium chloride and 0.001 M disodium (ethylenedinitrilo) tetraacetate (EDTA) and was stored at 4°. Before use, the pH of the extracting solution was adjusted to 7.2-7.3 with 0.5 M KOH (Bird et al., 1969).

The homogenization procedure described by Whiting et al. (1975) was used with a few modifications. Immediately following the excision, 3.7 g of the tissue pieces were transferred to an ice chilled cylindrical

smooth walled glass tube 30 x 170 mm. This tube and teflon pestle that was fitted to the internal diameter of the tube were the components of a Potter and Elvehjem (1936) type of homogenizer. The plastic weighing pan was rinsed with cold (4°) extracting solution which was transferred to the homogenizer tube. Ratio of tissue to extracting solution was 1:4 (w/v).

The tissue pieces were homogenized by submitting them to three, 2 sec bursts with a Tissumizer homogenizer (Tekmar Co., Cincinnati, Ohio) while the homogenizer tube remained in ice. A quick up and down pass with the teflon pestle rotating at 1,000 rpm while the tube was kept in ice completed the homogenization. The Tissumizer consisted of a cylindrically shaped, slotted, external stator and a revolving internal rotor and was comparable to that described by Caldwell and Grosjean (1971). For this work the Tissumizer was used at maximum rotating speed of 20,000 rpm.

Fractionation

The homogenate was fractionated by the scheme described by Whiting et al. (1975). As with the other operations, the centrifugations were conducted in a cold room at 4°. A Servall centrifuge was used, fitted with a rotor type SS-34.

After being transferred to a cylindrical centrifuge tube (2.8 x 10.2 cm), the homogenate was centrifuged at 1,000 x G for 10 min to remove

insoluble cellular material. The supernatant was decanted into another centrifuge tube through two layers of cheese cloth wetted with the extracting solution to prevent floating debris from being transferred. The pellet was resuspended in the same tube with 10 ml of cold extracting solution by first mixing with a glass rod and then by giving a short (approximately 0.5 sec) burst with the Tissumizer homogenizer. The suspension was centrifuged under the same conditions as above and 3-5 g of pellet were obtained. Floating debris were also usually present after the second centrifugation. The resulting supernatant was decanted through two fresh layers of wetted cheese cloth into the same centrifuge tube containing the supernatant of the first centrifugation. After the combined supernatants were mixed with a glass rod and centrifuged at 27,000 x G for 20 min, the pink supernatant (15-20 ml) was decanted through a fresh layer of wetted cheese cloth to remove a floating pelicle of debris. The small pellet (0.05-0.15 g) was transferred with a spatula into another centrifuge tube. When a large number of samples was required in the same trial, both sides of the fish were homogenized and fractionated separately and both pellets combined for the study.

Nomenclature of Whiting *et al.* (1975) was followed when naming the fractions. The pellet obtained from the second 1,000 x G centrifugation was termed sediment; supernatant was the designation adopted for the supernatant obtained in the 27,000 x G centrifugation; whereas the

pellet from this centrifugation was termed lysosomal pellet. The designation of this fraction as lysosomal pellet was not intended to imply that the pellet consisted only of pure lysosomes. Although this pellet contained lysosomes as shown by Whiting et al. (1975), it also contained other subcellular particles including mitochondria, microsomes and others, some of these organelles probably even in higher numbers than the lysosomes themselves. For misuse of this designation, see the review by de Duve (1963).

Enzyme Preparations

The whole lysosomal pellet and about 0.4 g of the sediment were separately resuspended in cold extracting solution. The resulting suspensions were designated sediment preparation and lysosomal preparation, respectively. In both cases, resuspension was accomplished by giving the suspensions a short burst (approximately 0.5 sec) with the Tissumizer. The amount of extracting solution used was a function of the weight of the pellet resuspended. In general, the sediment was resuspended in 4.0 ml whereas the lysosomal pellet was resuspended in 3.0 ml. The supernatant was used with no further treatment.

Immediately following the resuspension, the enzyme preparations were added to the reaction mixture and the incubation started. When three fish were used in the same trial usually about 5.5 hr elapsed

from the time the fish were sacrificed until the incubation period started. In the preparation of rat liver lysosomes de Duve et al. (1955) reported an elapsed time of about 6 hr.

Enzyme Activity Measurement

The procedures and conditions described below were used throughout the present work except where otherwise specifically indicated and are referred to as standard procedures or standard conditions, respectively.

Composition of the Reaction Mixture

The reaction mixture consisted of 0.80 ml buffer, 0.20 ml 15% (w/v) Triton X-100 (octyl phenoxy polyethoxyethanol), 0.25 ml substrate dispersion and 0.25 ml enzyme preparation. In the blanks, buffer was substituted for the substrate dispersion and, after the reaction was stopped, 0.25 ml of the substrate dispersion was added to the reaction mixture. The buffer was 0.1 M citrate-phosphate at pH 4.2-4.3. Final concentration of Triton X-100 (2% w/v) in the reaction mixture was based on the work of Bilinski et al. (1971).

In the preparation of the substrate dispersion, the procedure reported by Hayase and Tappel (1970) was followed. Tridecanoylglycerol (approximately 99% pure) was used as substrate since maximum activity of lysosomal lipase of rat liver was reported with this substrate

(Mahadevan and Tappel, 1968). Sufficient triacylglycerol to give a concentration of 5 mM in the final dispersion was dissolved in a small volume of chloroform. Cold 10% gum arabic solution prepared in the incubation buffer was added and the mixture blended for 1 min with the Tissumizer homogenizer rotating at maximum speed (20,000 rpm). The mixture was kept in ice during homogenization. Chloroform was evaporated by placing the dispersion in a water bath (50°) for 20 min while passing a stream of nitrogen (prepurifyed) through the dispersion. The resulting milky dispersion was used within 24 hr of preparation and was held at 4°.

Soon after resuspension of the pellets, protein determinations were performed on aliquots of the enzyme preparations according to the procedure of Lowry et al. (1951) with absorbance determinations at 600 nm (Whitaker and Bernhard, 1972). No correction for the presence of sucrose or EDTA was made since these substances were present in very small concentrations in the diluted aliquots. Bovine serum albumin was used in the preparation of the standard curve.

The protein concentration in the reaction mixture was usually 1.0-1.2 mg/ml for the supernatant, 0.4-0.6 mg/ml for the sediment and 0.2-0.4 mg/ml for the lysosomal preparation.

Incubation Conditions

Blanks and samples were incubated in duplicate. The reaction

mixture in teflon lined screw capped (16 x 150 mm) test tubes was incubated for 3 hr in a water bath at 37°. Throughout the incubation, the test tubes were held at an angle of approximately 60° and shaken at approximately 200 oscilations per min.

Before starting the reaction, the components of the reaction mixture except the enzyme preparation were incubated in the water bath with no shaking for about 60 min. Besides providing for temperature equilibration this pre-incubation period allowed for changes in the physical state of the substrate, which otherwise would have occurred during the incubation period. In fact, during pre-incubation the reaction mixture lost much of the turbid, milky appearance and became more opalescent and translucid.

The reaction was started by addition of the enzyme preparation. When the incubation time was completed, the reaction was stopped by addition of 2.5 ml of copper reagent (Hayase and Tappel, 1970) followed by mixing for 30 sec in a vortex mixer. The copper reagent consisted of a mixture of three solutions: 1 M 2,2',2" nitrilotriethanol, 1 N acetic acid and 6.45% (w/v) $Cu(NO_3)_2 . 3H_2O$ in a ratio of 9:1:10. This reagent was held at 4° and was used within 7 days of its preparation. Duncombe (1963) reported that aging of this reagent causes erroneous results. Mahadevan *et al.* (1969) however, found the reagent to be stable for up to one week at room temperature.

After the reaction was stopped, 0.25 ml of substrate dispersion

was added to the blanks followed by mixing with the vortex mixer for 10 sec.

Enzyme Assay

The enzyme assay used was a combination of the assay systems reported by Hayase and Tappel (1970) and Mahadevan et al. (1969). The fatty acids liberated in the reaction mixture and later transformed into copper soaps by mixing with the copper reagent were extracted from the reaction mixture with chloroform. Either 7 or 10 ml of chloroform were used depending on the amount of the free fatty acids anticipated. Following addition of chloroform, the tubes were recapped and shaken manually 100 times. After releasing the internal pressure by slightly unscrewing the caps, the phases were allowed to separate at 4° overnight.

About 5 ml of the lower (chloroform) phase were transferred to 15 ml graduated conical glass centrifuge tubes. Once the transfer process was started, any delay in the handling of the samples was avoided to minimize chloroform evaporation. This measure applied to all steps in the enzyme assay procedure described below. The transfer process was accomplished by using long, fine, hypodermic needles (6 in, 20 gauge) attached to 5 ml glass syringes. Individual sets of needles and syringes were used for each sample. The chloroform solution was aspirated without the upper (aqueous) phase removed (Mahadevan et al.,

1969). Care was taken to insure that the needles did not touch the inner wall surface of the tubes where small blue drops of the aqueous (copper) phase sometimes collected. Other measures exercised to avoid contamination of the chloroform solution with copper included: discarding the first few drops of the aspirated solution, wiping the needles before delivering the solution into the recipient tubes and being careful that the needles did not touch the inner walls of the recipient tubes.

To further insure that no contaminant copper was present in the chloroform phase, an additional measure was taken (Mahadevan et al., 1969). About 2 ml of distilled water were added without mixing on top of the chloroform solution in the centrifuge tubes. These were then centrifuged at room temperature at approximately 510 x G for 10 min. Using a long, large diameter needle (6 in, 16 gauge), the water layer was oompletely removed along with some of the chloroform phase.

After centrifugation, a fluffy layer was frequently observed at the interface between the two phases. Duncombe (1964) also observed a similar layer when assaying for free fatty acids in plasma. Sometimes this layer was observed even before centrifugation. If the layer was inadvertidly broken during removal of the water, some of the material went into the chloroform layer causing turbidity. In this case, absorbance readings of the chloroform solution, the basis for the determination of the products of the reaction, would not be reliable. To prevent this from happening, the tip of the needle was carefully

placed at the fluffy layer, which could be completely aspirated along with some water and chloroform phase in one operation. The remaining water was then aspirated along with some additional chloroform phase. Well mastered, this technique was faster and simpler than the one reported by Duncombe (1964).

When the ambient temperature was below 21°-22°, a turbidity usually developed in the chloroform phase upon addition of the water layer. Immersion of the tubes for a few seconds in warm (about 40°) water usually cleared the solution.

After removal of the water phase and the fluffy layer, aliquots of the chloroform solution were transfered into small (13 x 100 mm) test tubes and submitted to a free fatty acid determination. Enzyme activity was measured by determining the amount of fatty acids liberated colorimetrically by the method reported by Mahadevan et al. (1969). One ml of complexing agent solution was added to the 2 ml aliquots of the chloroform solution and the mixture was immediately mixed for about 10 sec in the vortex mixer. Exactly 5 min following the beginning of the addition of the agent to a particular sample, the absorbance of that sample was read at 540 mm in a Perkin Elmer double beam spectrophotometer model 550. As reagent blanks in the spectrophotometer, 2 ml of chloroform substituted for the chloroform solution of the copper soap. The presence of fluffy layer particles was checked by reading the absorbance also at 4 min. A change in absorbance of

more than about 0.020 units between the 4 and 5 min readings was a strong indication of interference. A normal change generally ranged between 0 and 0.008 units with the most common values being between 0.001 and 0.004. The complexing agent was prepared by dissolving 1,5 diphenolcarbohydrazide in methanol (nanograde purity) to give an azide concentration of 0.5% (w/v). This solution was used within 2 hrs of preparation. Reagent grade methanol could not be used since it caused the development of a color in the complexing agent solution. The color was visible as early as 2 hrs after the solution was prepared and the intensity increased with time. This phenomenon did not occur when nanograde methanol was used.

The average absorbance value of the duplicate reaction mixture blanks was subtracted from the average value of the duplicate samples. The blank values represented the lypolytic activity against endogenous lipids present in the enzyme preparation. Samples to which the addition of the enzyme preparation was omitted both during incubation and after the reaction was stopped gave only negligible absorbance values (0.002-0.005). This indicated that the substrate dispersion was essentially free of free fatty acids. The difference between samples and reaction mixture blanks was referred back to a standard curve to estimate the amount of fatty acids liberated. The standard curve was prepared according to Mahadevan et al. (1969).

To obtain the activity of the enzyme preparation under test, the

amount of free fatty acid determined from the standard curve was multiplied by the appropriate dilution factor and the result divided by the amount of protein present in the reaction mixture.

Total activity of a particular experimental unity was the sum of the activities of the three enzyme preparations (sediment, supernatant, and lysosomal) and was expressed in terms of the nanomoles of fatty acids liberated per mg of protein during the incubation period.

RESULTS AND DISCUSSION

Establishment of the Conditions for the Enzyme Assay

Determination of the pH Optimum

The first step in determining the assay conditions for the lateral line muscle lipase activity of rainbow trout was to observe the effect of pH. Two peaks were observed, one at pH 4.2-4.5 and the other at pH 7.8, when the pH of the assay mixture was varied from pH 3.6 to 9.0 (Figure 1). This reproduces almost exactly the results reported by Bilinski et al. (1971) on homogenates of dark lateral line tissue of rainbow trout in spite of the use of a different substrate and assay procedure by those authors. However, this agreement is not completely casual. Besides the use of an identical enzyme source, the concentration of Triton X-100 in the reaction mixture and the type of buffers were identical to that used in the present work.

Purified preparations of rat liver lysosomes were shown to contain a triacylglycerol lipase with optimum pH 5.0-5.2 (Hayase and Tappel, 1970). However, a shift in the peak position to about pH 4.2 was observed when Triton X-100 was added to the reaction mixture at the concentration of 2%, the same as used in the present work. This result does not conform with the findings of Teng and Kaplan (1974) who observed an optimum pH of about 4 in the absence of Triton X-100

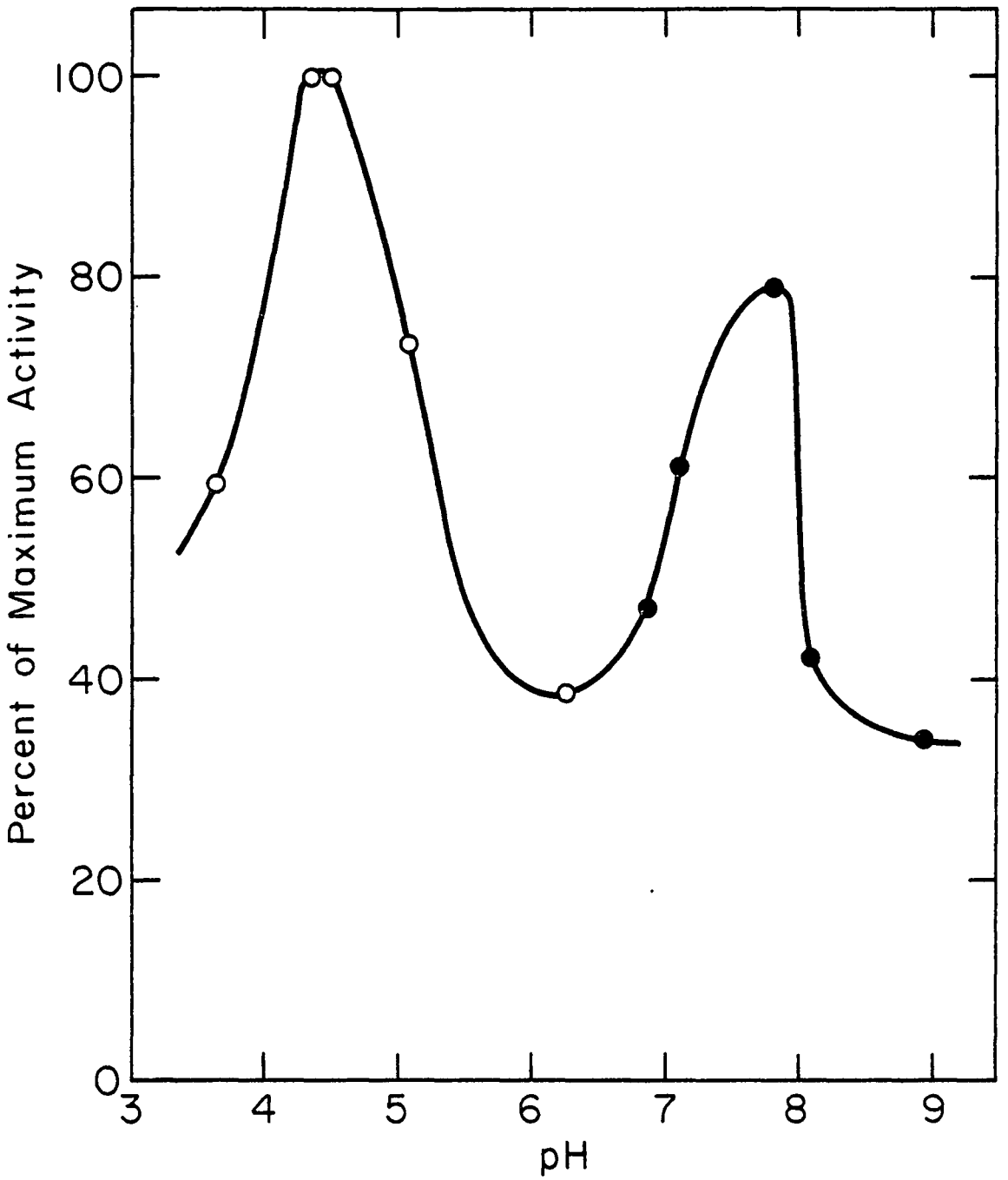

Figure 1. pH-activity curve for the lipase of lysosomal preparation. pH determined at 37° C. Each point represents an average of two replications in duplicate. Each replication corresponds to a different fish. ○ 0.1 M citrate-phosphate buffer, ● 0.1 M Tris-HCl buffer.

for the lysosomal lipase of rat liver. In the work of Hayase and Tappel (1970) a reduction in lipase activity accompanied the shift in pH optimum. This is in contrast with the results obtained in the present work and presented below in the section dedicated to the effects of Triton X-100 on the lipase activity.

Working with purified preparations of rat liver and kidney lysosomes, Mahadevan and Tappel (1968) obtained a single peak at pH 4.2. When these authors assayed microsomal and supernatant fractions, maximum activity was observed above pH 7. Similar results for the microsomes were also reported by Hayase and Tappel (1970). Lipolytic activity against triacylglycerol in the alkaline range has also been observed in the mitochondria by Waite and van Deenen (1967) and in mitochondria and microsomes by Carter (1967).

The lysosomal preparation used in the present work, not being purified, contained other classes of organelles including microsomes and mitochondria. Therefore, the peak obtained in the alkaline range could probably have been due to the lipase activity in the contaminant particles. Another possibility, which does not exclude the first, is the monoacylglycerol activity contained in the lysosomes. A single peak of monoacylglycerol activity in the alkaline range was found by Hayase and Tappel (1970) when 1-monodecanoylglycerol was exposed to purified preparations of rat liver lysosomes. Activity against monoacylglycerol was also observed by Fowler and de Duve (1969) in

purified preparations of rat liver lysosomes. These authors reported that while only the acid activity was observed when triacylglycerol was used as substrate, two peaks (one at pH 4.1 and another "at or above pH 5.7") were observed when 1-monopalmitoylglycerol was the substrate.

Whether the same or different enzymes are responsible for the activity against both tri- and monoacylglycerols can only be ascertained by running purified preparations of the lysosomal lipase. Such enzyme has been purified to a great extent from rat liver by Teng and Kaplan (1974). Unfortunately, their pH-activity curve covered only the acid range.

Based on the above considerations, a pH of 4.2-4.3 was chosen for the standard reaction mixture to assay for triacylglycerol lipase.

Effect of Incubation Time on the Activity

The enzyme activity was found to be proportional to the incubation time for a period of at least 4 hr (Figure 2). However, the plot suggests that the line does not go through the origin. Separate trials were run using different fish in each. The agreement of the results from the separate trials can be appreciated in Figure 2. The coefficient of determination for the straight line was found to be 0.999.

Since the linear portion of the curve shown in Figure 2 extended from 1 to at least 4 hr, any incubation period between those could be chosen for the standard assay. The lysosomal fraction and, in some

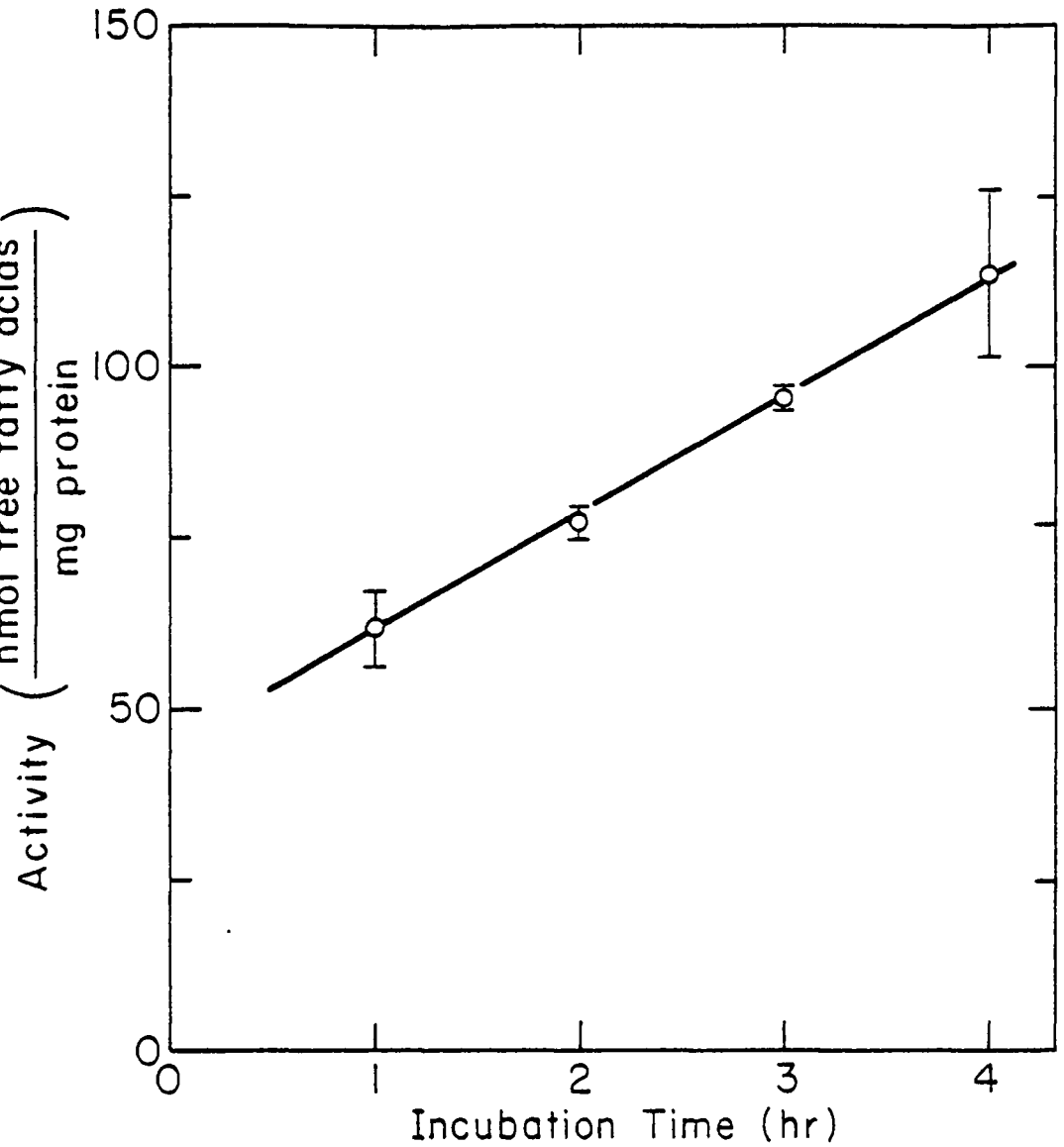

Figure 2. Effect of incubation time on the activity of the lipase of lysosomal preparation. Each point represents an average ± standard error of three replications in duplicate except for the 4-hr incubation period (two replications). Each replication corresponds to a different fish.

cases, also the supernatant fraction usually yielded meaningful results in 1 hr. However, the sediment fraction required 3 hr of incubation due to low activity. For this reason, 3 hr was chosen as the incubation time for the standard assay. The need for such an apparent long incubation period was not only due to the usually low activity of muscle lysosomal enzymes but also to the fact that a comparison among the activities of the three fractions (supernatant, sediment and lysosomal) ran in parallel was required in many of the experiments.

Long incubation periods in the assay of muscle lysosomal enzymes are not uncommon. Assay for fish muscle cathepsins and α-glucosidase have included 2 hr incubation periods (Whiting et al., 1975; Bird et al., 1969). A 4 hr incubation period was reported for the rat liver lysosomal phospholipase (Mellors and Tappel, 1967).

Lipolytic enzymes are also noted by their requirement for long incubation times. For these enzymes, incubation may vary from 2 to 4 hr, longer times being required by the phospholipases. Besides the rat liver lysosomal phospholipase mentioned, other examples include a phospholipase A (Jonas and Bilinski, 1967) and a lecithinase (Bilinski and Jonas, 1966), both assayed in homogenates of rainbow trout lateral line muscle. The incubation times were 4 and 2 hr, respectively.

An incubation period of only 1 hr was reported by Bilinski et al. (1971) for a lysosomal lipase similar in many aspects to the one dealt

with in the present work. This shorter incubation time was possible by the use of labelled substrate. This technique enabled the exclusion of the activity on endogenous lipids. However, the assay system used was not considered satisfactory for the present work due to the very low levels of activity reported (less than 1 nanomol of fatty acid liberated per 350 mg of homogenate in 1 hr of incubation).

Effect of Protein Concentration on the Activity

To study the effect of protein concentration on the activity, dilutions of the same enzyme preparations were assayed. The dilutions were prepared by replacing a certain volume of the enzyme preparation by buffer in the reaction mixture. All samples had the same volume of enzyme preparation (diluted or not) as indicated in the standard assay (0.25 ml).

The enzyme activity was found to be proportional to the protein concentration over the range studied (Figure 3). A very slight deviation from the linearity was observed in both trials when the curves were extrapolated to the origin.

A similar deviation in the range up to 0.3 μg/ml protein can also be observed in the results reported by Teng and Kaplan (1974) in the study of a purified lysosomal lipase of rat liver. The results of Mahadevan and Tappel (1968) for the same but non purified enzyme do not show any such deviation at the lower protein concentrations.

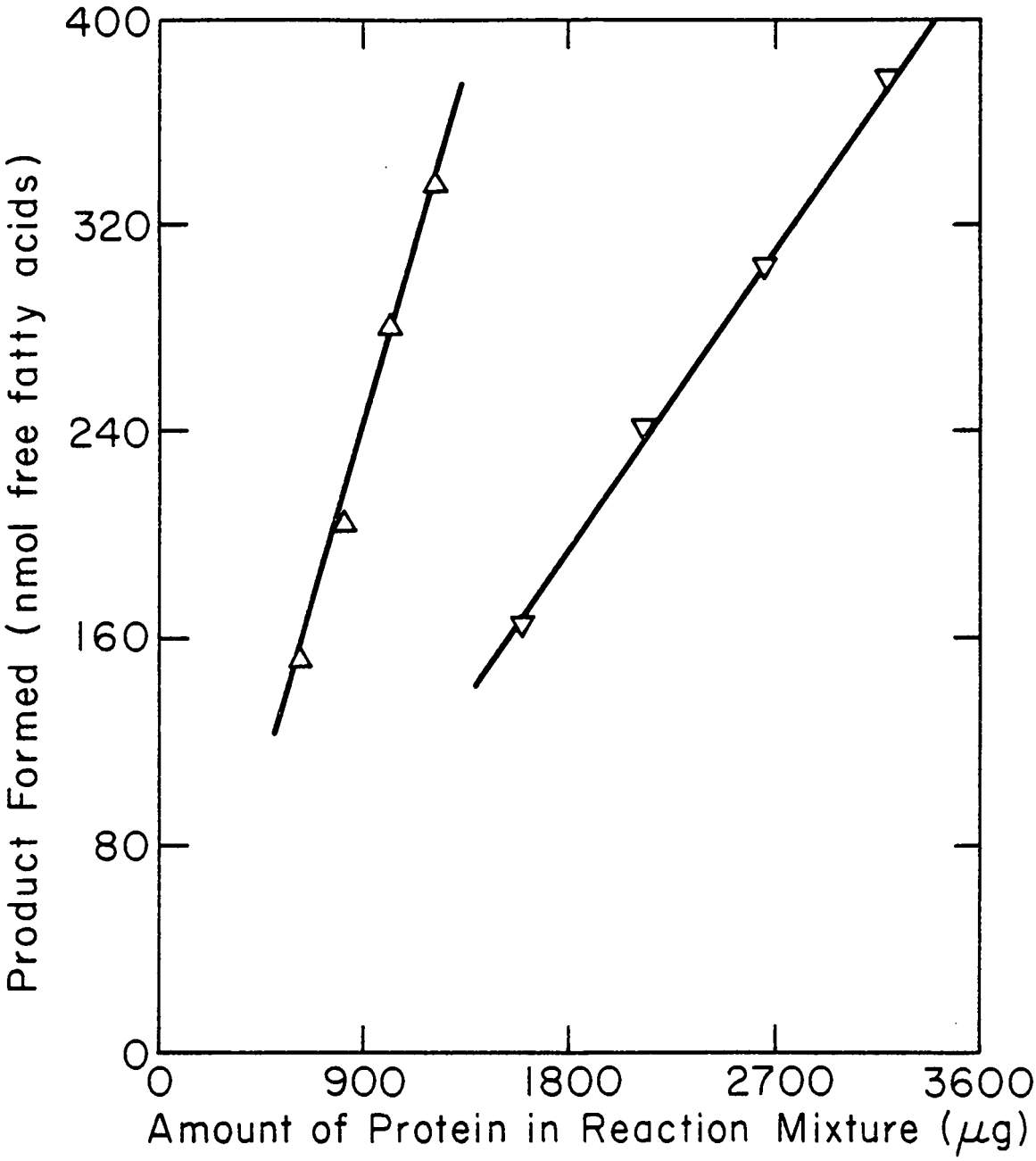

Figure 3. Effect of protein concentration on product formation by the lipase of lysosomal preparation. Each line corresponds to a different fish and each point represents average of duplicates.

However, in contrast to the results shown in Figure 3, these workers found a deviation from linearity at the higher (above 1.8 mg) levels of protein when tridecanoylglycerol was used as substrate. No such inhibitory effect was observed when trimyristoylglycerol was the substrate.

Effect of Triton X-100 on the Activity

As opposed to the determination of soluble or free enzyme activity, the lysosomes must be disrupted prior to the measurement of the total activity. In a study of the effectiveness of different disrupting procedures, Wattiaux and de Duve (1956) found the addition of Triton X-100 to the reaction mixture at a concentration of 0.1% (v/v) to be more advantageous than other methods studied. These methods included blending for 3 min, twelve freezing-thawing cycles and incubation at pH 5 for 3 hr at 37°. Wattiaux and de Duve (1956) claimed that the advantages for treatment of the preparation with Triton X-100 were: a) required minimal quantities of material, b) required no preliminary treatment of the granules and, c) insured complete activation without denaturation or inhibition of the enzymes.

Triton X-100 concentrations in the range of 0.01% to over 5% (w/v) have been used in studies of lysosomal enzymes. Following are some examples of studies of rat liver lysosomal lipase. A combination of mechanical disruption with 2% Triton X-100 in the reaction mixture

was used by Teng and Kaplan (1974). A high concentration of Triton X-100, about 5.3%, was found by Mahadevan and Tappel (1968) to be required for maximum hydrolysis whereas very little activity was found at the 0.13% level. In the absence of Triton X-100, the activity against triacylglycerols could not be detected by Fowler and de Duve (1969). However, in the presence of 5% (w/v), these authors observed a strong stimulation of the triacylglycerol lipase activity and a considerable increase of the diacylglycerol activity whereas the activity against monoacylglycerols was greatly reduced. On the other hand, results of Hayase and Tappel (1970) showed a reduction of more than 85% in the triacylglycerol activity in presence of 2% (w/v) Triton X-100 when compared to no detergent present. Triton X-100 has been found to also inhibit the activity of the triacylglycerol lipase of the adipose tissue (Gorin and Shafrir, 1964), a non lysosomal enzyme.

Concentrations as low as 0.01% and 0.02% have been used in the study of the beef lysosomal enzymes cathepsin and β-glucuronidase respectively (Moeller et al., 1976). Total activity of cathepsins of fish muscle has been determined in presence of 0.1% (Reddi et al., 1972) and 0.2% (Whiting et al., 1975) Triton X-100.

Similarly to the results reported by Hayase and Tappel (1970), Bilinski et al. (1971) also found the effect of Triton X-100 on the lipase activity to be dependent on pH. Studying a lysosomal lipase of fish muscle, Bilinski et al. (1971) observed maximum activity in the

alkaline range in absence of Triton X-100 whereas a very limited activity was found at pH 3.5-5. The presence of 0.2% Triton X-100 in the reaction mixture resulted in the appearance of a peak in the acid range and a reduction of the activity above pH 7. When the detergent concentration was further increased to 2%, the highest lipolytic activity was observed at pH 4-4.5 whereas an inhibition of 80% was noted in the alkaline activity. Increasing the concentration to 8% did not result in a further increase of the activity. It was concluded that a concentration of 2% was sufficient to provide a complete release of the enzyme. A lower level of Triton X-100, 0.2%, was found by Bird et al. (1969) to completely release four other lysosomal hydrolases of fish muscle.

The enzyme studied by Bilinski et al. (1971) was a lysosomal lipase of the lateral line muscle tissue of rainbow trout. Since that enzyme has many similarities to the one dealt with in the present work, a level of 2% (w/v) was chosen to be tested regarding a possible inclusion in the standard reaction mixture. The activity of samples containing 2% (w/v) Triton X-100 in the reaction mixture was compared to that of samples in which the detergent was replaced by buffer. The results are shown in Figure 4a. Statistical analysis revealed a significantly higher activity in the samples containing Triton X-100 (total activity, supernatant and lysosomal fraction: $p \leq 0.01$; sediment fraction: $p \leq 0.05$). For this reason, 2% (w/v) Triton X-100 was adopted for the

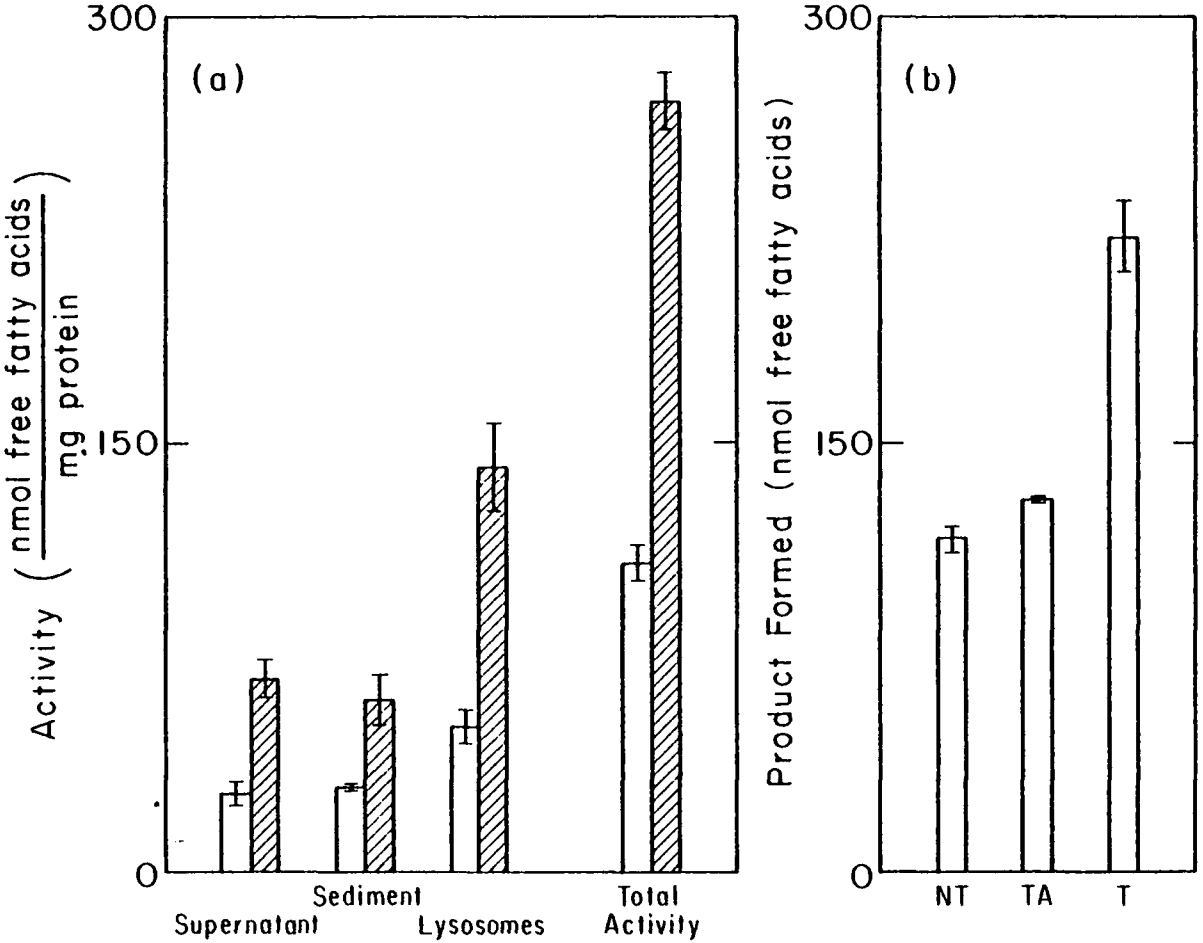

Figure 4. Effect of Triton X-100 on the lipase activity. Average ± standard error of three replications in duplicate. Each replication corresponds to a different fish. a) ▨ 2% Triton X-100 in the reaction mixture; □ no Triton X-100. b) NT: Triton X-100 replaced by same volume of buffer during incubation. TA: Same as NT except Triton X-100 added after reaction was stopped. T: Standard conditions: 2% (w/v) Triton X-100 present during incubation. Same protein concentration in each of the three replications. Only lysosomal preparations were assayed.

standard reaction mixture.

Since the controls in the above experiment had no Triton X-100, there was the possibility that the observed increase in activity could have been due to interference caused by the detergent on the activity values. Results shown in Figure 4b suggest that this was not the case. Activities of assays where either no Triton X-100 was added or the Triton X-100 was added after incubation were similar, whereas the results where Triton X-100 was present during incubation were significantly higher ($p \leq 0.01$).

Even though the principal use of Triton X-100 in the study of lysosomal enzymes is to serve as a disruption agent, the results suggest that this was not the only role played by the detergent under the assay conditions of the present work. When free (soluble) activity of lysosomal enzymes is to be determined, conditions that prevent lysosomal membrane disruption must be met. The assay should be run under adequate osmotic protection for the lysosomes and at a pH higher than 5 for a time no longer than 10 min when the incubation temperature is 37° (de Duve, 1963). These conditions were exceeded in the present work since total activity was the parameter sought. In fact, incubation at pH 5 for 3 hr at 37° was found by Wattiaux and de Duve (1956) to be as effective as treatment with Triton X-100 for the complete release of five hydrolases from lysosomes. Thus, it would be expected that the lysosomes were all disrupted under the assay

conditions of the present work, even in the absence of Triton X-100. Therefore, the dramatic increase in activity in the Triton treated samples cannot be explained solely on the basis of the lysosomal disrupting properties of this detergent. Since interference in the colorimetric determination of free fatty acids is excluded as shown in the results displayed in Figure 4b, other roles played by Triton X-100 remain to be determined. Alterations of some of the physico-chemical properties of the assay system seems to be an attractive possibility. In this respect, speculations could be directed to changes related to an improvement in the substrate emulsification, a better extraction from the reaction mixture of the liberated fatty acids or also a better separation of the chloroform and water phases. This latter possibility is hinted by the observation that, in absence of Triton X-100, the separation of the phases was always more difficult. The shaking operation that preceeds the phase separation step had to be much gentler in absence of Triton X-100 if a complete separation was to be accomplished in a reasonable amount of time.

Effect of Fasting of the Fish on the Activity

In the study of a lysosomal phospholipase of rat liver, the animals were not fed for 24 hr before being sacrificed (Mellors and Tappel, 1967). Fish were also maintained without food for about 20 hr before sacrifice in studies of both the alkaline lipase (Bilinski and Lau, 1969)

and of the lysosomal lipase (Bilinski et al., 1971) of the dark lateral line tissue of rainbow trout. In this tissue, the overall lipolytic activity was shown to increase during starvation of the fish (Bilinski, 1969).

To determine if fasting would have an influence on the lysosomal lipase activity, enzyme preparations obtained from fish maintained without food for different periods were studied. The results are shown in Figure 5. Increasingly higher total activities resulted from longer fasting periods. The total activity obtained after the first 24 hr of fasting was found to be significantly higher ($p \leq 0.05$) than that of non-fasted fish. The total activity at the 72-hr period was also found to be significantly higher than those of both fish fasted for 24 hr ($p \leq 0.05$) and non-fasted fish ($p \leq 0.01$).

Non significant differences ($p \geq 0.05$) were observed between the activity of the non-fasted fish and that of the 24-hr fasted fish in both the supernatant and the sediment fractions. On the other hand, the lipase activity of the lysosomal fraction experienced the largest increase in the same period (significant at $p \leq 0.05$) which indicates that this fraction was the major contributor to the large increase in the total activity observed in the first 24 hr of fasting.

Comparing the activities of the 72- and the 24-hr fasted fish a significant increase ($p \leq 0.01$) with time was observed in both the supernatant and the sediment fractions whereas the activity of the

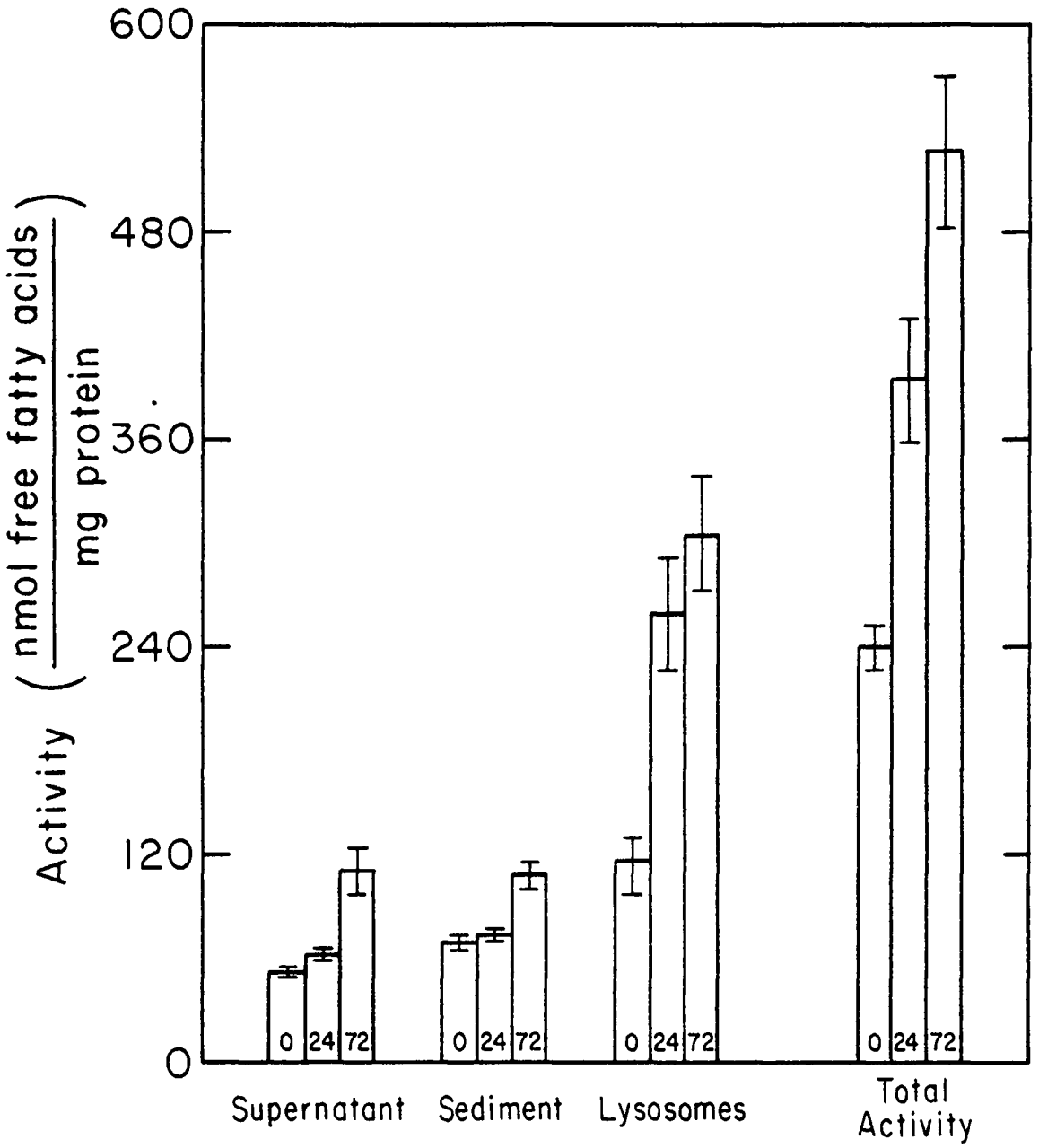

Figure 5. Effect of fasting of the fish on the lipase activity. Fasting time (hr): number of fish analyzed: 0:3, 24:12, 72:11. Zero time: fish fed $\frac{1}{2}$ hr before sacrifice. Average ± standard error of three replications in duplicate. Each replication corresponds to a different fish.

lysosomal fraction showed a non significant change ($p \geq 0.05$) in that period. Therefore, both the sediment and the supernatant fractions were the major contributors to the increase in the total activity between 24 and 72 hr of fasting.

When the lipase activity of the non-fasted fish and that of the 72-hr fasted animals were compared, the latter was found to be significantly higher in all the three fractions (supernatant and sediment: $p \leq 0.05$; lysosomal: $p \leq 0.01$).

Since the total activity was higher in the fasted fish, it was decided to adopt as a standard procedure to use fish fasted for either 24 or 72 hr. Caution was exercised, however, that in all trials of any single experiment all fish utilized were fasted for the same period of time.

Distribution of Activity Among the Fractions

As a test for the effectiveness of the procedure employed in the preparation of the enzyme suspension, the distribution of activity among the fractions was examined. Relative proportions of 60.6, 21.5, and 17.9% of the total activity were found in the lysosomal, sediment and supernatant fractions, respectively (Figure 6). A high activity in the lysosomal fraction indicated the mildness of the homogenization method, the effectiveness of the fractionation procedure and the adequateness of the osmotic protection provided to the lysosomes.

Presence of activity in the supernatant and sediment fractions

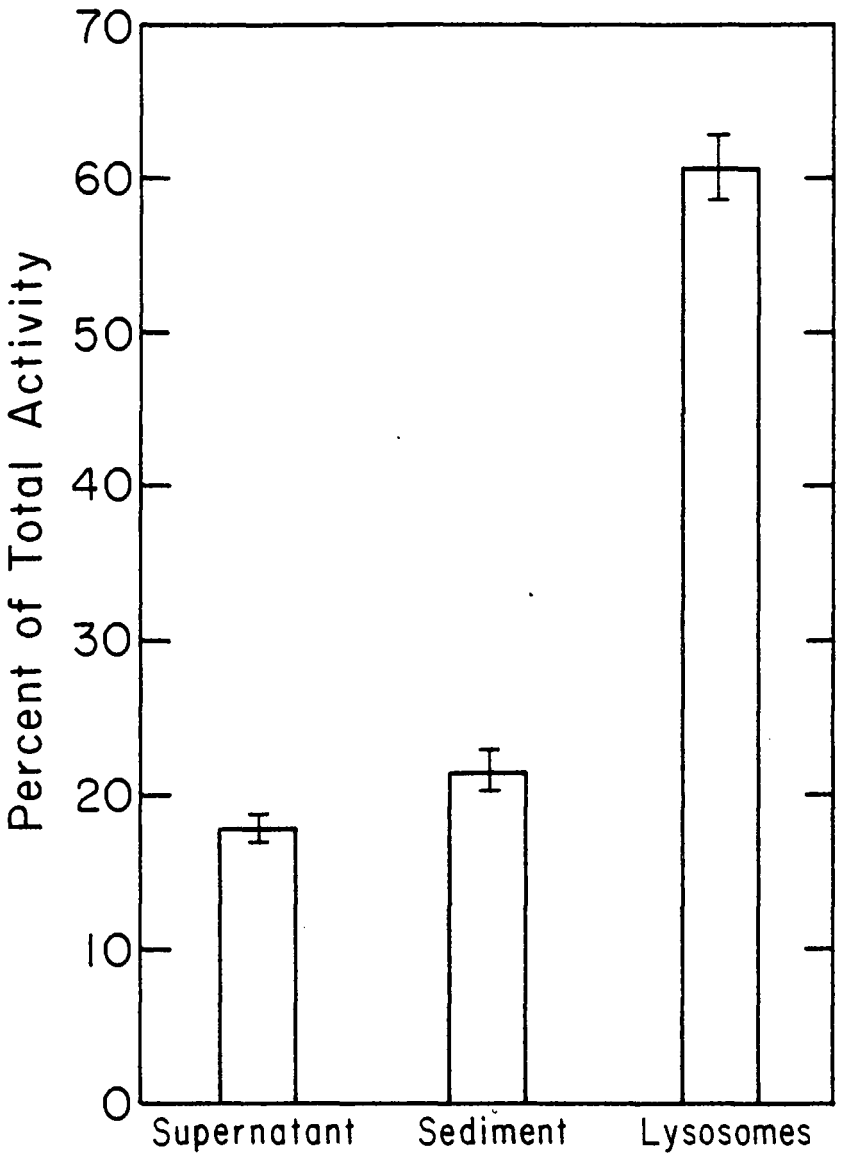

Figure 6. Distribution of the lipase activity among the fractions. Average ± standard error of 18 replications in duplicate. Each replication corresponds to a different fish. Total activity: 372.2 ± 28.7 nanomoles free fatty acid/mg protein.

was not surprising considering the unavoidable damage to the lysosomal membrane caused by the homogenization, fractionation and handling involved. Verity et al. (1968), for instance, observed 39.4-51% of the total activity of three lysosomal hydrolases to be in the supernatant fraction.

Fractionation and homogenization procedures used in the present work were, with a few modifications, those reported by Whiting et al. (1975) in a study of lysosomal cathepsins of rainbow trout white muscle. However, a much lower proportion of the cathepsin total activity was found in the lysosomal fraction in that study. The relative proportions were 23, 54 and 23% for the lysosomal, sediment and supernatant fractions, respectively. Possible factors that could have accounted for this would include the above mentioned differences in the nature of both the enzyme studied and the tissue that served as source of the enzyme. Another factor could be related to the modifications introduced in the homogenization procedure and in the preparation of the suspension medium. Before homogenization with the Tissumizer, Whiting et al. (1975) used a hand extruder to grind the muscle. This step was omitted in the present work. A 2 sec burst with the Tissumizer was employed by Whiting et al. (1975) to disrupt white muscle of rainbow trout. To homogenize the dark muscle used in the present work, three 2 sec bursts had to be used. Caldwell and Grosjean (1971) found a higher activity in the lysosome-rich pellet when

chicken skeletal muscle was disrupted by a single although longer burst as compared to several short bursts. De Duve (1963) reported that slight changes in the homogenization procedure resulted in large alterations in the ratio of sedimentable to non-sedimentable activity.

Following the classical work of de Duve et al. (1955) on lysosome isolation from rat liver, most of the work on lysosomes extraction utilizes unbuffered 0.25 M sucrose solutions containing 1 mM EDTA. However, as pointed out by de Duve (1963), this may not have been the best suspension medium for other tissues. Therefore, modifications in the composition of this solution have been employed by some. The work of Caldwell and Grosjean (1971) showed that the optimal suspension medium for lysosomes isolation from chicken skeletal muscle was a phosphate buffer pH 7.2 containing 0.44 M sucrose and 1 mM EDTA. Bird and colleagues extracted lysosomes from rat liver and muscle (Bird et al., 1968), from goldfish muscle (Bird et al., 1969) and from rainbow trout muscle (Milanesi and Bird, 1972) with unbuffered 0.25 M sucrose containing 1 mM EDTA solutions whose pH had been adjusted to 7.2. Ono (1970, 1971) used an extracting solution consisting of 0.25 M sucrose and 0.175 M potassium chloride to isolate lysosomes from bovine skeletal muscle.

Whiting et al. (1975) found that a solution of 0.25 M sucrose - 0.175 potassium chloride in 0.001 M EDTA resulted in better yields of rainbow trout muscle lysosomes than the solution without potassium

chloride. Sucrose concentrations of 0.25 and 0.50 M were also compared by these authors but the higher concentration elevated the lysosomal activity to only 15% of the total activity as compared to the value of 13.5% for 0.25 M sucrose solution. Based on the above information, it was decided to use the extracting solution of Whiting et al. (1975) but with the pH adjusted to 7.2. This pH adjustment could be another reason for the higher lipase activity in the lysosomal fraction obtained in the present work as compared to the work of Whiting et al. (1975). Prior to the pH adjustment, the solution had a pH of approximately 5 and de Duve (1963) reported that the lysosomal membrane was unstable at pH values below 5.

Redistribution of the Lipase Activity Among the Fractions Following Low Temperature Treatments

Several investigators reported on the release of acid hydrolases from lysosomes in muscle foods held at above freezing low temperatures (for references, see section on the effect of storage on ice). However, lipase was not included in the experiments.

Having no previous knowledge of how the distribution of the lysosomal lipase of fish muscle would be affected by the autolysis process, the fish used in the experiments described below were submitted to the respective treatments soon after sacrifice. This is not the case in commercial operations where the fish is unavoidably

held on ice usually for up to several days before processing.

Experiments described below were designed to determine if low temperature treatments such as those commonly used in the fish processing industry would have an effect on the release of the lipase from the lysosomes. A treatment was considered to have an effect on the release of lipase if a decrease in the activity of the lysosomal fraction with a correspondent increase in the activity of the supernatant and/or sediment fractions resulted from the treatment.

A fillet from one side of each fish was cut soon after the animal was sacrificed. The fillets were packed in heat sealable plastic bags which were vacuumized, sealed and given the treatment intended. To serve as controls, unless otherwise specifically indicated, the dark lateral line muscle of the other side of the fish was excised immediately after the sacrifice and enzyme preparations obtained and assayed.

Where thawing was involved, this was accomplished by submerging the sealed bags in running tap water (16-18^{o}) for 10-15 min. The importance of using a common rate of thawing for all treatments was shown by Lugovoi and Kravchenko (1976). Those investigators noticed a larger release of enzymes from frozen lysosomes with longer thawing times.

To determine the effect of a treatment on the release of lipase, the percentage of the total activity of the treated samples and that of

their correspondent controls were compared. The results were analyzed statistically by the paired t test (Ingram, 1974). This approach was similar to that adopted by Moeller et al. (1976) in a study of the effect of high temperature conditioning of beef on the release of lysosomal enzymes.

Effect of Storage on Ice

Several investigators have been able to detect the release of lysosomal enzymes during the conditioning or aging period at 4° to which meat is commonly submitted for tenderization purposes. Ono (1971) found the release of β-glucuronidase, β-galactosidase and acid ribonuclease to be almost complete at the fourth day of the aging period. Dutson and Lawrie (1974) also detected the release of β-glucuronidase. Eino and Stanley (1973) noticed an increase in the proteolysis up to 6 days post-mortem and suggested that the cause was a breakdown of lysosomal membrane with a resulting release of cathepsins. On the other hand, Valin (1970) observed only a small rate of liberation of cathepsin and β-glucoronidase until the eighth day of the aging period.

In rainbow trout white muscle, Whiting et al. (1975) reported a considerable decrease of cathepsin activity in the lysosomal pellet with a concurrent increase in the supernatant fraction during aging of the muscle stored both at 4° and 15°.

Since fish is normally held on ice for several days prior to processing, an experiment was undertaken to determine if lipase was released from lysosomes during storage on ice.

The plastic bags containing the fillets were packed in ice and kept at an air temperature of 4° for 7 days. From time to time, the water from the melting ice was drained and the ice replenished to the original level. At the end of the storage period, the lateral line muscle was excised and the lipase in the different fractions assayed.

The results are shown in Table 1. The difference in the percentage of total activity between controls and treated samples was not significant ($p \geq 0.05$) in all fractions (paired t test). These results show that the lipase was not released from the lysosomes during the 7 day storage period. Acid hydrolases have been shown not to be released concomitantly from the lysosomes. During beef aging, Valin (1970) observed a pattern of cathepsin D release different from that presented by β-glucuronidase. While the release of cathepsin D proceeded through the rigor mortis period and later decreased, the release of β-glucuronidase started after rigor was resolved. In the present experiment, rigor was resolved long before the storage period was over (Whiting et al., 1975). Therefore, any comparison between the lack of release of lipase and the pattern exhibited by the β-glucuronidase in the experiments of Valin (1970) is excluded.

One possible explanation for the observed absence of release of

Table 1. Effect of storage on ice on the distribution of the lipase activity.

Nature of the sample[a]	Percent of total activity[b]		
	S	D	L
C	17.5 ± 0.3	17.1 ± 1.1	65.4 ± 0.9
T	16.0 ± 1.2[c]	16.0 ± 1.1[c]	68.0 ± 0.1[c]

(a) C (controls) and T (treated samples) are from preparations obtained from opposite sides of the same fish.

(b) Values in each line correspond to the average ± standard error of two replications in duplicate. Each replication corresponds to a different fish. Fractions: S (supernatant), D (sediment), L (lysosomal).

(c) Not significantly different from correspondent control by paired t test ($p \geq 0.05$).

the lipase could be that the enzyme remained bound to the damaged lysosomal membrane, sedimented with it and, though not necessarily consequently, remained inactive toward the added substrate. It has been shown that the various lysosomal enzymes were not released at the same time and with the same ease when the organelles were disrupted (Stagni and de Bernard, 1968; Romeo et al., 1966; Caldwell and Grosjean, 1971; Sawant et al., 1964a,c). Sawant et al. (1964c) found that some enzymes remain associated with the particulate fraction even after extensive damage to the lysosomal membrane. These researchers submitted lysosomes to 10 freezing-thawing cycles and found that, whereas arylsulphatase and β-glucuronidase were solubilized by the treatment, 27 and 45% of the total activities of ribonuclease and acid phosphatase, respectively, remained in the particulate fraction. Some lysosomal enzymes such as acid phosphatase have been reported to be available to act on added substrates even when the enzyme is sedimentable with the damaged lysosomes (Berthet et al., 1951). Thus, the reason for the observed absence of the release of lipase remains to be determined.

Effect of Temperature Fluctuation During Frozen Storage

Taylor (1932) expressed the opinion that maintenance of constant temperatures during storage of frozen fish was of great importance. According to this author, fluctuating temperatures of the fish, and not

necessarily only of the freezer, would cause ice crystals to grow and the same adverse effects of slow freezing would be observed in a fish frozen at an adequate freezing rate. The suggestion was made that temperature fluctuation during the storage would also cause a gradual aggregation of the proteins with a resulting increase in the amount of separable fluid upon thawing (drip) and a tougher texture.

On the other hand, Pottinger (1951) intentionally fluctuated the temperature of frozen fish fillets between -17.8° and -9.4° and found no adverse effects on the quality of the product as judged by the results of taste panels, visual examinations and other physical measurements. This author suggested that the average storage temperature would be more important in determining the quality of the stored product than the range of fluctuating temperatures.

To satisfy a request made by local fish processors that temperatures encountered during transport of frozen fish be as low as those of the warehouses, the Canadian railways asked for proof of the necessity of such a demand. Investigators of the Fisheries Research Board of Canada concluded that maintenance of a constant temperature was important to fish quality (Dyer et al., 1957). Frozen cod fillets, which were submitted to 2 week storage periods at -9.4° after previous and subsequent storage at -17.8°, were found to deteriorate more rapidly than fillets held constantly at -17.8°

Besides transport, other causes of temperature fluctuation

encountered under commercial conditions include the use of storage rooms for freezing, overloading of refrigeration equipment, power failure, equipment breakdown, improper setting of control devices and the transfer of frozen products from one storage room to another (Pottinger, 1951).

The experiment described below was designed to determine if the release of the lysosomal lipase would be enhanced by fluctuating temperatures during frozen storage of fish.

To serve as controls, fillets obtained from one side of the fish were packed in plastic bags in the same manner as the treated fillets. Both the controls and the treated samples were frozen in a freezer at -23°. While the controls remained at this constant temperature throughout the experiment, the corresponding fillets taken from the other side of the fish were submitted to artificially induced temperature fluctuations. This was accomplished by using two additional freezers, one at -11° and the other at -40°. The fillets were moved to the various freezers to provide for the desired temperature fluctuations. The time of transfering from one freezer to another was minimized (30-60 sec). After 4 days of storage, both the controls and the samples were thawed and enzyme preparations were obtained and assayed.

Taylor (1932) has stated that "the only temperature fluctuation that has any effect on the fish is that of the fish itself" and not that of the air in the freezer. The temperature profile of the fillets (Figure 7) as

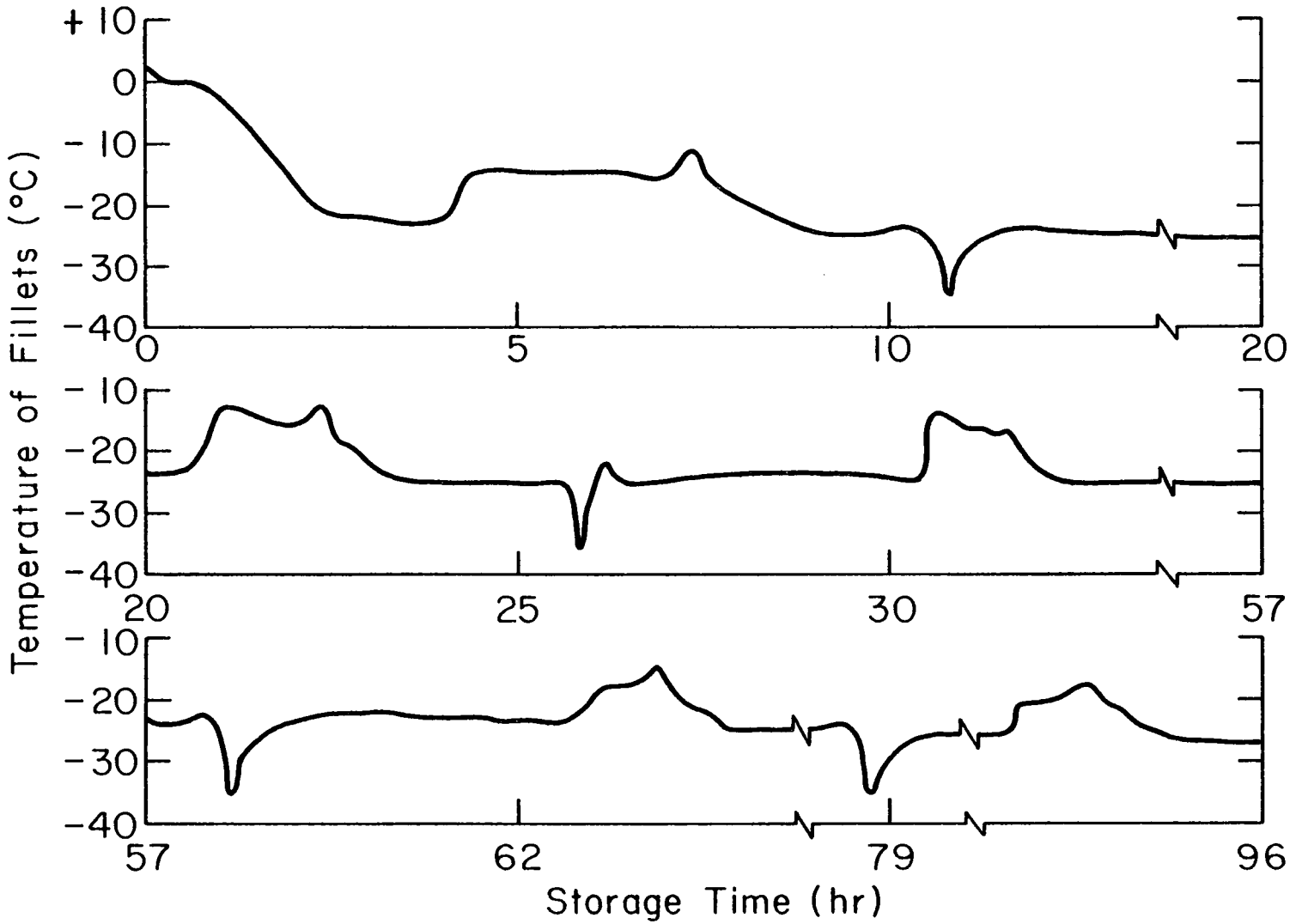

Figure 7. Temperature fluctuation of the fillets.

well as of the freezers were monitored by means of thermocouples connected to a tele-thermometer (Yellow Springs Instruments, model 42SF, Yellow Springs, Ohio). One thermocouple was used to monitor the air temperature of the freezers while another was used to monitor the temperature of the fillets. Assuming identical temperature variation among the fillets, the temperature profile of only one of the fillets was monitored. So that such an assumption would be valid, all fillets (controls and treated samples) had about the same thickness and were stored in the same area of the freezer. From Figure 7 it can be seen that the temperature fluctuated between about -10^{o} to -35^{o}.

Activity of the treated samples was compared with that of the correspondent controls and the results statistically analyzed by the paired t test (Table 2). While the sediment fraction showed no significant change ($p \geq 0.05$), a significant decrease ($p \leq 0.01$) in the activity of the lysosomal fraction was accompanied by a significant increase ($p \leq 0.01$) in the activity of the supernatant. These results show that drastic fluctuations in the storage temperature of frozen fish should be avoided if the release of the lipase from the lysosomes is to be prevented.

Effect of Freezing Rate

Although no significant release of the lysosomal enzymes studied was observed by Valin (1970) in aging meat, a simple freezing-thawing

Table 2. Effect of storage temperature fluctuation on the distribution of the lipase activity.

Nature of the sample[a]	Percent of total activity[b]		
	S	D	L
C	20.1 ± 1.7	21.9 ± 1.4	58.0 ± 3.0
T	33.6 ± 0.6[c]	23.4 ± 1.2	43.0 ± 1.6[c]

(a) C (controls) and T (treated samples) values are from preparations obtained from opposite sides of the same fish.

(b) Values in each line correspond to the average ± standar error of three replications in duplicate. Each replication corresponds to a different fish. Fractions: S (supernatant), D (sediment), L (lysosomal).

(c) Significantly different from correspondent control by paired t test ($p \leq 0.01$).

operation was reported to result in an increase of more than 50 and 30% in the activities of liberated cathepsin and β-glucuronidase, respectively.

Warrier et al. (1972) were not able to detect any significant release of five lysosomal hydrolases in fish muscle after freezing. The enzymes studied were arylsulphatase, β-glucuronidase, acid phosphatase, cathepsin and acid ribonuclease. However, those investigators observed increased activities of the enzymes in the drip, the liquid that flows spontaneously from the flesh during thawing. This indicates that freezing probably had an effect on the liberation of the hydrolases from the lysosomes. Difficulties intrinsic to the study of muscle lysosomes were probably the reason for the failure to detect the release of the enzymes. Problems of obtaining a homogeneously disrupted muscle without extensive damage to the intact lysosomes were admitedly encountered by those authors.

Rhodes and Lea (1961) and Rhodes (1961) reported that the onset of detectable lipase activity of lamb liver occurred after 2 days of storage at 15°. This lag period was eliminated when the tissue was frozen and autolytic degradation of the lipids in the liver was accelerated three to four times. Activity on endogenous triacylglycerols was more pronounced than on endogenous phospholipids. Rhodes (1961) suspected that the elimination of the lag phase by freezing could have been due to the release of the hydrolases from lysosomes.

Recently, Lugovoi et al. (1977) studied the release of several enzymes from isolated rat liver lysosomes. Whereas incubation at 0-4° caused only less than 6% of the enzymes to be released, more than 50% was liberated upon freezing to -30°.

The significance of the rate at which fish is frozen on the quality of the product is a controversial question. Reviewing the subject, Cutting and Spencer (1968) concluded that "it is generally accepted that slowly frozen fish suffers more denaturation than quickly frozen fish" and pointed out that British authorities established specifications for the rates at which fish should be frozen. The same authors, however, referred to some studies where the freezing rate of fish was concluded to be less important to quality than previously recognized.

Partmann (1975) also reviewed the influence of the rate of freezing on the quality of frozen foods and similarly indicated the divergence of opinions among the researchers about the subject. However, based on the references consulted, Partmann (1975) mentioned that "it is well established that the passage through the freezing zone should take no more than 30 min if the consequences of accelerated enzymic reactions are to be minimized."

The effect of the freezing rate on the release of the lipase from the lysosomes in muscle foods has not been studied. The experiments described below were intended to examine this subject in fish muscle.

Bags containing the fillets to be treated were divided into three

groups. One group was placed in a freezer room at -40° and the change in temperature of the fillets was monitored with a telethermometer as described above. The time taken for the temperature of the fillets to pass from -1° to -5° was recorded (thermal arrest time). Immediately upon reaching -5°, the fillets were transferred to a freezer room at -23°. Another group was placed in a freezer at -11° and was treated as the -40° group. A third group was placed in the freezer at -23° and therefore did not have to be transferred when the temperature reached -5°. The time required for the transfers to the -23° freezer was 30-60 sec. Fennema (1975) indicated that not only the freezing rate but also the final temperature of freezing was important when the activity of enzymes in frozen systems was studied.

Thermal arrest times were 6, 56 and 193 min, respectively, for the samples frozen in the -40°, -23° and -11° freezers and the corresponding freezing rates are hereafter referred to as fast, intermediate and slow, respectively. The fillets remained in the -23° freezer for less than 48 hr before being thawed and assayed. By this time the temperature of the fillets of all treatments was equilibrated with that of the freezer.

Results (Table 3) of the paired t test showed that the percentage of the total activity of controls and treated samples were not significantly different ($p \geq 0.05$) in all lipase fractions of fish frozen either at a fast or intermediate rate. On the other hand, significant changes were observed in the lipase activity of the supernatant and lysosomal

Table 3. Effect of freezing rate on the distribution of the lipase activity.

Freezing rate[a]	Nature of the sample[b]	Percent of total activity[cd] S	D	L
Slow	C	18.1 ± 1.1	20.6 ± 1.4	61.3 ± 2.3
	T	26.7 ± 0.7[e]	23.0 ± 1.3	50.3 ± 1.9[f]
Intermediate	C	21.1 ± 1.8	22.8 ± 1.2	56.1 ± 3.0
	T	23.4 ± 0.4	24.4 ± 1.4	52.3 ± 1.7
Fast	C	17.0 ± 0.6	20.0 ± 0.7	63.1 ± 1.2
	T	20.3 ± 2.0	19.3 ± 1.4	60.5 ± 3.4

(a) The time for the internal temperature of the fillet to pass from -1^{o} to -5^{o} was 6, 56, and 193 min for the fast, intermediate, and slow rate of freezing, respectively.

(b) C (controls) and T (treated samples) values are from preparations obtained from opposite sides of the same fish.

(c) Values in each line correspond to the average ± standard error of three replications in duplicate. Each replication corresponds to a different fish. Fractions: S (supernatant), D (sediment), L (lysosomal).

(d) Values with superscripts are significantly different from correspondent controls by paired t test (e: $p \leq 0.01$; f: $p \leq 0.05$).

fractions of the slowly frozen fish. Lipase activity increased significantly ($p \leq 0.01$) in the supernatant and decreased significantly ($p \leq 0.05$) in the lysosomal fraction. Non significant change ($p \geq 0.05$) was observed in the sediment activity of the slowly frozen fish. These results indicated that slow freezing caused lipase to be released from the lysosomes in fish muscle. In contrast, no lipase release was detected in the fish frozen either at a fast or at an intermediate freezing rate.

The extent of release of lipase was also compared among the three freezing rates. The parameter compared was the difference between the percentage of total activity of each of the fractions of treated samples and their correspondent controls. This difference represented the change in distribution of the lipase brought about by the various freezing rates and is displayed in Figure 8. In a first step, the differences were submitted to an analysis of variance one-way classification (Ingram, 1974). When such analysis revealed an overall significant difference among the effects of the freezing rates, the results were further analyzed statistically by t tests (Ingram, 1974) to determine which treatment was different from the others.

Results of the analysis of variance one-way classification showed that the three freezing rates did not differ significantly ($p \geq 0.05$) in their effects on the lipase activity recovered in the sediment fraction. On the other hand, the effects of the three freezing rates on the lipase

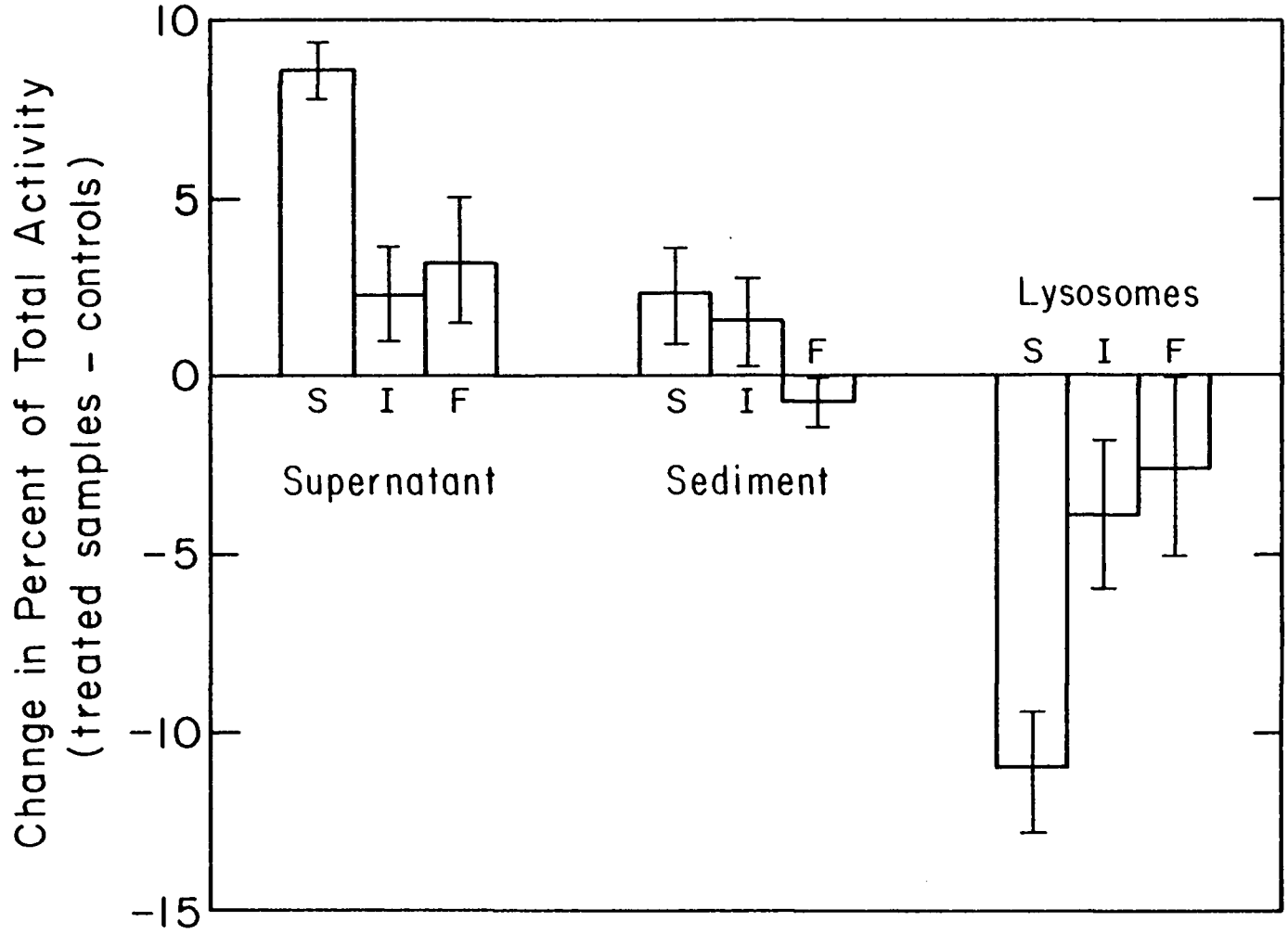

Figure 8. Influence of freezing rate on the distribution of lipase activity. Freezing rates: slow (S), intermediate (I), fast (F). Average ± standard error of three replications in duplicate. Each replication corresponds to a different fish.

activity recovered in both the supernatant and lysosomal fractions were found to be significantly different ($p \leq 0.05$).

Since the activity of the sediment fraction was equally affected by the three freezing rates, only the results obtained for both supernatant and lysosomal fractions were further analyzed by t tests (Table 4). The statistical analysis showed significantly higher changes in the lipase activity recovered in supernatant (increase) and lysosomal (decrease) fractions when the fish were frozen slowly than when either an intermediate or a fast rate of freezing was employed. No significant differences ($p \geq 0.05$) between the two latter treatments were observed with respect to the lipase activity recovered in either the supernatant or lysosomal fraction.

These findings indicated that, while both intermediate and fast freezing rates were considerably superior to slow freezing, fast freezing provided no advantage over an intermediate rate of freezing.

Effect of Time and Temperature of Frozen Storage

Studying the effect of prolonged frozen storage of fish, Shaw and Botta (1977) observed that the major sensorial, as well as, chemical changes were more the result of the freezing and thawing operations rather than of frozen storage. Those authors noticed a liberation of fatty acids in the fish even at a storage temperature of -23°.

The group of Dyer (Peters et al., 1968) investigated the effect

Table 4. Comparison of freezing rate effects on the distribution of the lipase activity.

Freezing rate[a]	Change in percent of total activity[bc]		
	S	D	L
Slow	8.6 ± 0.8^{de}	2.4 ± 1.3	-11.0 ± 1.7^{df}
Intermediate	2.3 ± 1.4^{d}	1.5 ± 1.2	-3.9 ± 2.1^{d}
Fast	3.3 ± 1.8^{e}	-0.7 ± 0.7	-2.6 ± 2.5^{f}

(a) The time for the internal temperature of the fillet to pass from -1^{o} to -5^{o} was 6, 56, and 193 min for the fast, intermediate, and slow rate of freezing, respectively.

(b) Percentage of total activity of each of the fractions of treated samples minus percentage of total activity of each of the correspondent fractions of controls. Values in each line correspond to average $\pm$ standard error of three replications in duplicate. Each replication corresponds to a different fish. Fractions: S (supernatant), D (sediment), L (lysosomal).

(c) In a column, values sharing common superscripts are significantly different by t test (d and f: $p \leq 0.05$; e: $p \leq 0.01$).

of different temperatures during frozen storage of fish and noticed that the amount of lipid hydrolysis increased when the storage temperature was raised from -23° to -18° or -12°. They concluded that

> "There is reason to suspect that this is due to an enzyme activation, or, more likely, to a disruption of membranes or other tissue structure by the melting of ice occurring during the temperature rise and the resulting accelerated diffusion of reactants to active sites where chemical or enzymatic reaction can occur."

The experiment described below was designed to determine if the lysosomal lipase was released during frozen storage and if the release was a function of storage time and temperature. Combinations of three storage temperatures and two storage times were studied. To insure that the fillets were all treated similarly, they were frozen at a common freezing rate. This was accomplished by placing the bags containing the fillets in a freezer at -23° for 10 hr. At that time, two groups were transferred to each of two additional freezers, one at -40° and the other at -11°. The remaining group was not transferred from the -23° freezer. Half of the samples in each group was stored for 30 days and the other half for 120 days. At the end of the storage time, the fillets were thawed in the same manner and enzyme preparations obtained and assayed.

When the percentage of the total activity recovered in each fraction after storage was compared with that of their correspondent controls by paired t tests (Table 5), all treatments resulted in a significant

Table 5. Effect of storage temperature and storage time on the distribution of the lipase activity.

Storage temperature (°C)	Storage time (days)	Nature of the sample[a]	Percent of total activity[b] S	D	L
-11	30	C	19.3 ± 1.4	21.8 ± 0.8	58.9 ± 1.7
		T	39.0 ± 1.5[e]	24.8 ± 1.9	36.2 ± 0.6[e]
	120	C	20.2 ± 0.8	24.5 ± 0.7	55.3 ± 1.4
		T	45.1 ± 1.7[d]	28.4 ± 1.7	26.6 ± 3.3[d]
-23	30	C	17.5 ± 1.0	22.2 ± 2.8	60.3 ± 3.8
		T	29.1 ± 2.1[d]	25.8 ± 1.5	45.1 ± 2.0[d]
	120	C	17.2 ± 0.6	21.1 ± 1.2	61.7 ± 1.8
		T	33.8 ± 0.7[f]	24.4 ± 1.7	41.8 ± 2.3[e]
-40	30	C	15.7 ± 0.8	18.1 ± 0.2	66.2 ± 0.6
		T	23.5 ± 0.6[d]	21.1 ± 1.3	55.4 ± 1.9[d]
	120	C	15.5 ± 0.2	17.6 ± 0.2	66.9 ± 0.3
		T	25.5 ± 1.1[e]	20.9 ± 1.0	53.6 ± 2.0[d]

(a) C (controls) and T (treated samples) values are from preparations obtained from opposite sides of the same fish.

(b) Values in each line correspond to the average ± standard error of three replications in duplicate. Each replication corresponds to a different fish. Fractions: S (supernatant), D (sediment), L (lysosomal).

(c) Values with superscripts are significantly different from correspondent controls by paired t test (d: $p \leq 0.05$; e: $p \leq 0.01$; f: $p \leq 0.001$).

decrease ($p \leq 0.05$) of the lipase activity in the lysosomal fraction. Since the sediment activity was not significantly affected ($p \geq 0.05$) by any of the treatments studied, the significant increase in lipase activity ($p \leq 0.05$) observed in the supernatant fraction resulted from the release of lipase from the lysosomal fraction.

Since the control values in Table 5 refer to enzyme preparations obtained from fish that had not been frozen, the results actually represent a combination of the effects of freezing, storage, and thawing. However, no significant lipase release was detected in the experiment described above (Effect of Freezing Rate) when the fillets were both frozen and thawed at rates identical to the ones employed in the present experiment. Therefore, most of the change observed in the enzyme distribution pattern was ascribed to the storage parameters. It is also noteworthy that fish frozen at an intermediate rate of freezing, stored at a low temperature (-40^{o}) for only 30 days and quickly thawed, showed a significant release of the lipase.

The absolute values of the contribution of the storage parameters to the release of lipase cannot be assessed from the results shown on Table 5 since the controls had not been frozen. However, since all treated samples were frozen and thawed in the same manner, the relative contribution of the various storage treatments to the release of the enzyme can be compared.

To be able to compare these storage effects (temperature and

time), the effects were isolated from each other and were analyzed separately. The parameter compared in both cases was the difference between the percentage or total acitvity of each fraction of the treated samples and their correspondent controls (Figure 9).

Effect of the Storage Temperature

In a first step, the differences between the percentage of total activity of each fraction of treated samples and their correspondent controls were submitted to an analysis of variance one-way classification (Ingram, 1974). When such analysis revealed an overall significant difference among the effects of the three temperatures, the results were further analyzed statistically by t tests (Ingram, 1974) to determine which treatment was different from the others.

Results of the analysis of variance one-way classification showed that the lipase activity recovered in the sediment fraction was not significantly different ($p \geq 0.05$) among the three temperatures at both periods of storage. On the other hand, the changes caused in the lipase activity recovered in both lysosomal and supernatant fractions were found to be significantly different ($p \leq 0.05$) among the three temperatures at both the 30-day and 120-day storage period. The statistical significance levels were $p \leq 0.05$ and $p \leq 0.01$ for both the lysosomal and sediment fractions after 30 and 120 days of storage, respectively.

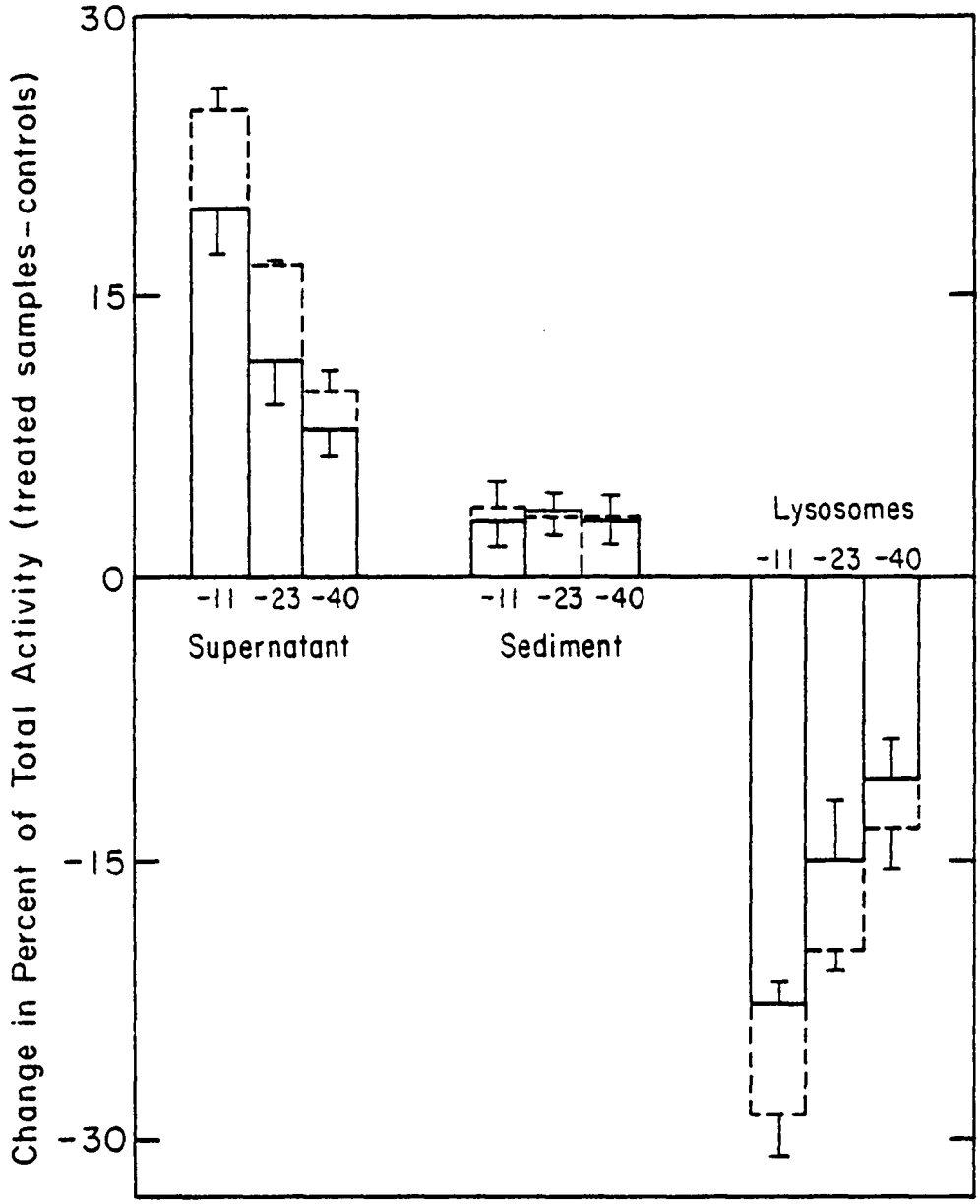

Figure 9. Influence of storage time and temperature on the distribution of lipase activity. Storage temperatures: -11°, -23°, -40°. Storage times: 30 (□) and 120 (⁚⁚) days. Average ± standard error of three replications in duplicate. Each replication corresponds to a different fish.

Since the sediment fraction was equally affected by the three temperatures, only the results obtained for both lysosomal and supernatant fractions were further analyzed by t tests (Table 6).

Similar patterns were found for lipase activity of both lysosomal and the supernatant fractions at the 30 as well as at the 120 day storage period. After 30 days of storage, no significant differences ($p \leq 0.05$) were observed in the change of lipase activity of the samples stored at -23° and those stored at -40°. However, the changes in lipase activity of samples stored at -11° were significantly higher than the changes noticed in samples stored at both -23° ($p \leq 0.05$) and -40° ($p \leq 0.01$).

The differences between the activities of the treated samples and controls were also pronounced at the 120 day storage period. Not only was there a significant difference between the -11° and the -23° samples ($p \leq 0.01$) and between the -11° and the -40° samples ($p \leq 0.001$) as observed at the 30 day storage period, but a significant difference was also noticed between the -23° and -40° samples ($p \leq 0.01$).

These results indicated that release of the lipase was dependent on the storage temperature and that lower temperatures resulted in a lower release of the enzyme.

Table 6. Comparison of time and temperature effects on the distribution of the lipase activity during frozen storage.

Storage time (days)	Storage temperature (°C)	Change in percent of total activity[ab] S	D	L
30	-11	19.7 ± 2.5^{cd}	3.1 ± 1.4	-22.7 ± 1.2^{cdg}
	-23	11.6 ± 2.3^{cg}	3.6 ± 1.3	-15.2 ± 3.3^{c}
	-40	7.8 ± 1.4^{d}	3.0 ± 1.1	-10.9 ± 2.3^{d}
120	-11	24.9 ± 1.0^{de}	3.8 ± 1.4	-28.7 ± 2.0^{dfg}
	-23	16.6 ± 0.2^{dfg}	3.3 ± 1.2	-19.9 ± 1.0^{cd}
	-40	10.0 ± 1.1^{ef}	3.3 ± 1.2	-13.3 ± 2.2^{cf}

(a) Percentage of total activity of each fraction of treated samples minus percentage of total activity of the correspondent fraction of controls. Values in each line correspond to the average ± standard error of three replications in duplicate. Each replication corresponds to a different fish. Fractions: S (supernatant), D (sediment), L (lysosomal).

(b) Within the six blocks, values sharing common superscripts are significantly different by t test (c: $p \leq 0.05$; d and f: $p \leq 0.01$; e: $p \leq 0.001$). Within the three columns, values sharing the common superscript g are significantly different by t test ($p \leq 0.05$).

Effect of the Storage Time

When the effects of the two storage times were compared at each of the three temperatures by t tests, no significant changes ($p \geq 0.05$) were observed between the two periods for the majority of the treatments (Table 6). Even though the differences between the activities of the treated samples and controls were higher at 120 days (with exception of the sediment fraction at -23°), these differences were found to be non significant with two exceptions: the lysosomal fraction at -11° and the supernatant fraction at -23° ($p \leq 0.05$). In these cases, the changes were not paralled by corresponding changes in the other two fractions.

These results suggested that most, if not all, of the release of the lipase from the lysosomes occurred within the first 30 days of storage with little change thereafter. This agreed with the results of Podeszewski and Jasinska (1974) who noticed that the greatest increase of catheptic activity occurred during the first 30 days of storage of frozen herring.

SUMMARY AND CONCLUSIONS

Conditions were established for the assay of an acid lipase that sedimented with the lysosomal fraction following differential centrifugation of homogenates prepared from the dark lateral line muscle of rainbow trout. Validity of the assay conditions was verified by the criterion of linear relationship between lipase activity and incubation time and protein concentration.

Under the assay conditions employed, an optimum pH of 4.2-4.5 was observed. Triton X-100 greatly enhanced the lipase activity in a manner that suggested an additional role of this detergent in the assay system other than acting as a lysosome disrupting agent. Fasting the fish for 24 or 72 hr was found to result in an increase of the lipase activity.

When homogenates were fractionated into lysosomal, sediment and supernatant fractions, 61, 21, and 18% of the lipase activity were found to be associated, respectively, with these fractions.

Homogenization, fractionation and assay procedures employed were found to be appropriate for use in the study of in situ release of acid lipase from lysosomes of fish muscle following low temperature treatments of fillets.

Storage of fillets for 7 days on ice caused no detectable release of the lipase from the lysosomes. On the other hand, temperature

fluctuation of fillets in the range of -10° to -35° during 4 days was found to promote the release of the enzyme.

Considerably less lipase liberation from the lysosomes was observed when the fillets were frozen either at a fast or at an intermediate rate of freezing as compared to those frozen slowly. No significant difference in release of lysosomal lipase was noted between the fast and intermediate freezing rates. Rates of freezing studied were 6 (fast), 56 (intermediate) and 193 (slow) min for the internal temperature of the fillets to be reduced from -1° to -5°.

When fillets were stored at -11°, -23° or -40° for 30 or 120 days, a significantly larger lipase release from the lysosomes was noticed at the higher temperatures. Extending the storage period from 30 to 120 days caused no additional release of the enzyme.

It was concluded that a lipase was released from the lysosomes in fish muscle when fillets were subjected to conditions similar to some of those encountered in the fish processing industry. Such conditions included slow freezing and drastic temperature fluctuation of the frozen product. Time and temperature of frozen storage also played a role in the release of lipase. Most of the release occurred within the first month of frozen storage and decreased with lower storage temperatures.

Next steps in the assessment of the significance of the release of lysosomal lipase in frozen fish should include a) the demonstration

that this enzyme is able to hydrolyze fish lipids *in situ* at low temperatures and b) the determination of the relative contribution of this lipase to fatty acid liberation in both dark and white muscle as compared to other lipolytic enzymes present in these tissues.

The nature of the substrate and the concentration used in the assay system employed in the present work were derived from studies conducted with lysosomal lipase of rat liver. Further studies are needed to determine if these conditions also result in optimum *in vitro* activity for the lysosomal lipase of fish muscle. Purification of the lysosomal lipase of fish muscle followed by studies of the properties of the enzyme would increase our knowledge of the mechanism of hydrolysis of lipases.

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