FATTY-ACYL AMIDASES FROM THE SLIME MOLD DICTYOSTELIUM DISCOIDEUM SPECIFIC FOR BACTERIAL LIPOPOLYSACCHARIDE

by

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by

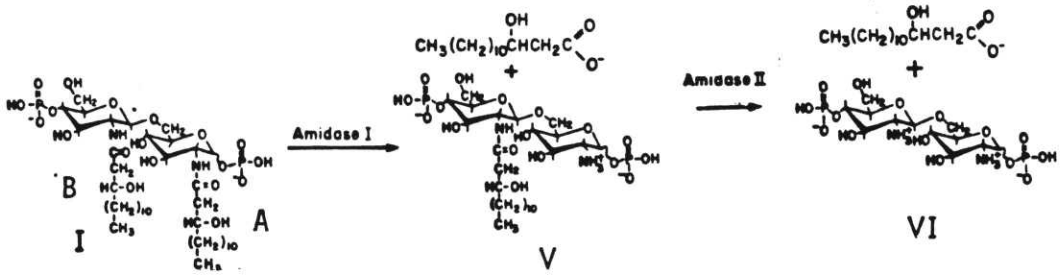
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ABSTRACT

From lysosomal acid hydrolases of the slime mold Dictyostelium discoideum, two fatty-acyl amidases have been isolated that sequentially hydrolyze the two β-hydroxymyristoyl amides on lipopolysaccharide (LPS) and its derivatives. LPS is a unique glycolipid in the outer membrane of gram-negative bacteria, responsible for many characteristic properties of their cell envelope; it is also the primary endotoxin. Amidase I hydrolyzes the amide linkage at site A of the de-esterified lipid A derivative designated as compound I. Amidase II cleaves the other linkage (B) but not before the first fatty acid chain has been removed by prior action of amidase I. The enzymes are highly specific for their designated fatty amides; even upon prolonged incubation, amidase I does not proceed to hydrolyze the linkage at site B, and amidase II cannot liberate the chain at site A.

Amidase I was prepared from lysates of D. discoideum strain NC4, cultured on lawns of E. coli. Vegetative amoebae contain much amidase I, acid phosphatase and esterases, all of which degrade LPS. They are, however, comparatively deficient in amidase II. Amidase I was purified by a sequence of heat treatment, to suppress phosphatase activity, ion-exchange chromatography on DEAE cellulose and gel filtration on Sephacryl S200. In the course of purification of amidase I, two phosphatases and also two esterases were resolved. The phosphatases differ in their lability to heat treatment, inhibition by KF and ability to hydrolyze p-nitrophenyl phosphate. The resulting amidase I preparation was free of phosphatase activity but retained traces of one esterase isozyme. Chromatographic behavior of the esterase and amidase I along with other characteristics suggests that these activities belong to separate enzymes.



Amidase II was isolated from the extracellular fluid of axenic cultures of strain AX-3. Though rich in amidase II and one phosphatase isozyme, the medium lacks amidase I activity. Amidase II was purified by ion-exchange chromatography on DEAE cellulose and gel filtration on Sephacryl S200.

Amidase I activity was observed in AX-3 myxamoebae grown on Aerobacter aerogenes and in NC4 grown on E. coli, but it is absent in axenically grown AX-3 amoebae. Thus, expression of the amidases is modulated by culture conditions as for other acid hydrolases of D. discoideum.

With compounds I and V as respective substrates, amidases I and II were found to have Km's of 3 and 20 uM at pH 3.0, the optimal pH. Both enzymes are inactivated by SDS but retain their activity in various non-ionic detergents and in buffers containing >40% ethylene glycol.

Amidase I is inhibited by di-N-acetyl-chitobiose, by N-acetyl-glucosamine-α-1-phosphate and by compound V, its product; however, sugars with cationic ammoniums such as α-glucosamine-1-phosphate do not inhibit. The enzyme does not hydrolyze monosaccharide substrate analogs N-myristoyl-glucosamine α-1-phosphate or 6-phosphate, indicating a need for the second acyl sugar for productive binding to the enzyme and hydrolysis. Amidase I cannot cleave a formyl amide substituted for β-hydroxymyristoyl at site A, attesting to a preference for long chain amides. Despite this requirement of long chains for hydrolysis and for a second saccharide moiety, N-acetyl glucosamine derivatives do inhibit the enzyme, raising the possibility of interactions with the pyranose ring. β-hydroxylations on the fatty amides is not essential since non-hydroxylated fatty amides are hydrolyzed.

Amidase II is not inhibited by acetylated and non-acetylated glucosamine derivatives; compound I is a potent inhibitor of amidase II despite this enzymes inability to degrade it. Amidase II does cleave fatty amide at site B even when site A is formylated. It can also deacylate N-myristoyl-glucosamine-α-1-phosphate.

Thus, amidase II recognizes a single acyl sugar on lipid A derivatives. Its inability to degrade compound I may lie in binding to site A in a non-productive fashion; note that it degrades a monosaccharide analog of site A. Amidase I, on the other hand, recognizes both acyl sugars of lipid A , which is consistent with di-N-acetyl-chitobiose inhibiting better than N-acetyl-glucosamine-1-phosphate. The different substrate determinants required by each amidase may be the basis for their sequential specificity.

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to my mother Lorraine

and to the memory of her mother before her,

Jeanne

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These days, I remember well my mother's steadfast love, her faith, that maintained me even in dismal times, her belief that what possibilities I might choose would be realized. I recall a grandmother who cherished me, sometimes despite myself, reminding me that I was one of her own. No expression of gratitude to these two could ever return the riches from which I profit, nor is return required. My sister, brothers and I were and remain one; challenging, engaging, hopeful and forever loyal, we engendered a home where inquiry, confrontation and love each played their parts. A special measure of my esteem always remains for Gabriel; nothing more need be said. Carmen, ma marraine to whom I may owe life itself, receives more than a moment of my thoughts. And I recall my father; what he gave I prize.

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LIST OF ABBREVIATIONS

A. Lipopolysaccharide and Derivavatives

LPS	lipopolysaccharide
OH-LPS	alkali-treated LPS
KDO	2-keto-3-deoxy-D-manno-octulosonic acid
formyl-V	N-formyl compound V
Myr-V	N-myristoyl compound V
formyl-VI	N-formyl compound VI (mono-formylated)

B. Chemicals, Materials and Units

EDTA	ethylenediaminetetraacetic acid
TEAB	triethylammonium bicarbonate
Tris	tris(hydroxymethyl)amino methane
Pi	inorganic phosphate
PPi	inorganic pyrophosphate
AMP	adenosine 5' monophosphate
SDS	sodium dodecyl sulfate
DEAE	diethylaminoethyl
DE-81	DEAE cellulose paper
GlcN	glucosamine
GlcNac	N-acetyl glucosamine
N-myr-glcN	N-myristoyl-glucosamine
1-P	1-phosphate
6-P	6-phosphate
S200	Sephacryl S200
DE-52	DEAE cellulose for column chromatography.
NMR	Nuclear magnetic resonance spectroscopy
IR	infrared spectroscopy
O.D.	optical density

BSA	Bovine Serum Albumin
BAP	Bacterial Alkaline Phosphatase
cpm	counts per minute
mu	millimicrons, nanometers

Chapter 1

GENERAL INTRODUCTION

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INTRODUCTION

The cell envelope of gram-negative bacteria consists of three discrete layers: an inner cytoplasmic membrane, a pepticoglycan sacculus and an outer membrane. Lipopolysaccharide (LPS) is a unique glycolipid of gram-negative cells and an important component of their outer membrane. LPS is characterized by two domains, a hydrophobic lipid A region inserted into the bylayer and an extensive, hydrophilic polysaccahride protruding into the extracellular aqueous phase. The outer membrane, absent in gram positive bacteria, endows the gram-negative cell envelope with its characteristic properties, especially impermeability to hydrophobic compounds including many antibiotics and detergents; these properties depend heavily on the structure of LPS. LPS is also the primary endotoxic agent of these cells and provokes wide-ranging responses from host organisms. These include modulation of the immune system and fever induction among vertebrates, and among invertebrates such phenomena as triggering Limulus amoebocytes to a clotting response (1,2).

Understanding the biology of LPS with respect to its role in the outer membrane and as an endotoxin requires analysis of LPS structure and approaches to specifically modify the molecule. Rosner (20,174) has identified a novel class of fatty-acyl amidases from the slime mold Dictyostelium discoideum, that specifically cleave amides on the disaccharide backbone of lipid A. These provide the means to modify the amides of LPS, heretofore resistant to hydrolysis under mild conditions. The amidases were applied by Rosner et al. (174) for the structural elucidation of the LPS of a heptose-less mutant E. coli D31m4. Their usefulness increases with purification and with understanding of their kinetic properties and of the range of substrates that they recognize.

THE OUTER MEMBRANE OF GRAM-NEGATIVE BACTERIA

The Gram-negative Cell Envelope.

The envelope of gram-negative bacteria consists of three layers: an inner or cytoplasmic membrane, a peptidoglycan sacculus or cell wall, and an outer membrane. While gram-positive bacteria have evolved elaborate cell walls incorporating lipoteichoic acids to resist hydrolases, gram-negative bacteria have adopted outer membranes. As a result the gram-negative peptidoglycan has remained relatively unmodified (37). The outer membrane, while permeable to nutrients , excludes many hazardous substances (38, 39), and enables these cells to colonize otherwise inhospitable environments, like the large intestines.

Composition of the Outer Membrane.

The outer membrane is an asymmetric structure (30,31,39-48), consisting of three major components: protein, phospholipid and LPs (37,42). Phosphatidyl ethanolamine comprises ∿70% of phospholipid while phosphatidyl glycerol and cardiolipin make up the rest (60).

The LPS Component.

Electron microscopic examination of the outer membrane show it to be a bilayer (29-31,42). With ferritin-conjugated antibody, Muhlradt *et al.*(40,45) demonstrated that LPS occupies strictly the outer leaflet of the outer membrane. Funahara and Nikaido (50) have recently shown that LPS of intact cells and purified LPS in solution are equally susceptible to galactose oxidase, thus endorsing the conclusion of Muhlradt and coworkers. LPS moves freely in the lateral plane. In filamentous *Salmonella*, rapid diffusion of rhodamine-labelled LPS was measured in fluorescence bleaching experiments (72). LPS was not anchored by other outer membrane components.

Phospholipid of the Outer Membrane.

Studies by Kamio and Nikaido (51) with phospholipase C and a PE-labelling

reagent, CNBr-activated dextran, found no accessible phospholipid on the outer surface of smooth strains. Based on this evidence and the composition of the outer membrane, they concluded that phospholipid occurs only in the inner leaflet of the bilayer. While in Rc and S strains, phospholipid of the outer membrane are cryptic to the above reagents, PE was susceptible to both reagents on the surface of deep rough mutants with extensive defect in the polysaccharide moiety (51). In a similar study with outer membrane protein (omp) mutants and with rough mutants, van Alphen et al.(46) implicated certain "major" proteins collaborating with LPS in shielding phospholipid from external reagents; they claimed that phospholipid occurs in both leaflets of the outer membrane

Outer Membrane Protein.

Many studies have examined the protein distribution in the outer membrane and have demonstrated a strong bias for the outer, LPS-bearing, leaflet. Freeze-fracture micrographs invariably find the overwhelming majority of particles in that leaflet (29,30,42). The majority of outer membrane protein can be labelled with membrane-impermeable reagents (47,48). Other studies have established strong interaction between LPS and outer membrane proteins; stable complexes have been characterized (43,57). van Alphen and coworkers (31) have examined multiple outer membrane protein (omp) mutants, lacking three of the major outer membrane protein: b, c, and d. Finding a 75% reduction in the density of particles in mutant membranes, they could provoke the appearance of new particles and pits with Ca^{+2} treatment. EDTA reversed this change and caused sloughing of LPS from the cells. Restoring any one these proteins, as in double mutants, also increased the particle density. They have suggested that the abundant particles, prominent in the outer leaflet, are hemimicelles of LPS, stabilized by divalent cations, that contain a molecule of any one of the major proteins. They also proposed that

these might be hydrophilic channels.

Influence of LPS on the Physical State of the Outer Membrane.

Physical examinations of gram-negative cell membranes and also of LPS and phospholipid vesicles endorse segregation of the two domains. Nikaido et al(43) probed outer and inner membrane and also vesicles of pure phospholipid and LPS with spin-labelled fatty acid. Their ESR studies found no difference in mobility of the probe between outer and inner membrane despite their finding in pure LPS vesicles that the probe was more restricted than in phospholipid vesicles. They have argued that independent phospholipid domains, not mixed with LPS, must exist in the outer membrane and that their probe prefers the phospholipid enviroment, where fluidity is comparable to the inner membrane. More recently, that group has shown that mixed liposomes of LPS and phospholipid also contain discrete domains (53).

Overath et al. (44) studied the melting transition of E. coli outer membranes by X-ray diffraction and by fluorescence of an N-phenyl-napthylamine probe. Both inner and outer membrane vesicles underwent similar thermal transitions with respect to breadth of transition and mid-transition temperature. However, according to the X-ray analysis, only 25-40% of hydrocarbon chains in outermembrane vesicles underwent transition while the overwhelming majority (60-80%) in the inner membrane melted. Cheng et al. (63) reported longer fluorescence lifetimes with a pyrene probe in outer membranes and also twin transitions as oposed to only one and shorter lifetimes in inner membrane vesicles. These results persuasively support segregation of phospholipid and LPS.

Other studies have challenged this notion. Rottem et al. applied ESR probes to Proteus mirabilis (60) and to E. coli.(61) membranes. In both systems, they found outer membrane to be less fluid than the inner one and have argued that LPS is interspersed whith phospholipid and that by such in-

teraction phospholipid chains are restricted. Monolayer studies by Fried and Rothfield (62) addressed the interaction of LPS and phosphatidylethanolamine (PE). Below a 16:1 molar ratio of PE to LPS, the equilibrium pressure was invariant at that value for pure LPS monolayers; above this ratio, pressures depended upon PE concentrations. These authors have suggested a shell of 16 PE's surrounding an LPS molecule. But it must be noted that such monolayers represent an artificial situation quite unlike the bilayer structure of the outer membrane, where two components can actually partition into distinct leaflets. Still, others have proposed that interaction with LPS shields PE from labelling reagents (46).

These multiple results from probes of the outer membrane can be rationalized in a manner consistent with Nikaido's model of separate phospholipid and LPS domains. Probes partitioning to some extent in the more viscous LPS domain must report lower fluidity for outer vs. inner membrane, as opposed to those dissolving in phospholipid domains, that must find the two membranes of comparable fluidity. Cheng's observation of two phase transitions with the pyrene probe indicates localization in the two distinct environments of the outer membrane. In comparing their ESR studies with those of Rottem et al., Nikaido has commented on the higher probe concentrations used in Rottem's experiments as well as on the use of different bacteria. Such drastic differences between E. coli and Salmonella membranes, despite nearly identical LPS would indeed be surprising. One interpretation lies in the possibility that at higher probe concentrations the membrane is perturbed and a significant fraction of the probe penetrates the LPS-rich and less fluid domain.

Effect of LPS Mutations.

Much evidence suggests that LPS and phospholipid occupy separate domains and furthermore, as proposed by Nikaido (42), phospholipid are restricted to the inner leaflet in the smoother strains. Ames et al. (65) have deter-

mined that deep rough mutants, lacking nearly all the polysaccharide moiety of LPS (see next section), fail to incorporate outer membrane protein to the extent found in less rough strains. Nikaido and coworkers (42) found this to depend on an important interaction disrupted by removal of the glucose residue directly linked to heptose, as in Rd through Re strains (fig. 2). Further studies revealed that the 60% decrement in protein is accompanied by a comparable (70%) increase in phospholipid along with a large increase in LPS (42,66 67). Considering the area and composition of the outer membrane, Smit et al. have calculated that less than sufficient phospholipid to complete one leaflet occurs in the outer membrane of S through Rc strains; deep rough mutants (Rd-Re) have enough phospholipid to complete one face and partially cover another.

Interaction of Cations with Outer Membrane.

The strongly anionic substituents on lipid A, i.e. phosphates and KDO along with multitudinous charged groups on the polysaccharide, lead to strong interaction with cations. EDTA treatment of E. coli provokes realease of LPS (68) and concomitant increase in sensitivity to hydrophobic antibiotics. Uptake of gentian violet by E. coli and Salmonella deep rough mutants was drastically reduced to wild type levels in cultures containing added Mg^{+2} or Na^{+}, although the protein composition did not revert (69). ^{31}P-NMR and freeze-fracture studies have documented the effect of Ca^{+2} on the structure of pure LPS bilayers (70) or in outer mmbrane prepared without lysozyme and EDTA (71). Though unaffected by added Ca^{+2}, spectra of outer membrane preparations responded to EDTA treatment. ^{31}P-NMR analysis also established that mixed liposomes of LPS and phospholipid were in the same physical state as isolated outer membrane.

Permeability of the Outer Membrane.

Permeability distinguishes bacterial outer membrane from all other biological membranes (37), and this largely influenced by the structure of the LPS component. Unlike phospholipid bilayers, outer membrane normally excludes

most hydrophobic compounds and behaves as a molecular sieve permitting non-specific passage to hydrophilic molecules up to a sharp exclusion limit at ~600 daltons.

Hydrophilic pores - Studies with polylysines (73) and oligosaccharides (38,74) have established molecular sieve properties of outer membrane and have explained the inability of E. coli to grow on high molecular weight peptides. These hydrophilic channels are non-specific, a fact arguing for their being aqueous pores rather than carrier proteins. On theoretical grounds, Inoue (75) proposed that the Braun lipoprotein aggregates in the bilayer to form aqueous channels of the proper dimensions. Reconstitution by Nakae (76,77) of vesicles permeable to small oligosaccharides argue against involvement of lipoprotein; rather they implicate several of the major proteins in the range of 33-38,000 daltons in cooperation with LPS. Mizushima and coworkers (55-57) have reconstituted vesicles of protein O-8 and O-9 (both ~35,000 daltons) along with LPS and have reestablished a lattice structure closely resembling SDS-extracted outer membrane. Hancock and Carey (78) have characterized the pore-forming ability of an outer membrane protein, D_1, inducible under conditions that also induce the high affinity glucose uptake system and have distinguished it from another porin, F, involved in low affinity uptake of glucose. Porin D_1 renders liposomes permeable to glucose , slightly so to the trisaccharide raffinose but not to inulin (5000 daltons). Clearly complexes of the porins must form in conjunction with LPS.

Exclusion of Hydrophobic Compounds - Gram-negative bacteria are immune to a large class of hydrophobic agents including antibiotics, detergents and dyes, that affect gram-positive organisms (37). This resistance, attributed to the outer membrane, has been linked to LPS in studies of rough mutants (79, 80). Mutations in LPS structure, as extensive as Rc, that affect 80-90% of the polysaccharide, do not decrease resistance to hydrophobic agents. How-

ever, with the absence of the first glucose residue, Rd through Re strains become highly permeable to these compounds.

It is thought that the LPS-based polysaccharide matrix prevents penetration of hydrophobic compounds by presenting a polar and highly hydrated barrier. Yet, such a mechanism would predict gradual loss of resistance as sugars are gradually removed by stepwise mutation. The sudden decline of this barrier upon removal of glucose-1 argues for involvement of other factors. As discussed earlier, the outer leaflet of the outer membrane is composed of LPS and protein but is apparently devoid of phospholipid, wherein hydrophobic molecules can easily penetrate. Deep rough mutants, on the other hand, incorporate phospholipid in their outer leaflet. Thus, it is by excluding phospholipid from the outer leaflet that gram-negative bacteria are impermeable to hydrophobic agents. High levels of divalent cations can decrease the sensitivity of rough strains apparently by tightening the LPS matrix (69).

Fusion of phospholipid vesicles to Salmonella is facilitated by rough mutations, probably because of exposure of phospholipid domains (82). A bactericidal protein from polymorphonuclear leukocytes displays greater activity toward rough mutants and binds more extensively to them (83,84). One property of this protein is to stimulate penetration of hydrophobic agents, such as actinomycin D. Cells are protected from both its bactericidal and permeabilizing activity by exogenous Ca^{+2} or heparin.

Function of LPS as a Phage Receptor.

Many phages recognize the LPS polysaccharide as receptor. In Salmonella anatum, phages ε15 and c341 bind to the O-antigen and possess a glycosidase that causes release of any phages bound to O-antigenic subunits farther up the chain (85). Injection of DNA requires this hydrolytic step. Some sugars inhibit the infectivity of various Pseudomonas-specific phages (86); purified LPS or exopolysaccharide does likewise (87). In the case of phage T_4, two

groups have shown that not LPS alone but a complex with an outer membrane protein acts as receptor (88,89). The polysaccharide is required since deep rough LPS was inactive.

Role of LPS in Rhizobial Symbiosis.

Purified LPS from legume-colonizing Rhizobia inhibit interaction between plant lectins and bacterial cell walls (91). Mutants have been characterized that bind to root hairs of specific legumes but fail to infect (92). These mutants bear wild type exopolysaccharide (EPS) but were defective in certain antigens on LPS. While strain specific binding to legumes could be attributed to EPS, invasiveness and nodule formation was a property of LPS. Bacteroids are more sensitive to detergents and lysozyme than free-living bacteria of the same strain (93). Comparison of their lipopolysaccharides by SDS-PAGE revealed as yet unidentified differences between the two forms, not related to a smooth to rough transition (94).

STRUCTURE OF LIPOPOLYSACCHARIDE

Tripartite Structure of LPS

Though much variation in LPS occurs among bacterial species (1,3), the structure of all such molecules may be resolved into three distinct domains (fig 1-1). A highly lipophilic region, designated lipid A, consists of a mono or disaccharide of amino hexoses, substituted with ester and amide-linked fatty acids (fig. 1-2). Elaborations on this basic framework abound. The core region of enteric bacteria comprises a short oligosaccharide of roughly ten residues attached directly to lipid A. Normal LPS ends with the long O-specific antigen, made of repeating oligosaccharide subunits, thus presenting a dense forest of polysaccharide at the bacterial surface and endowing a strain with its serological specificity.

As determinants of serological specificity, the O-antigen varies and distinguishes individual species of bacteria (1,6). The core region, on the other hand, is more conserved. Structural and serological analyses argue for a common core sequence among *Salmonellae* (4.5). *E. coli* and *Salmonellae* differ only by substitution of one glucose for galactose (6). Lipid A is by far the most conserved domain; only two broad classes have been described thus far. Differing genera, such as *E. coli* and *Salmonellae* share identical lipid A backbones (1,3,18).

Structures of Lipid A.

The range of lipid A structures has been reviewed by Galanos *et al.* (1) and by Rietschell *et al.* (3). The first of two classes of lipid A (fig. 1-2) was first elucidated in *Salmonella* and found in many other strains (7). It is characterized by a β(1→6)-diglucosamine backbone substituted with amide-linked β-hydroxyl fatty acids, especially β-hydroxymyristic acid, rarely found as structural components of other cell membranes. Estimation of β-

FIGURE 1-1

Organization of the Lipopolysaccharide of *Salmonella typhimurium* (222).

FIGURE 1-1

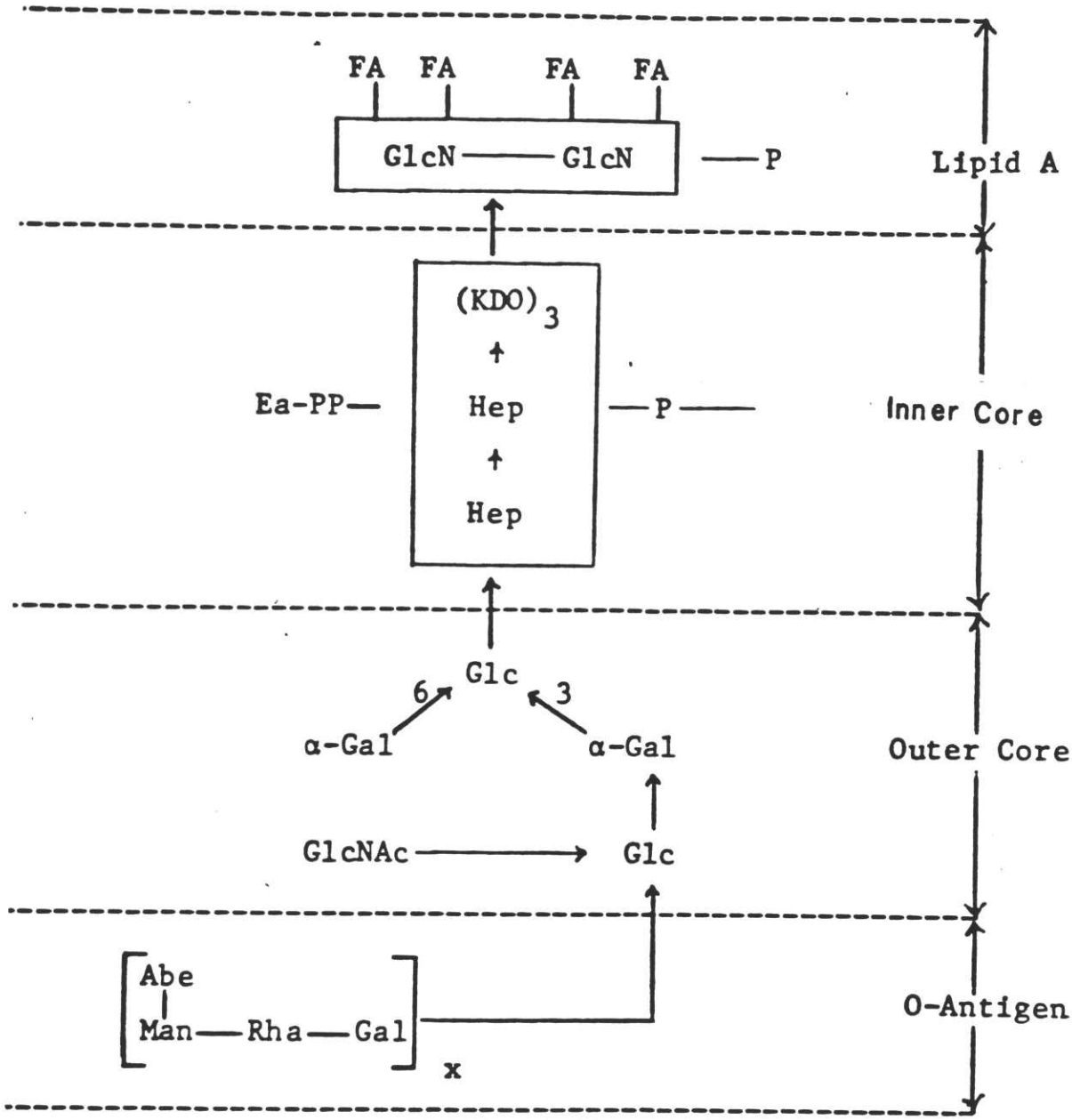
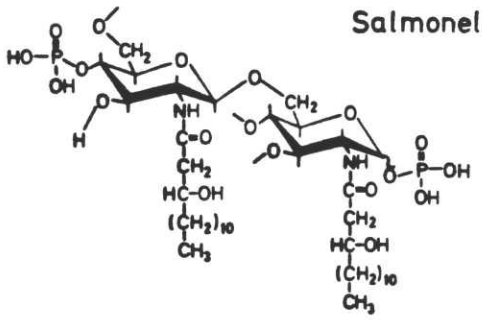

FA FA FA FA
GlcN — GlcN — P
Lipid A
(KDO)3
Ea-PP—
Hep
—P—
Hep
Inner Core
Glc
6
3
α-Gal
α-Gal
Outer Core
GlcNAc
Glc
Abe
Man—Rha—Gal
x
O-Antigen

FIGURE 1-2

Lipid A of vaious bacterial genera (1).

CLASS

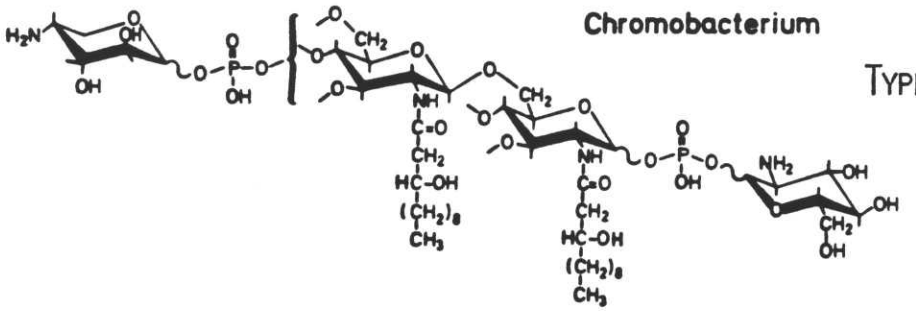
A



Salmonella

TYPE I

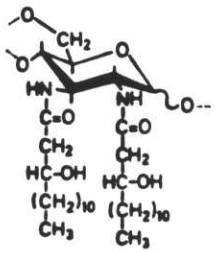
B



Chromobacterium

TYPE I

C



Rhodopseudomonas viridis

TYPE II

hydroxymyristic acid has served in specific quantitation of endotoxin (9). Beyond these common features, much diversity prevails. in most cases, hydroxyls on the backbone are esterified with fatty acids or phosphorylated. Among the fatty-acyl esters, both hydroxylated and non-hydroxylated fatty acids occur, also cyclopropane fatty acids (10,11). Branched chain fatty acids have been reported in nitrogen-fixing Rhizobia (13). C_1 and C_4' phosphorylation is consistently found among lipid A of this type, but Selenomonas ruminantium apparently omits these phosphates (12).

In the other class of lipid A, the diglucosamine backbone is replaced with a monosaccharide 2-3-diamino-2-3-dideoxy-D-glucose (fig. 1-2) (1,3). This form, observed in Rhodopseudomonas viridis and R. palustris, contains no esterified fatty acid but is acylated at both amino groups with β-hydroxymyristic acid. Such LPS does not cross-react serologically with the previous and are relatively weak endotoxins.

Phosphates of Lipid A.

Much research has been devoted to characterizing the phosphates of lipid A. Nowotny et al. (14) proposed a structure with phosphodiester bridges between disaccharide subunits. Luderitz and coworkers (15) latter suggested the existence of pyrophosphate linkages between lipid A monomers. However, ^{31}P-NMR of Salmonella lipid A revealed no phosphate bridges between monomers (16,17). With E. coli lipid A, Rosner and coworkers (18-20) arrived at the same conclusion from chemical analyses and ^{31}P-NMR. No evidence has emerged for covalent linkages between LPS monomers either by phosphodiester or pyrophosphodiester bridges; however, LPS molecules are bridged non-covalently by divalent cations coordinating phosphates of separate molecules (21)

Under certain growth conditions, substituents such as sugars, amino acids or ethanolamine block both phosphates on the diglucosamine backbone making implausible covalent bridging between LPS monomers. Osborn and cowork-

ers have isolated and characterized a Salmonella typhimurium mutant, temperature-sensitive in the KDO-8-phosphate synthetase (see Biosynthesis of Lipid A). When grown in Merk medium, an acidic precursor predominates with its phophates unsubstituted (24). But in Loeb medium, a neutral precursor accumulates, which has 4-amino-arabinose linked to the C_1' and C_4' phosphates respectively (8). Rosner (18-20) has demonatrated heterogeneity in LPS with respect to the phosphate groups of E. coli lipid A. Two forms of LPS occur, in varying relative abundance depending on growth conditions, with either phosphate or pyrophosphate linked to the reducing end of the disaccharide.

Structure of the Core Oligosaccharide.

The diversity of core oligosaccharide sequences defies simple classification, but close resemblances persist among related bacteria. The keto sugar, 2-keto-2-deoxy-octulosonic acid (KDO), is common to most core structures and occurs as a trisaccharide linked at C_3' of lipid A. Some rare exceptions, such as Pseudomonas cepacia, have been reported to lack KDO (36). The acid labile ketosidic linkage of this sugar facilitates hydrolytic cleavage of the polysaccharide from lipid A. Another residue,linked directly to the KDO trisaccharide, L-glyceromanoheptose is also widely found. The core sequence of Salmonella is depicted in figure 1-1;

Structure of the O-antigenic Polysaccharide.

The O-specific antigen is characteristic of each species and is, therefore, highly variable. Bacteria have evolved elaborate means for altering their surfaces by incorporating unusual sugars in the O-antigen. The number of repeating units in Salmonella LPS were determined by SDS-PAGE. Studies of Goldman and Leive (27) and of Mumford et al. (28) show much heterogeneity in the number of repeating units (0-40 units), resulting in a rather irregular topography of the outer membrane. Charge diferences were not responsible

for this heterogeneity since a unique band was observed by isoelectric focussing (27). In contrast, mutants unable to elaborate the O-antigen produce LPS of a single molecular weight, according to SDS-PAGE analysis.

Mutants in LPS Structure.

Mutants that fail to complete the polysaccharide structure are known. These are designated according to the extent of sugar deficiency on LPS. Deep rough strains lack all but the KDO trisaccharide of the inner core while smooth, wild-type, strains elaborate the core and the O-antigenic polysaccharide. Bacteria do not tolerate mutations eliminating the KDO inner core; the only stable mutations in KDO biosynthesis are temperature-sensitive (22). Major pertubations in the outer membrane result from deep rough mutations (29-31). A detailed nomenclature of LPS mutants is presented in figure 1-3.

Specific, Stepwise Degradation of LPS.

For structural studies, procedures which specifically modify LPS have been designed (8,18). Stepwise degradation of the molecule, as developed by Rosner and coworkers is outlined in fig. 1-4 for the LPS of a heptose-less deep rough E. coli mutant. Esterified fatty acids were removed with alkali without disruption of phosphates, amide-linked fatty acids and KDO trisaccharide. The resulting OH-LPS is composed of two species distinguished by either glycosidic phosphate or pyrophosphate. Due to the acid lability of the ketoside, mild acid hydrolysis degrades the KDO trisaccharide and also cleaves the equally labile glycosidic pyrophosphate. Under slightly harsher conditions, glycosidic phosphate is hydrolyzed. Alkaline phosphatase preferentially excizes the C_4' phosphate and attack at C_1 is inhibited with SDS. In SDS micelles, the neighboring fatty-acyl amide protects the glycosidic phosphate from alkaline phosphatase.

Amide linkages on lipid A resist specific hydrolysis under mild condi-

FIGURE 1-3

Nomemclature of Lipopolysaccharide-mutations in Salmonella. (37)

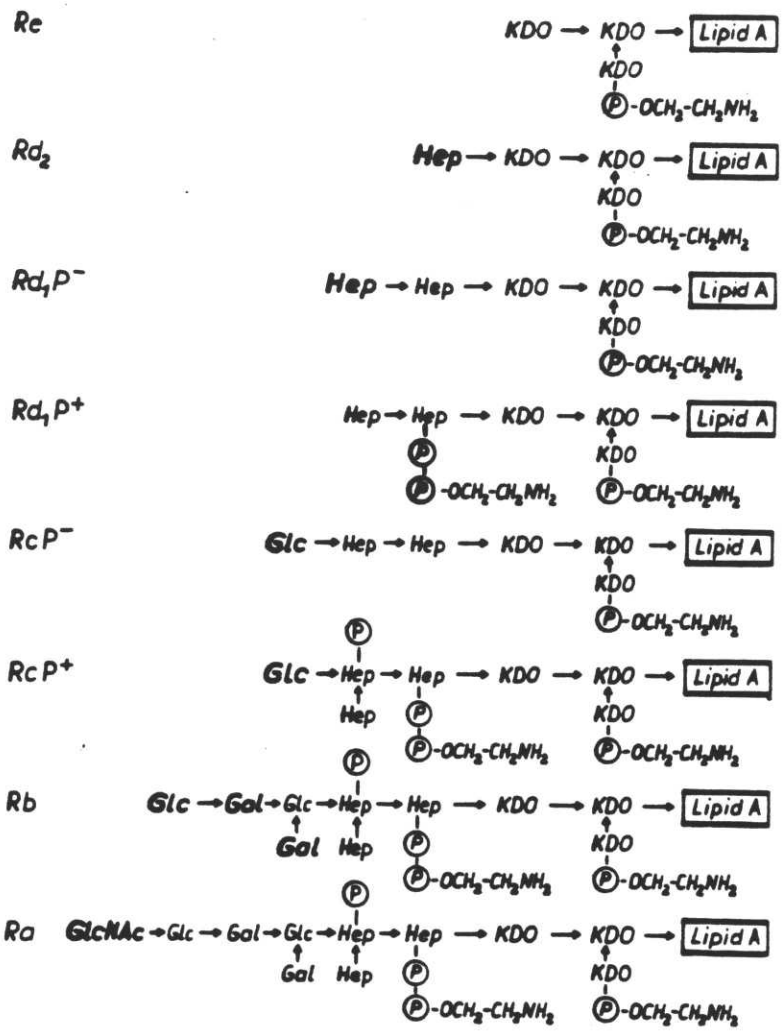

Re
KDO → KDO → Lipid A
KDO
P-OCH2-CH2NH2
Rd2
Hep → KDO → KDO → Lipid A
KDO
P-OCH2-CH2NH2
Rd1P−
Hep → Hep → KDO → KDO → Lipid A
KDO
P-OCH2-CH2NH2
Rd1P+
Hep → Hep → KDO → KDO → Lipid A
P
P-OCH2-CH2NH2
KDO
P-OCH2-CH2NH2
RcP−
Glc → Hep → Hep → KDO → KDO → Lipid A
KDO
P-OCH2-CH2NH2
RcP+
P
Glc → Hep → Hep → KDO → KDO → Lipid A
Hep
P
P-OCH2-CH2NH2
KDO
P-OCH2-CH2NH2
Rb
P
Glc → Gal → Glc → Hep → Hep → KDO → KDO → Lipid A
Gal
Hep
P
P-OCH2-CH2NH2
KDO
P-OCH2-CH2NH2
Ra
P
GlcNAc → Glc → Gal → Glc → Hep → Hep → KDO → KDO → Lipid A
Gal
Hep
P
P-OCH2-CH2NH2
KDO
P-OCH2-CH2NH2

FIGURE 1-4

Stepwise degradation of Lipopolysaccharide according to M. Rosner (20).

This diagram was executed by Dr. Robert Rosner.

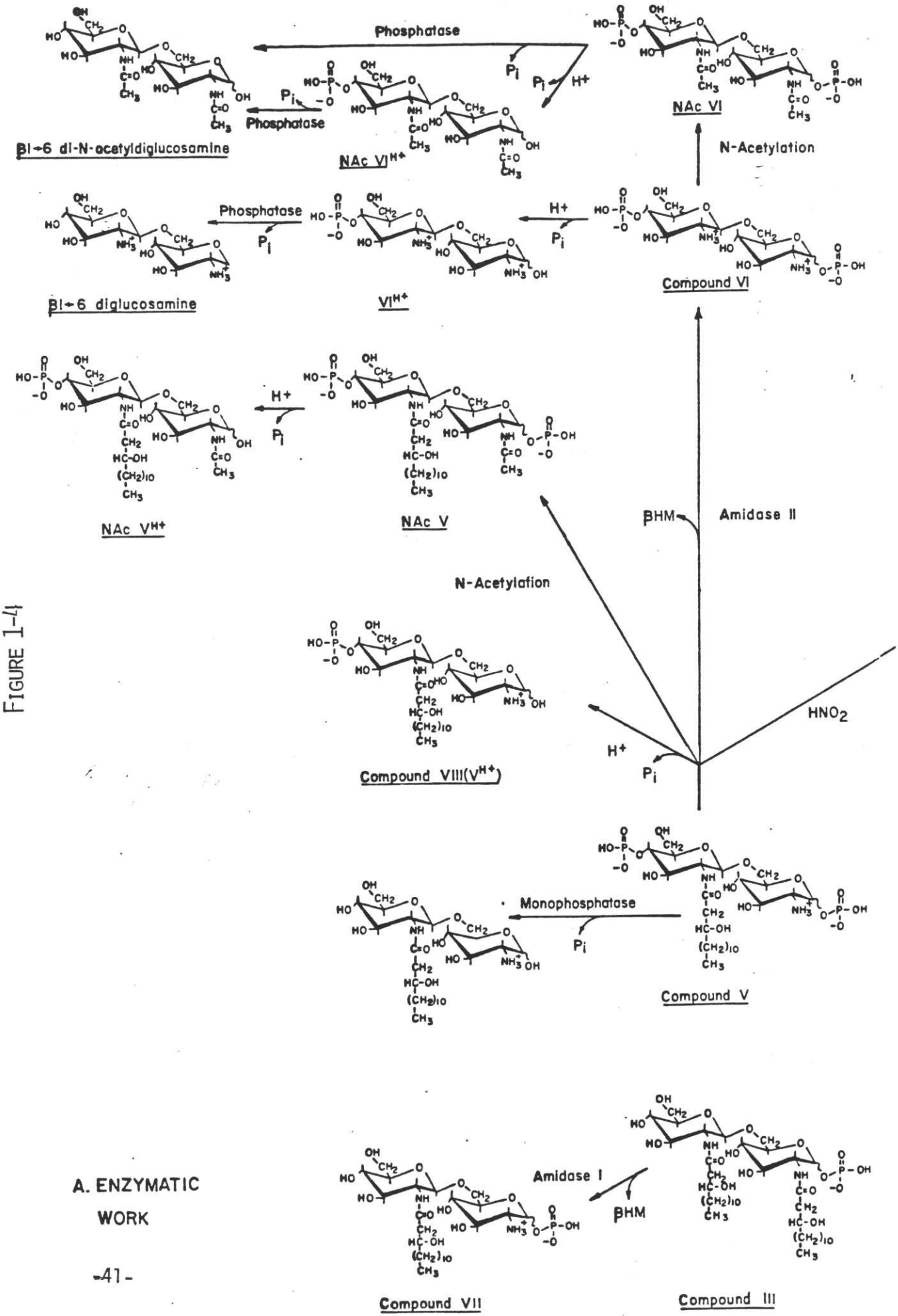
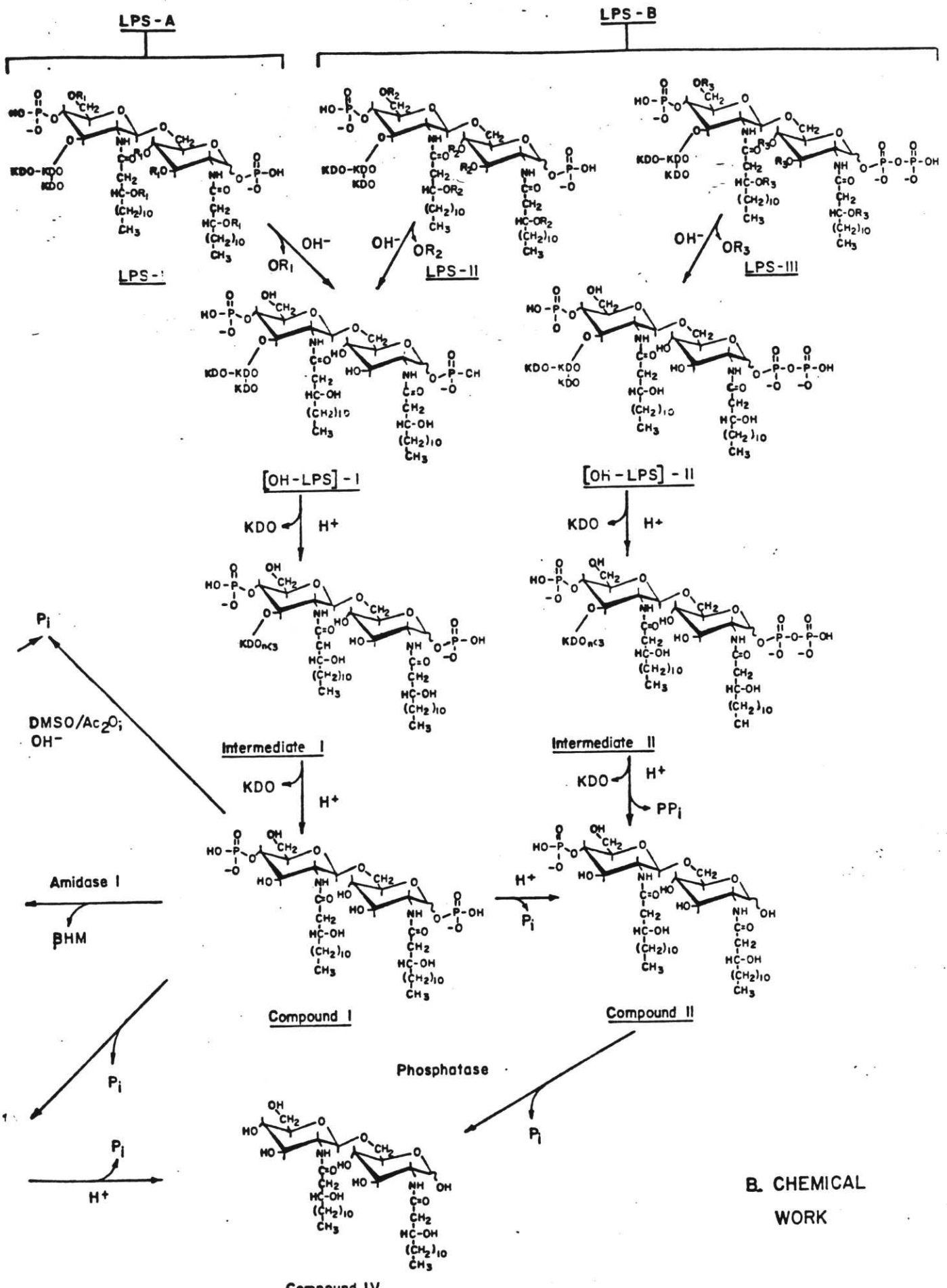


FIGURE 1-4

A. ENZYMATIC WORK



B. CHEMICAL WORK

FIGURE 1-4

tions. Hydrazinolysis has been applied by Hase and Rietschel (7), but other functionalities succumb to some extent to this treatment. The two fatty-acyl amidases introduced by Rosner (174) allow sequential and specific excision of the fatty-acyl amides in compound I.

LPS-like Endotoxins from Gram-Positive Bacteria.

Wexler and Oppenheim (159) have reported an LPS-like component from Envelopes of Lysteria monocytogenes, a gram-positive bacterium. This compound exhibits endotoxic activity; it is pyrogenic to rabbits and active in the Limulus amoebocyte lysate (LAL) gelation assay. The Lysteria toxin is extractable by the classical phenol-water protocol and doe not resemble the teichoic acids. Sugar and fatty acid composition suggest that it is LPS, especially in light of the occurence of β-hydroxymyristic acid. Electron mycroscopy of Lysteria uncovers no structure vaguely resembling an outer membrane.

Isolation of Lipopolysaccharide.

By far the most succesful method for preparing LPS has been the phenol-water procedure described by Westphal (32). Here, cells are extracted with a hot phenol-water mixture, which separates into two phases upon cooling. LPS, usually recovered in the aqueous phase along with nucleic acids and other polysaccharides, is then purified by ultracentrifugation or gel filtration. In contrast to other extraction solvents, such as trichloroacetic acid (33) or aqueous ether (34), the above protocol yields LPS free of polysaccharide and protein of which some bear endotoxic activity.

Phenol-water extraction fails to effectively purify the more hydrophobic LPS of deep rough strains, which partition between both phases. A newer procedure, developed by Galanos et al. (35) utilizes a mixture of petroleum ether, chloroform and phenol. Once the more volatile solvents are removed, pure LPS precipitates from the phenolic solution upon addition of water.

BIOSYNTHESIS OF LIPOPOLYSACCHARIDE

Lipid A and Lower Core Sugars.

Synthesis of the diglucosamine backbone and the origin of the hydroxylated fatty acids remain unclear. Humphreys et al. (95) have shown in vitro that lauryl CoA results in β-hydroxylaurate substituted on lipid A, but the process of hydroxylation was not clarified. The length of β-hydroxy fatty acids in lipid A of Selenomonas ruminantium was found to depend on the length of exogenous fatty acid supplied in culture (96). Taylor and Heath (97) failed to demonstrate transfer of β-hydroxymyristate from acyl-carrier protein into de-esterified lipid A, although transfer to lysophosphatidyl ethanolamine did occur.

Study of a temperature-sensitive KDO-8-phosphate mutant, Salmonella typhimurium PRX22H9, has clarified the steps of KDO and fatty acid coupling to the disaccharide. Under non-permissive conditions, this strain accumulates a precursor, phosphorylated at C_1 and C_4' (see Phosphates of Lipid A) and acylated at both aminos and the C_6 hydroxyl with β-hydroxymyristate (23). Return to permissive temperatures allows conversion of precursor to complete LPS (98). Heath et al (99) had reported incorporation of KDO from the nucleotidyl sugar CMP-KDO. In the hands of Osborn and coworkers (100), a membrane-associated KDO-trasferase transfered two KDO residues from CMP-KDO into the precursor and also into saponified lipid A. Possible involvement of polyisoprenyl carrier lipid was refuted by the activity of lipid-free transferase solubilized in detergent. These studies established that non-hydroxylated fatty acids are added only after the core KDO's.

Core sugars and O-antigen.

Eidels and Osborn (101) have demonstrated the origin of L-glycero-D-manoheptosyl residues from seduheptulose-7-phosphate. The other core sugars,

glucose and galactose, are derived from their UDP-sugars. Water-soluble glucosyl and galactosyl transferases, involved in core synthesis, were shown by Rothfield and coworkers (102) to require phospholipid, either phosphatidyl ethanolamine, phosphatidyl glycerol or phosphatidic acid to activate the enzymes.

The O-antigenic polysaccharide is built of repeating units. Individual units are synthesized on polyisoprenyl carrier and transfered as blocks to naked core or growing LPS chains (103,104).

Translocation of Lipopolysaccharide.

We know from Muhlradt et al. (49) that newly synthesized LPS is transported from the cytoplasmic membrane to the outer membrane via some 200 structures resembling the adhesion sites of Bayer (105), where outer and inner membrane appear to coincide. Ferritin-conjugated antibody, specific for LPS, decorated those sites in galactose-deficient mutants when a pulse of galactose allowed synthesis of LPS bearing antigenic determinants.

Osborn's group has established that translocation is irreversible. In fusing mixed vesicles of LPS and phospholipid to Salmonella, they found free diffusion of exogenous phospholipid into the inner membrane while LPS remained in the outer envelope (82). This stands in light of evidence of rapid, lateral diffusion of LPS throughout the outer membrane as shown in fluorescence bleaching and recovery experiments (72). In their temperature-sensitive KDO mutant, Osborn et al. (106) have also observed weak translocation of lipid A precursor to the outer membrane. Shift to permissive temperature allows completion only of precursor still in the cytoplasmic membrane; precursor already translocated cannot return and, therefore, remains unmodified.

HOST RESPONSES TO LIPOPOLYSACCHARIDE

Introduction.

Perhaps the most striking aspects of LPS are its endotoxic properties comprising a long list of apparently unrelated effects on higher organisms (1,2). Early, it was recognized that the endotoxic domain resides in lipid A and not in the highly antigenic polysaccharide. Thus, endotoxic LPS varying widely in core and O-antigenic polysaccharides bear strong similarities in lipid A. Mild acid hydrolysis, cleaving at the base of the core, yields a non-endotoxic polysaccharide and a highly endotoxic lipoidal moiety.

The range of endotoxic activities of LPS have been frequently reviewed (1,2). These include action on invertebrates such as clotting of Limulus blood. In vertebrates, the most profound responses and probably the largest category of responses pertain to immunity. In most responses, a case may be raised for the involvement of reticuloendothelial cells, most notably macrophages (107,108). This repertoire may comprise such disparate responses as pyrogenicity, shock, Schwartzman reaction and the adjuvancy of LPS.

The Responder and Non-Responder Phenotypes.

The occurrence of mutant mouse strains unresponsive to LPS in most of its biological manifestations has facilitated resolution and characterization of cell types responsible for various responses. Sultzer (109) first described a non-responder, C3H/HeJ; since then other strains of different MHC haplotypes but similar phenotypic response to LPS have been isolated (31,111).

Watson and coworkers (112,113) have extensively analyzed the genetic

basis for the non-responder phenotype. By analyzing recombinant strains, they have localized this pleiotropic mutation to a single locus, termed Lps, and furthermore have mapped it between the Ps gene for polysyndactyly and the Mup-1 locus for a major urinary protein, both on chromosome 4. Failure of another non-responder, C57BL/10.ScCr, to complement the C3H/HeJ defect in matings of the two clearly shows that the former bears its defect also at the Lps locus (114). Although unresponsive to Lps, these strains respond to endotoxic activities of other outer membrane components (115). Crosses of LPS non-responders with responders are intermediate in all responses to LPS endotoxicity, implying co-dominant expressions of the alleles.

Another mouse, CBA/N, fails in mitogenic response of its B-cells to LPS. This mutation is unrelated to the Lps locus, but arises from an X-linked defect in maturation of B-cells (113). CBA/N mice are fully responsive to other endotoxic activities of LPS such as macrophage stimulation. CBA/N x C3H/HeJ progeny are fully responsive to LPS stimulation.

Modulation of the Immune System.

(A) B-Lymphocytes.

Immunization with smooth or rough LPS evokes not only anti-LPS immunoglobulins but also high levels of non-specific antibodies due to polyclonal stimulation of the B-cell repertoire (116,132). Experiments with lipid A confirmed that it was the mitogenic moiety (117,118). Despite earlier reports to the contrary (117), later studies did conclude that saponification of the lipid, releasing esterified fatty acid, did not nullify mitogenic activity (119). Luderitz et al. (120) observed mitogenic activity also in the acidic precursor of Osborn and coworkers (22-24), that lacks the non-hydroxylated fatty acids, but they found that removal of all fatty acids abolishes mito-

genic activity.

LPS-responsive B lymphocytes comprise a distinct subgroup of the entire repertoire. The nature of this subpopulation has been reviewed by Morrisson and Ryan (116) among others. Inhibiting cell division in LPS-stimulated spleenocytes with colchicine or cytosine arabinoside distinguishes two further subdivisions among responsive cells (121). Other B-cell-specific mitogens, including the purified protein derivative of tuberculin (PPD) (122, 123) and pokeweed mitogen (124), stimulate other B-cell populations distinct from, yet overlapping with the LPS responsive subset. Another B-cell mitogen, dextran sulfate, cooperates synergistically with LPS (125).

While in mice both peripheral and spleenic lymphocytes respond to LPS, human peripheral lymphocytes respond weakly (126,127). Earlier, Miller et al. (128) had reported LPS-stimulated proliferation of human peripheral lymphocytes if they were cultured for five or more days with serum, preferably human, prior to stimulation. Ilfeld and coworkers (126) observed that incubation of these lymphocytes in culture for at least 24 hours prior to LPS stimulation renders them responsive. These observations suggest the need for proliferation of helper activity or elimination of suppressor or both. Such a proposition is supported by a report of Nespoli et al.(127) that treatment with con A, a T-cell mitogen, endows responsiveness among peripheral lymphocytes; yet the target of this treatment is unclear since T-cell products can trigger other effectors of B-cell function.

It must be noted that other components of the outer membrane are B-cell mitogens (129), whose action does not depend on the Lps locus. Lipid A is known to trigger other cell lines, notably chick embryo fibroblasts and dog kidney cells (1).

(B) The LPS Receptor

Many studies have attempted to establish specific binding of LPS to B-lymphocytes and appear to contradict themselves. Eldridge (130) has recently reviewed this extensive list and categorizes them according to the concentration range examined. He contends that failure to detect specific binding to B-cells compared to spleenocytes not bearing Ig (T or MΦ) occurs because high concentrations of LPS (>50μg/ml) were used. In the range of 1-20 ug/ml, Watson and Riblet (131) reported specific binding of synthetically tritiated LPS to B-cells, and observed no distinction between B-lymphocytes of responders and non-responders. Zimmerman et al. buttressed this conclusion with studies using ^{125}I-labelled LPS at concentrations below 1 ug/ml. Combining immunofluorescence with autoradiography, Gregory et al. (133) were able to show that only a subpopulation (∿25%) of Ig-bearing cells (B-cells) were responsible for specific binding of LPS. Comparable levels of binding were found between B-cells of responder and non-responder strains while autologous T-cells bound weakly. In his immunofluorescence studies, Eldridge (130) has confirmed these findings but also documented low levels of LPS-binding to Thy-1.2 positive (T) cells and also to null cells (Ig^- and Thy-1.2^-). These studies established that non-responders are fully competent in specific binding of LPS and that their defect must rest at some subsequent step, possibly cell triggering. They also stongly suggest the existence of an LPS receptor on ∿25% of B-cells, absent on most thymocytes. Nigren et al. (134) have electron microscopic evidence of such a receptor on erythrocytes that bind LPS coupled to horseradish peroxidase.

Forni and Coutinho have prepared an antiserum to responder (C3H/Tif) B-lymphocytes and absorbed it on non-responder (C3H/HeJ) B-cells (81). The resulting antiserum distinguished between responder and non-responder B-cells

and detects specific determinants on responder B-cells not on non-responders (C3H/ eJ or C57BL/10.ScCr). They have proposed that the serum recognizes an LPS receptor; the nature and specificity of their antiserum remains unclear and what B-cell determinants it recognizes is not established.

C) T-Lymphocytes.

Thymocytes proliferate weakly, if at all, in response to LPS (135). One study of chimaeric mice with thymus grafts detected donor antigens on only 1.2% of cells dividing in response to LPS (136). Although not mitogenic to most T-cells, LPS synergistically enhanced the effect of T-specific mitogens con A (137) and PHA (138), thus requiring specific interaction of LPS with T-cells. LPS-responsive thymocytes were found to be among a subclass of low buoyant density (137) previously described by Konda et al. (139). These encompass ∿10% of thymocytes and are relatively deficient in Thy antigen and relatively rich in H-2 markers. Decrement in Thy (Θ-antigen) raises suspicion that these are immature thymocytes or else possibly null cells contaminated with some T-cells. The involvement of macrophages in T-cell response cannot be dismissed.

D) Macrophages.

A crucial, regulatory role for macrophages in response to endotoxin may be inferred from their multiple reactions to LPS and their capacity to modulate other cells through their various secretions. In vitro, macrophages respond to LPS with enhanced pinocytosis (140) and phagocytosis (141), secretion of prostaglandin E_2 (PG-E_2) and lymphocyte activating factor (LAF) (141) among others, and cytotoxicity to tumor cells (142). Higher concentrations of LPS (1-50 ug/ml) are toxic to them and inhibit phagocytosis of opsonized substrates (141,143). In vivo treatment with LPS endows mice with non-specific immunity to novel bacterial challenge despite concurrent suppression of

specific immunity. Galleli et al.(144) have correlated this protective effect to ability of non-immune macrophages to inhibit bacterial proliferation. Both activation and toxic response of macrophages are regulated by the Lps locus; C3H/HeJ macrophages are unresponsive.

Cooperation of lymphocytes in LPS stimulation of macrophages has been observed in mice. In their studies of complement-mediated phagocytosis of sheep erythrocytes, Nowakowski and coworkers (110) noted that injection of responder spleenocytes along with LPS into C3H/HeJ, non-responder, mice led to activation of formerly unresponsive macrophages. Thus, they argued that macrophage activation is mediated by primary interaction of LPS with LPS-sensitive lymphocytes, most likely B-cells. However, Pace and Russel(145) showed that lymphokines in the cell-free supernatant of con-A-stimulated spleenocytes greatly potentiated sensitivity of murine macrophages in vitro, therefore, implying direct action of LPS on macrophages. In the related guinea pig system, Schubert et al.(140) found macrophages stimulated in pinocytotic activity despite absence of detectable B-cells in their preparations. One must consider that lymphocytes modulate the macrophage response but also that LPS interacts directly with the latter.

In the reverse order, endotoxin-stimulated macrophages secrete product that potentiate antigenic stimulation of B-cells (146). They are also potent effectors on other immunological cell types. In C3H/HeJ, chemotaxis of polymorphonuclear leukocytes to an interperitoneal challenge of LPS can be restored by adoptive transfer of responder maacrophages (147). Macrophages secrete interleukin-1, necessary for proliferation of thymocytes (148).

E) Adjuvant and Immunosuppressive Effects of LPS.

LPS can either potentiate or suppress an immune response depending on the schedule of administration. Administering LPS a few days prior to antigenic challenge results in diminished antibody and plaque-forming-cell res-

ponse, while simultaneous administration with antigen increases specific response (149).

McGhee et al. have shown that LPS adjuvancy depends on macrophages and T-cells (150,151). They reconstituted the adjuvant response with responder macrophages and T-cells along with non-responder B-cells; the converse was ineffective. Farrar et al. (152) have postulated a model whereby LPS triggers macrophages to secrete interleukin-1; IL-1 then provokes helper T-cells to proliferate.

Uchiyama and Jacobs (153) showed that LPS-injection 1-3 days prior to sacrificing mice reduced the anti-sheep-erythrocyte plaque-forming-cell response. Suppression was mediated apparently by T-suppressor cells as shown by transferability of suppression in culture via T-cell enriched preparations, and also by susceptibility of the suppressive population to anti-Thy serum. This refutes the possibilty that helper cells were being eliminated. Other authors have argued a role for B cells in LPS-immunosuppression (154,155). One for macrophages cannot be overlooked. LPS also suppresses generation of cytotoxic lymphocytes (156), inhibits cell-mediated rejection of skin grafts (157) and reduces the autoimmune reaction in experimental allergic encephalomyelitis (EAE) (158).

DICTYOSTELIUM DISCOIDEUM

Introduction - Catabolism of LPS.

Malchow and coworkers (172) have demonstrated degradation of LPS by the slime mold Dictyostelium discoideum grown on gram-negative bacteria. From the medium of vegetative amoebae, they isolated a 15,000 dalton polysaccharide derived from LPS. Their analysis revealed complete deacylation including removal of amide-linked β-hydroxymyristic acid. Despite an abundance of glycosidases, many purified by Every and Ashworth (173), the polysaccharide was recovered relatively intact, including O-specific sugars, according to molecular weight determination and detection of O-acetylated galactose.

Rosner et al. (20, 174) identified in vitro two fatty acyl amidases sequentially deacylating the amides of lipid A and its derivatives. These enzymes became important to the elucidation of LPS structure (174). They have also observed phosphatases and esterases in D. discoideum extracts that degrade LPS.

Life Cycle and Growth of D. Discoideum.

First described in 1935 by Raper (160), D. discoideum has been a popular system in which to explore many aspects of development. Two stages comprise the life cycle of this slime mold: a unicellular myxamoeba and a multicellular stage culminating in a spore-bearing fruiting body. Development of D. discoideum has been well reviewed by Loomis (161). When the density of nutrient bacteria is adequate for growth, the organism persists in unicellular amoeboid form. However, depletion of nutrient evokes morphogenesis beginning with chemotactic aggregation of cells under the influence of such secretions as cyclic AMP (162). A sheath covers the multicellular pseudoplasmodium as it migrates across the substrate by alternate rising and

toppling over. Further development continues within the pseudoplasmodium results in formation of a sorocarp or fruiting body containing a sporus of roughly $7x10^4$ spores on top of a stalk of vacuolized, cellulose encapsulated cells.

Wild type D. discoideum grows by phagocytosis of bacteria. Vogel et al. (163) isolated mutants defective in various aspects of phagocytosis and have distinguished three intrinsically different and saturatable modes of substrate apposition to the membrane: receptor (lectin) mediated adhesion, non-specific adhesion of hydrophilic substrates and non-specific adhesion of hydrophobic substrates. In mutants defective in the second pathway, uptake of E. coli B/r was completely inhibited by glucose, also the terminal sugar of its rough LPS. Bacteria also enter by the second, non-specific, pathway, that is demonstraded by phagocytosis of protein-coated latex beads; this avenue is ineffective under the high shear forces present in stirred liquid culture. The third pathway was identified with unadulterated latex beads, whose entry could be negated by point mutation. Despite some increase in pinocytic rate, these mutants do not grow well in axenic culture of non-particulate nutrients, glucose, yeast extract and peptone.

Susman and Susman (164) first isolated an axenic mutant, AX-1, that grows in simplified medium by virtue of enhanced pinocytic uptake of medium. No channels other than endocytosis exist in D. discoideum for the accumulation of nutrients. Other axenic strains, exhibiting more synchronous development, have been described.

Modulation of the Lysosomal Enzymes.

Specific activity of lysosomal hydrolases varies through the developmental stages. Solomon and coworkers (165) have resolved at least 5 isozymes of acid phosphatase, and demonstrated their sequential appearance as cells proceed through the stages of development; they also resolved isozymes of

alkaline phosphatase and non-specific esterases, and they found developmentally-regulated modulation of these.

In examining the effects of growth conditions, Weiner and Ashworth (116) have compared growth of AX-2 on bacteria to growth in axenic medium and reported differences in enzymic composition and density of lysosomes. Specific activity of all acid hydrolases tends to be higher in axenically grown cells. Longmore and Watts (167) observed 3-4 fold higher specific activity of N-acetyl glucosaminidase in axenic myxamoebae compared to logarythmic cells cultured on gram-negative bacteria. Meanwhile cultures with gram-positive organisms resulted in specific activities comparable to axenic growth. They also discovered that cell envelope preparations of gram-negative bacteria, purified LPS or lipid A could just as well depress N-acetyl glucosaminidase specific activity in axenic cultures. Thus, it appears that LPS modulates the biology of the lowly slime mold as it does among higher life forms.

Burns, Livi and Dimond (168) have determined that the four earliest developmentally controlled activities in D. discoideum (i.e. N-acetyl-glucosaminidase, α-manosidase, leucine aminopeptidase and alanine transaminase) accumulate during exponential, axenic growth to levels at least as high as observed during starvation of amoebae cultured on gram-negative bacteria. They suggested that axenic conditions trigger amoebae to assume some aspects of early-developing cells. In light of this and the above-cited observations of Longmore and Watts (167), it is clear that the presence of certain bacterial components, notably LPS, signals the slime mold as to its proper developmental attitude.

Axenic and developing cells secrete their lysosomal enzymes. Dimond and coworkers (169) have resolved two, and possibly three, classes of lysosomal particles in terms of secretion of their contents. Secretion of acid phosphatase behaves differently in kinetics and response to inhibitors when

compared to certain glycosidases including β-galactosidase-1. Possibility of a third class was inferred from residual bodies containing β-galactosidase-2 but not the first. These and other studies (170) reveal a common antigenic determinant among many acid hydrolases. Dimmond and coworkers suggest that this may reflect a common post-transcriptional modification, possibly carbohydrate. A role for such carbohydrate has been described for receptor-mediated endocytosis of lysosomal enzymes in mamalian cells (171).

Catabolism of LPS by Other Organisms.

Degradation of LPS has also been documented in Physarum polycephalum (175). In this case esterified fatty acids were excized. However, unaffected were β-hydroxymyristic acid, KDO and immunodominant sugars of the O-region. Supernatants and plasmodial extracts were active against both purified LPS and material contained in heat-killed bacteria. Juices from the gut of the the snail Helix pomatia completely deacylated LPS but again left KDO and the extended polysaccharide intact (176).

In Dictyostelium, growth on gram-negative cells modulates lysosomal composition. One suspects that LPS may be an effector in slime mold as well as Physarum and Helix pomatia. Deacylation of LPS may serve to inactivate its endotoxic activities. Skarnes (177) has suggested that de-esterification may be a principal mode of detoxification in mamalian serum.

FATTY-ACYL AMIDASES

The fatty-acyl amidases identified by Rosner et al. (174) represent a new class of lipases. In this class can also be included the LPS-specific esterases observed in D. discoideum, P. polycephalum and Helix pomatia. With a saponified derivative of lipid A, designated compound I, the amidases sequentially hydrolize first the chain near the reducing end (site A) and then the distal chain (site B) (fig. 1-5). Amidase II, specific for site B, cannot act on the compound until site A has been hydrolyzed by action of amidase I. The enzymes exhibit optimal activity near pH 3 like other lysosomal hydrolases from D. discoideum (173).

The LPS-specific amidases rank among few other reports in the literature of other fatty-acyl amidases (178). By far the most intensively sudied enzymes of this category are lysosomal ceramidases [3.5.1.23] first studied by Gatt (182,183) that hydrolyze amide linkages on sphingolipids. They are highly specific and distinguish D-erythro sphingosine (or dehydroshpingosine) from D-threo and L-erythro isomers. They also do not hydrolyze short chain compounds such as acetyl sphingosine.

Relatively non-specific alkyl amidases [3.5.1.4] derived from mamalian tissues were studied by Bray and coworkers (179). These hydrolyzed N-monosubstituted amides with maximal activity towards compounds of 6 to 8 carbon chain lengths; they were inactive towards compounds with chains 4 carbons or shorter. Methyl and phenyl substituents on nitrogen were tolerated. Chen and Dauterman (180) characterized in sheep liver a similar activity [3.5.1.39], in this case, active towards N-N-disubstituted as well as monosubstituted amides. Similar chain length tolerances and optimal pH $\sim$ 9 suggest that enzymes identified by Bray et al. and those of Chen and Dauterman are the same. Park et al. (181) have studied acyl-lysine deacylase

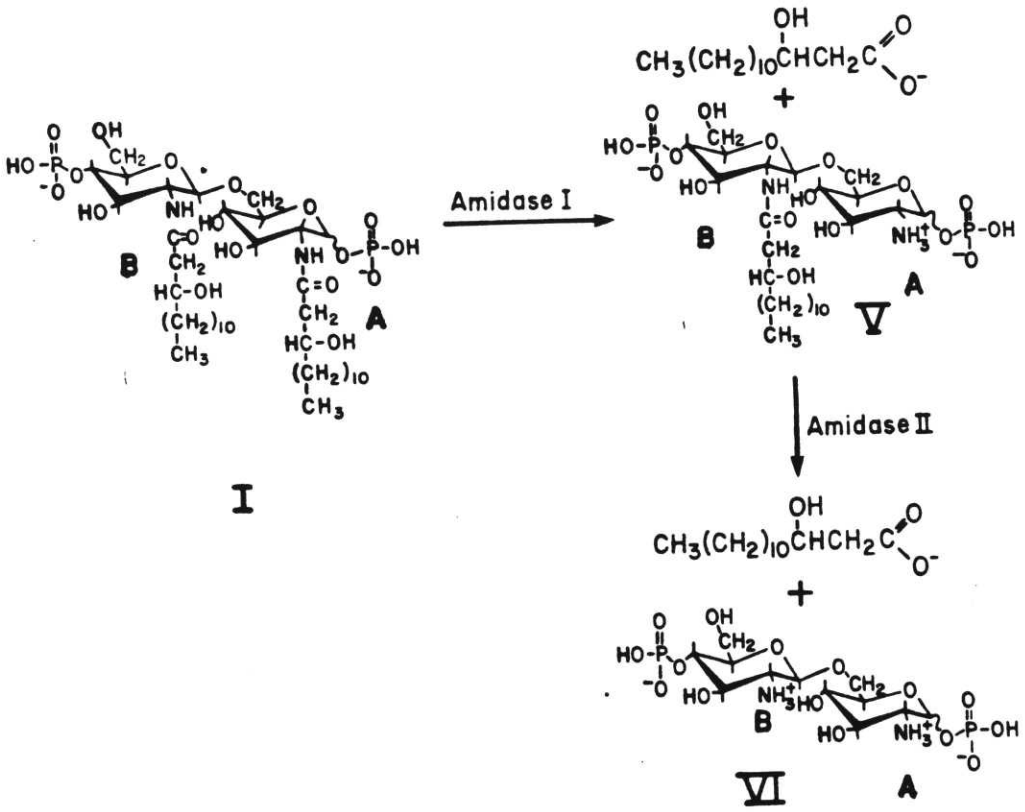
FIGURE 1-5

Sequential action of amidase I and II on the lipid A derivative compound I.

Site A indicates the amino group or amide near the reducing end.

Site B indicates the distal amino group or amide.

NOTE THAT AMIDASE II CANNOT DIRECTLY HYDROLYZE COMPOUND I


Amidase I
Amidase II
B
A
I
V
VI

[3.5.1.17] active toward long chain fatty-acyl amides on the ε-amino. This enzyme is highly specific for amides on L-lysine and not for the D isomer.

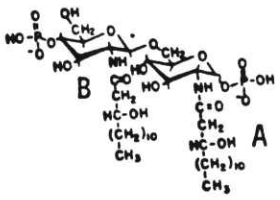
Also of interest in comparaison to the LPS-amidases are chitin deacetylases [3.5.1.4.] able to hydrolyze the amides on N-acetyl glucosamine residues (184). Unlike the amidases, they do not act on long chain fatty-amides.

SUMMARY OF THE THESIS

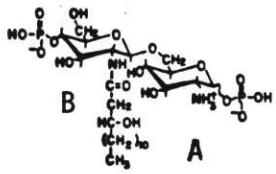
Amidase I, hydrolyzing site A of lipid A derivatives, was isolated from D. discoideum NC4 grown on bacteria and was purified >110 fold by a sequence of heat treatment, ion-exchange chromatography and gel filtration. Amidase II, specific for site B of lipid A derivatives, was isolated from axenic culture of D. discoideum AX-3 among the extracellular hydrolases and was purified ∿ 12-fold by ion-exchange and gelfiltration.

NC4 extract was rich in amidase I but is deficient in amidase II. Two phosphatases were resolved during ion-exchange chromatography. They were also distinct with respect to heat-lability and ability to hydrolize p-nitrophenyl phosphate. Two esterases were also resolved.

AX-3 cells cultured axenically produced no detectable amidase I, while secreting much amidase II. Only one of the phosphatases observed in NC4-extract was found among the AX3 extracellular hydrolases. AX-3 myxamoebae cultured on the gram-negative bacterium Aerobacter aerogenes did produce amidase I.



COMPOUND I



COMPOUND V

Amidase I was found to hydrolyze compound I at site A with a Km of 3 uM at pH 3.0. Amidase II cleaved compound V at site B with a Km of 20 uM at pH 3.0. Their activity is stable in hydrophobic media such as 40% ethylene glycol or non-ionic detergents; they are inactivated, however, by SDS. The cmc of compound I, substrate of amidase I, was measured at 15 uM. No

singularity was observed in the kinetic profiles that might indicate dependence on monomer-to-micelle transition of substrates.

Specific modification of lipid A derivatives show that neither amidase requires the C_1 and C_4' phosphates substituents in order to properly recognize a molecule as substrate and to hydrolyze the appropriate amide. Also, β-hydroxylation of the amide-linked fatty acids are not essential for excision by the amidases. Amidase I cannot hydrolyze monosaccharide analogs of compound I; amidase II can hydrolyze some monosaccharide analogs, albeit rather slowly.

Amidase I was unable to hydrolyze formyl amide at site A, indicating a requirement for long chain acyl substituents. The presence of a blocking formyl group at site A did not prevent hydrolysis of the site B amide by amidase II.

Parameters important to sequential action of the amidases and to their recognition of substrates are discussed.

Chapter 2
MATERIALS AND METHODS

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MATERIALS

Ingredients for Culture Media.

Special reagents for cultivation of bacteria and slime molds were obtained from Difco Laboratories; these were peptones, tryptone, casamino acids, antibiotic medium 3 and agar. Amino acids were purchased either from Sigma or from Calbiochem. Buffer components were derived from the most readily available sources and were at least of reagent grade.

Sugars and Analytical Reagents.

Glucosamine, α-glucosamine-1-phosphate, glucosamine-6-phosphate and their acetylated derivatives were purchased from Sigma; so were KDO, p-nitrophenyl-phosphate (sodium or Tris salt) and p-nitrophenyl-N-acetyl-β-D-glucosamine. Dimethylaminobenzaldehyde and 2-thiobarbituric acid were obtained from Eastman. Ninhydrin came from Pierce. M. Rosner provided a sample of di-N-acetyl-chitobiose. β-Hydroxymyristic acid was obtained from Applied Science Laboratories, Inc.

Alkaline phosphatase was purchased from Worthington Biochemicals Corp.; type F bacterial alkaline phosphatase was used throughout.

Radiochemicals.

^{32}P-orthophosphoric acid in 0.02M HCl (carrier-free) and dry $^{14}C_1$-sodium acetate (50 mCi/mmol) were procurred from New England Nuclear.

Buffers for Column Chromatography.

Buffer A: 20 mM Tris Cl, pH 7.8 (4^{o}C), 0.1 mM EDTA, 50 mM KCl

Buffer B: 5 mM sodium phosphate, pH 6.5, 0.1 mM EDTA

Buffer C: 10 mM sodium citrate, pH 5.5

Buffer D: 10 mM sodium citrate, pH 3.0

Buffer E: 50 mM sodium acetate, pH 4.5

Triethylammonium bicarbonate (TEAB) was prepared according to the proto-

col of Gillum (214). Chlorosulfonic acid was added to triethylamine (10gm of acid / liter of TEA), and the mixture was refluxed for 6-12 hours. TEA was first distilled, then redistilled over KOH (10gm/liter). It was stored at 4 ^{o}C. To prepare buffer, 170 ml of purified TEA in 400 ml of deionized water was acidified by bubbling CO_2 gas until pH fell below 8. The final concentration, always near 2 M, was established by titration against HCl with methyl red indicator.

Materials for Chromatography and Electrophoresis.

DE-52 cellulose and floc-grade DE-1 were obtained from Whatman. Pharmacia provided Sephadex G100 and Sephacryl S200. Biogel P4 (200-400 mesh) and hydroxylapatite were from Biorad, as were reagents for gel electrophoresis. Alkyl agarose minicolumns were purchased from Myles (Yeda).

For thin layers, glass-backed HR/DEAE cellulose plates were from Analtech Inc. while plastic-backed ones were from Brinkman Instruments. Silica TLC plates (Silica gel 60) were obtained from EM Reagents either fluorescent or not, plastic or glass-backed.

For paper electrophoresis and chromatography, rolls of DE-81 and sheets of Whatman #1 were purchase from Whatman Inc.

Polyethylene Glycol Coating (PEG).

As described by Rosner (20), all glassware and plastic tubes that were used to contain LPS derivatives were presoaked in 10% solution of PEG (Carbowax, 20,000, Fisher) and rinsed with copious deionized water. This helped to reduce adsorption of these hydrophobic compounds.

INSTRUMENTS AND EQUIPMENT

Liquid scintillation counting of radioactive samples was performed on Beckman LS250 counter.

Absorbance in the ultraviolet and visible range was normally measured

on a Zeiss PMQ II double beam spectrophotometer. Scanned spectra in the UV-Vis. range were recorded on a Cary 15 machine. Infrared spectra were monitored on a Beckman IR 4210, NMR on a Hitachi R24B.

Preparative, low speed centrifugation was performed on Sorvall RC2B and RC5 centrifuges. Beckman L2 machines were used for ultracentrifugation. An Eppendorf microcentrifuge (Brinkman Instruments) served for small scale work.

Polypropylene microcentrifuge tubes (1.5 and 0.7 ml) were utilized for small scale reactions and obtained from Sardsted, Inc.

To concentrate protein samples, Amicon ultrafiltration cells were used with PM filters.

Evaporation of large volumes of solvents was accomplished either on a Buchi rotary evaporator or Buchler evapomix. Smaller samples were evaporated on a Savant Vac-concentrator (vacu-fuge). Samples were lyophilized on a New Brunswick refrigerated apparatus.

Column eluants were recovered with LKB-7000 fraction collectors.

High voltage paper electrophoresis was executed with electrophoretors manufactured by Gilson, Inc.

Cell cultures were counted with a Petroff-Hauser haemocytometer using a Zeiss phase contrast microscope. Bacterial cell cultures were normally followed with a Klett-Summerson colorimeter.

GENERAL METHODS

Thin Layer Chromatography.

Samples were analyzed by TLC either on HR/DEAE cellulose with solvent I (isobutyric acid, concentrated NH_4OH, water; 66:33:1) or on silica gel plates with solvent II (ethanol, concentrated NH_4OH; 6:4). Removal of fatty-acyl

chains or posphates from LPS derivatives cause predictable changes in mobility on these TLC systems. Loss of a fatty-acyl chain reduces mobility as shown by the sequence of compounds I, V and VI (fig. 3-15). Removal of a phosphate, on the other hand, increases mobility (fig. 6-3).

To resolve non-hydroxylated fatty acid from β-hydroxymyristic acid in assays of esterase, silica plates were developed with diethyl ether (solvent III).

Electrophoretic Analysis.

LPS derivatives were also examined by electrophoresis with 0.3 M sodium phosphate, pH 7.1. On Whatman DE-81, samples were fractionated at 2.75 kilovolts for 45 minutes. Alternatively, they were electrophoresed on Whatman #1 for 30 minutes at 3.0 kilovolts. Opposite to the effect on TLC, removal of an acyl chain by amidase action increased mobility of compounds while excision of phosphate retarded them (figs. 3-2, 6-2, 6-11).

Colorimetric Spray Reagents for Chromatograms.

Phosphate was visualized by the method of Hanes and Isherwood (210). To develop color, hydrogen sulfide was generated with sodium sulfhydrate crystals to which concentrated HCl was added.

KDO was stained with 2-thiobarbiturate as described by Warren (211).

Ninhydrin spray for amino groups was prepared with 0.25gms of ninhydrin in 100 ml of acetone-lutidine (9:1) as described by Work and Work (212).

To visualize all components on silica gel plates, TLC's were sprayed with 25% ammonium sulfate and charred at 110-130 ^{o}C. With UV illumination of fluorescent plates, charring afforded sensitivity comparable to the above specific reagents.

Colorimetric Chemical Analyses.

Phosphate was determined by the method described by Ames (204) including his protocol for ashing with magnesium nitrate.

Glucosamine was quantified by the Elson-Morgan procedure as modified by Stominger et al. (205).

Reducing sugar was estimated by ferricyanide reduction established by Park and Johnson (206).

KDO was determined with 2-thiobarbituric acid by the protocol of Weissbach and Hurwitz as modified by Osborn (207).

Protein was quantitated with the Folin-Ciocalteau reagent as described by Lowry et al. (208) in order to establish specific activities of enzymes. To monitor column eluants, the extinction at 280 mμ was followed in the manner described by Layne (209)

Quantifying Radioactivity.

The specific activity of radiolabelled substrates was established by determining concentration with the above phosphate or glucosamine assays and radioactivity in a Beckman LS250 liquid scintillation counter. ^{32}P-labelled compounds were applied to filter paper and counted in toluene-based scintillation fluid containing 4 gm/liter of PPO (2-5-diphenyoxazole) and 0.1 gm/liter POPOP (1-4-bis[2-(4-methyl-5-phenyloxazoyl)]-benzene). These reagents were either from Fisher Scientific or New England Nuclear (NEN). ^{14}C-labelled compounds were counted in Hydrofluor (NEN).

In monitoring column purifications of ^{32}P-labelled compounds, fractions were counted without scintillant by Cerenkov light using the ^{3}H window, By this method, a counting efficiency of 42% was found when compared to counting in toluene-based scintillant.

Autoradiography.

Chromatograms and electrophoretograms of radioactive mixtures were visualized by autoradiography on Kodak XR5 or XAR-5 film. ^{32}P-labelled compounds were enhanced with Dupont Lightning Plus intensifying screens. Autoradiography of ^{14}C-labelled compounds was accelerated with Enhance spray of

New England Nuclear Corporation.

SDS-Polyacrylamide-Gel Electrophoresis.

SDS-PAGE was executed according to the method of O'Farrell (213) restricted to the second dimension.

CULTURE OF E. COLI D31m4

Lipopolysaccharide for these studies was derived from a heptose-less mutant E. coli K12 D31m4 introduced by Monner et al (201). LPS from this strain was characterized by Rosner et al. (18-20). This mutant is also auxotrophic for three amino acids: tryptophan, histidine and proline. Cultures of D31m4 were routinely tested for deep rough phenotype by their inability to grow on 1.5% agar plates of 1.75% antibiotic medium 3 containing the 0.1% deoxycholate; wild type cells proliferate in this concentration of bile detergent. Purity of the strain was also monitored by routinely verifying amino acid requirements on minimal medium plates (M9 salts) with 5 mg/ml solutions of the amino acids applied two at a time. The strain was preserved at $4^{o}C$ on stabs of 1.75% antibiotic medium 3 and 1.5% agar.

Growth of Radiolabelled Cells.

^{32}P-labelled cells were grown in low phosphate X121 medium (215): 0.2% bactopeptone, 0.1% casamino acid, 0.05% tryptophan, 0.08% glucose and 67 uM sodium phosphate in X121 salts. To a 20 ml culture, 10-20 mCi of carrier-free ^{32}P-orthophosphoric acid was added at the beginning of growth. Cells doubled roughly every 50 minutes and incorporated more than 70% of the radiolabel. ^{14}C-acetate label was incorporated in the above low phosphate medium to which 1 mCi of C_1-labelled sodium acetate was added; the resulting ^{14}C-LPS tends to be of rather low specific activity. Incorporation may be biased for the fatty acyl chains with 1:1 low phosphate and Silbert medium as described Rosner (20).

Growth of Unlabelled Cells.

Unlabelled cells were cultivated in a 25 liter fermentor on the rich medium described by Boman and Monner (202): 1.3% nutrient broth, 0.1% tryptone, 0.2% glucose and medium E. All cultures were harvested at the end of logarythmic growth either by continuous flow with a Szent-Gyorgi-Blum unit (Sorval) or by normal low-speed centrifugation.

Delipidation of Cells.

Immediately after harvest, cells were washed twice with deionized water, twice with ethanol, once with acetone and finally once with diethyl ether. Between washes, cells were recovered by centrifugation at 3000xg for 15 minutes. Dried cells could be stored indefinitely, preferably dessicated under vacuum.

PREPARING LPS AND ITS DERIVATIVES

Extraction of Lipopolysaccharide.

LPS was extracted from dried cells essentially by the procedure of Galanos et al. (35) with PCP (14 gm crystal phenol, 1.1 ml water, 25 ml chloroform and 40 ml petroleum ether). Unlabelled cells were suspended in ∿20 ml PCP per gram of dried cells with temperature strictly maintained between 5 and 20 ^{o}C. Supernatant fluid was separated from cell debris by centrifugation at 3000xg for 15 minutes within the prescribed temperature range and filtered through glass wool. The more volatile solvents chloroform and petroleum ether were evaporated on a rotary evaporator or evapomix depending on the size of the preparation. Dropwise addition of water to the phenolic solution led to precipitation of LPS. Care must be taken to avoid excess water and resulting phase separation. LPS was isolated by centrifugation at 3000xg for 15 minutes, washed with cold 80% phenol (∿2 ml/gm of dried cells) and twice with diethyl ether. LPS was dried under vacuum and redissolved

in 0.2 M TEAB.

Dried ^{32}P or ^{14}C-labelled cells from 20 ml cultures were extracted with 2 ml of PCP. The radioactive supernatant was filtered through glass wool and combined with supernatant from 0.5 gm of unlabelled cells. LPS was precipitated as above and solubilized in 0.2 M TEAB.

Saponification of LPS and Preparation of OH-LPS.

Esterified fatty acids were removed by hydrolysis in 1 N NaOH at room temperature for 15 hours. The alkaline solution was neutralized by passage through a small column of pyridinium Dowex-50, then chromatographed on Agarose 0.5 M (fig. 2-1a) with 20 mM aqueous TEAB. A peak of contaminant, not derived from LPS, emerges with varying intensity after OH-LPS; when it occurs it must not be confused with authentic OH-LPS. TLC either on silica or on HR/DEAE cellulose distinguishes the contaminant from OH-LPS. Note that LPS isolated from this column is a mixture of two species OH-LPS I and II characterized by glycosidic phosphate and pyrophosphate respectively. After lyophilization, The OH-LPS mixture was redissolved in deionized water and stored at -20 ^{o}C.

Resolution of OH-LPS I from OH-LPS-II.

Purified OH-LPS mixture was fractionated into its two components by ion-exchange chromatography on DE-52 cellulose with a linear gradient of TEAB (10-300 mM) in 50% ethanol (fig. 2-1b). Due to the close proximity of the peaks, the early OH-LPSI peak retains traces of OH-LPS II, but the later peak contains only OH-LPS II.

Acid Hydrolysis of OH-LPS, Preparation of Compounds I and II.

Hydrolysis of OH-LPS at 100 ^{o}C for 30 minutes in 10 mM acetate, pH 4.5, served to release the KDO trisaccharide. Two major products result: the diphosphate compound I derived from OH-LPS I and the monophosphate compound II

FIGURE 2-1

A. Fractionation of alkali-treated LPS on an Agarose 0.5 m column (2x75 cm) with 0.02 M aqueous TEAB. Flow rate @ 30 ml/hr.

B Fractionation of OH-LPS mixture to resolve species OH-LPS I and II on a DE-52 cellulose column (1x23 cm) with a 500 ml gradient of TEAB in 50% ethanol (0-300 mM). Flow rate 13 ml/hr.

C. Fractionation of an acid hydrolyzate (pH4.5, 30 min., 100 ^{o}C) of OH-LPS mixture on Sephadex LH-20 (2x90 cm) with 0.1 M TEAB in 40% methanol. Flow rate @ 34 ml/hr.

D. Fractionation of the hydrolyzate of compound I prepared with amidase I, by gel filtration on Biogel P4 (200-400 mesh) (1.5x50cm) with 0.1 M aqueous TEAB. Flow rate @ 17 ml/hr.

FIGURE 2-1

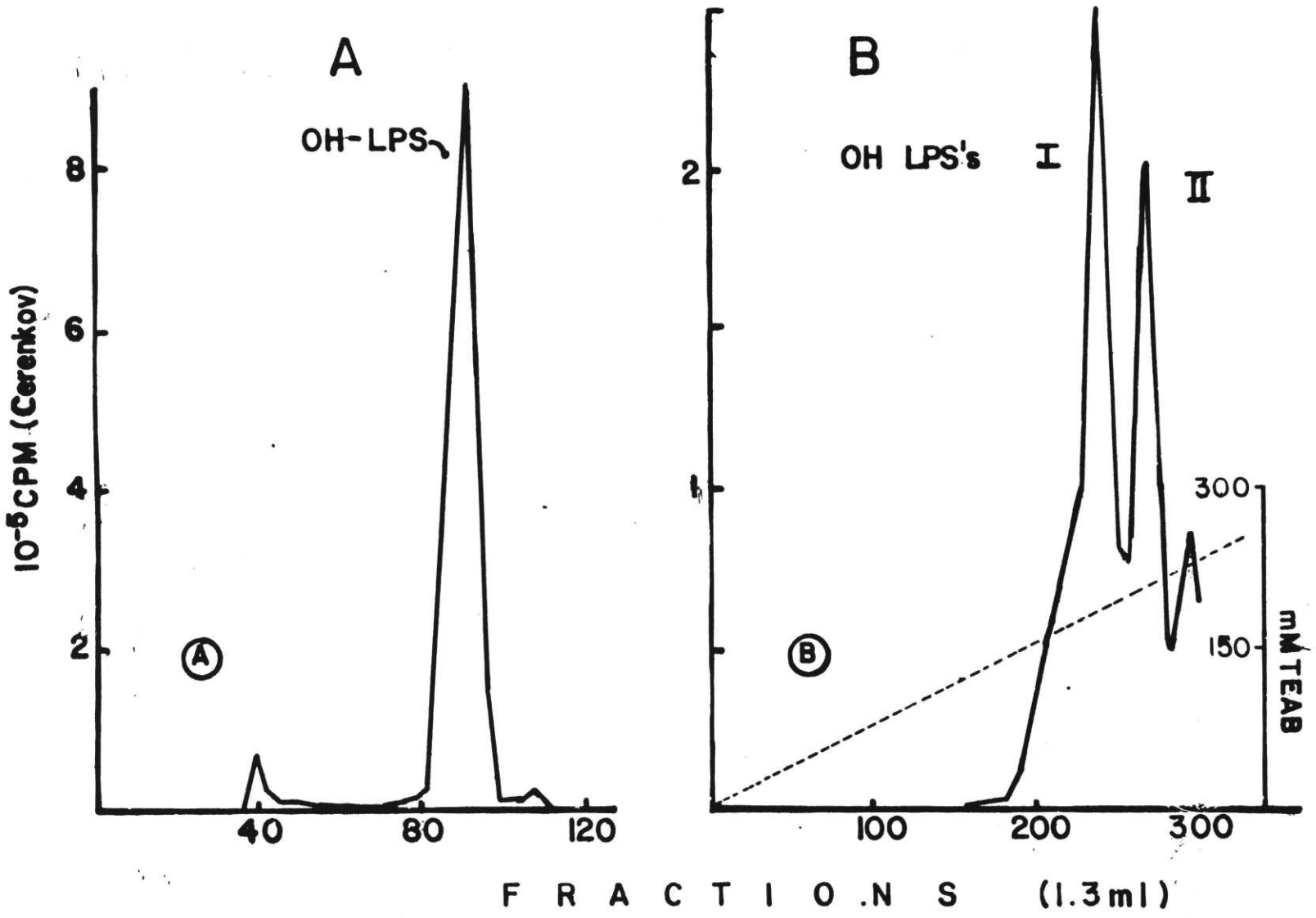
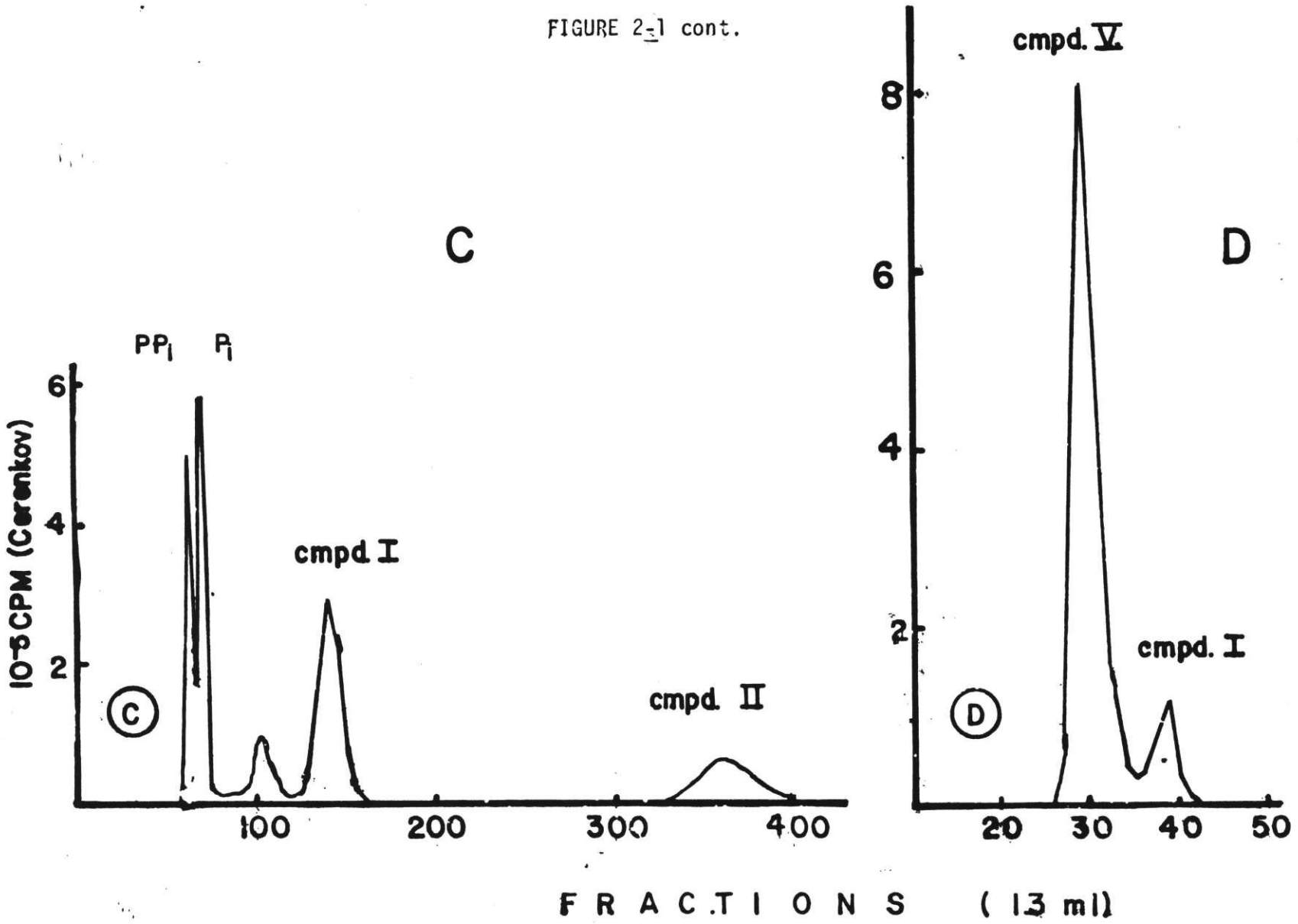

A
OH-LPS
B
OH LPS's
I
II
10^{-5}CPM (Cerenkov)
8
6
4
2
2
1
300
150
mM TEAB
40
80
120
100
200
300
FRACTIONS (1.3ml)

FIGURE 2-1 cont.



-75-

with a free reducing terminal arising from coincident hydrolysis of the glycosidic pyrophosphate of OH-LPS II. The hydrolyzate was fractionated on LH-20 with 0.1 M TEAB in 40% methanol (fig. 2-1c). Solvent was removed on a rotary evaporator using PEG-treated glassware. Because of their hydrophobicity, these compounds bind to surfaces even treated with PEG; therefore, solutions were never evaporated to dryness, but the last few milliliters were lyophilized in PEG-coated plastic centrifuge tubes. They were, then, more easily dissolved in deionized water and stored at -20 ^{o}C.

Alternate Preparation of Compound II.

Hydrolysis of compound I at 100 ^{o}C for 30 minutes in 0.1 M HCl also yields compound II. This offer a clearer solution of compound II, much more susceptible to the amidases. However, yields are extremely low as the compound precipitates irreversibly on to vessel walls at this low pH.

Preparation of Compound V.

In the standard amidase I reaction mixture, described below, compound I was hydrolized with sufficient enzyme at 37 ^{o}C. Amidase I was prepared as described in this text (fig. 3-1) up to the DEAE cellulose stage. After 2 hours of reaction, the mixture was chromatographed on Biogel P4 with aqueous 0.1 M TEAB (fig. 2-1d). This column effectively removes buffer components and protein from compound V; it also resolves compound V from residual precursor compound I.

Preparation of Compound III.

Dephosphorylation at C_4' of compound I by alkaline phosphatase is described in chapter 6 (fig 6-2). For studies of the amidases, crude compound III prepared with BAP without SDS was used. Omitting SDS allows some hydrolysis at C_1 resulting in the fully dephosphorylated derivative.

ACYLATIONS

Formylation of Amino Groups.

LPS derivatives were formylated at free aminos with 5 to 10-fold excess of ethyl formate in absolute ethanol and 5% triethylamine (TEA). Reactions were allowed to sit overnight at room temperature; complete formylation was observed. Methanol was removed on a vacu-fuge concentrator. The compound was then hydrolyzed for 6 to 12 hours in 10% aqueous TEA to cleave any formyl esters. Formyl amides on glycosamine derivatives were stable at room temperature, and were stored at -20 ^{o}C.

Synthesis of ^{14}C-Myristic Acid.

In 0.5 ml of dimethyl sulfoxide (DMSO), ^{14}C-NaCN (10 mCi) was allowed to react with excess bromotridecane (87 ul) for 18 hours at room temperature. According to TLC analysis, 90% of the label was incorporated into the fast-moving nitrile. After extraction from DMSO into petroleum ether, ^{14}C-tetradecanitrile was hydrolyzed at 110 ^{o}C in concentrated HCl and ethanol (9:1). After 24 hours, 20% conversion to fatty acid occurs. Fatty acid was purified first by extraction of nitrile into petroleum ether from an alkaline aqueous suspension, followed by extraction of fatty acid into petroleum ether from the reacidified aqueous phase.

Synthesis of Anhydride.

Myristic anhydride was prepared by coupling free acid in carbon tetrachloride solution via 0.55 equivalents of dicyclohexylcarbodiimide. Anhydride was taken up in petroleum ether while the insoluble dicyclohexyl urea (DCU) remained behind. Such anhydride was free of DCU according to IR. The anhydrides were kept dessicated over P_2O_5 under vacuum.

As for ^{14}C-myristic acid, it was combined with cold carrier to a specific activity of 10^4 cpm/nmole and dried by repeated dissolution in benzene and evaporation. The anhydride was synthesized in the manner for unlabelled

fatty acid.

Fatty-Acylation of Amino Groups.

Free aminos of compound V or of glucosamine phosphates were myristoylated in methanol containing 5% TEA. Four aliquots of anhydride, each containing roughly one equivalent dissolved in dichloromethane, were introduced over the course of four hours. Complete acylation was established by TLC on silica. After evaporation, the dry products were saponified overnight in 0.1 N NaOH, then acidified and extracted with petroleum ether to remove fatty acid. Gel filtration on Biogel P4 (1x50 cm) in 0.1 M aqueous TEAB served as a final purification and desalting step. The resulting fatty-acylated derivatives were devoid of detectable fatty acid.

AFFINITY CHROMATOGRAPHY RESINS

Preparation of Di-N-Acetyl Chitobiose.

Crab shell chitin was fused with KOH as described by Horowitz et al. (216) and purified by repeated precipitation from 5% acetic acid with KOH. The resulting chitosan was then hydrolized in 10 N HCl at 53 ^{o}C for 24 hours as opposed to 48 hours recommended by Distler and Roseman (217). Chitobiose was isolated from a Dowex column eluted with a gradient of HCl (217). Fractions were examined by paper chromatography on Whatman #1 with 1-butanol, pyridine and water (6:4:3,v/v/v). The resulting chitobiose contained some contaminating triose and tetraose (<10%). The sugar was N-acetylated in water with 3 equivalents of acetic anhydride and 7.5 equivalents of TEA added in alternate aliquots to maintain the pH near 9.5. Passage through Dowex-50-(H^{+}) served to isolate acetylated sugars. The products were ninhydrin negative and gave the expected NMR spectra.

Preparation of Affinity Resins.

Separose 6B (Pharmacia) was washed free of preservatives with deionized water on a buchner funnel and excess water was filtered off to yield a compact, wet pad. The gel was activated to the epoxide-agarose according to the protocol of Sundberg and Porath (218), and washed with copious water until the pH reached 7. The extent of activation was determined by titrating with HCl a small amount of gel that had been allowed to react for 30 minutes with 1.5 volumes of 1.3 M sodium thiosulfate. The indicator was phenolphthalein. This established a concentration of 43.5 umoles of epoxide per gram of wet gel.

In each of three flasks, 4 gm of activated resin was allowed to react in 8 ml of 0.1 M NaOH with either 200 mg of N-acetyl glucosamine, 269 mg of di-N-acetyl-chitobiose or nothing as control. After 24 hours, the resins were washed with 500 ml each of 0.1 M borate, pH 8, followed with copious water until a pH of 5-6. Preweighed samples of gel were hydrolyzed at 110 ^{o}C in 4 N HCl for 4 hours, dried down, then resuspended in deionized water and analyzed for glucosamine (205). Assuming a density of 1 gm/ml, N-acetyl-glucosamine-coupled agarose had 7 mM ligand when swollen. The di-N-acetyl-chitobiose resin showed a lower extent of coupling ($\sim$200 uM).

CULTURE OF DICTYOSTELIUM DISCOIDEUM

D. Discoideum NC4.

For isolation of amidase I, NC4 was grown on solid medium from a suspension of spores and E. coli B/r in stainless steel trays (27x40 cm). Medium consisted of 0.1% yeast extract, 1% glucose, 1% peptone, 0.1% $MgSO_4$, 0.096% K_2HPO_4, 0.146% KH_2PO_4 and 2% agar (203). E. coli B/r was cultivated beforehand in the same medium lacking agar and glucose. The spores from one petri

plate of D. discoideum NC4 were added to 250 ml of saturated E. coli B/r. 3.5 ml of this suspension was used to innoculate each steel tray. Trays were incubated at 22 - 23 °C for ∿2 days until a nearly confluent surface of myxamoebae nearly depleted of bacteria covered the surface. This strain was conserved as spores on petri plates (9cm diameter) sealed with parafilm and refrigerated.

D. Discoideum AX-3.

Axenic cultures were grown in a modified version of HL5 medium developed by Watts and Ashworth (185): 1.43% bactopeptone, 0.715% yeast extract, 1.54% glucose, 100 ug/ml streptomycin sulfate, 7.2 mM Na/K phosphate, pH 6.7. Glucose was autoclaved separately and added to the other components. Streptomycin was filter sterilized. Na/K phosphate buffer was prepared by mixing 1 M solution of dibasic sodium phosphate and monobasic potassium phosphate to pH 6.7. Cultures, shaken at 200 rpm, were maintained at 22 °C. They doubled approximately evey 10 hours and reached saturation at about 1.5×10^7 cells per ml.

AX-3 cells were also cultivated at 22 °C on autoclaved bacteria (10^{10}/ml) in 16.7 mM potassium phosphate and 2 mM $MgSO_4$, pH 6.1. Either Aerobacter aerogenes or E. coli B/r were acceptable nutrients.

ENZYME ASSAYS

Assay of Amidase I.

Amidase I activity was determined at 37 °C in a solution of 4.8 mM potassium fluoride, 24 mM sodium citrate, pH 3.0. The substrate. ^{32}P-compound I was normally set at 100 uM for standard assays. Normally, 21 ul reaction mixtures were composed from which three 6 ul time points were spotted on DE-81 paper. Reaction was begun by adding substrate to a mixture of

enzyme and buffer that had been preincubated at 37 ^{o}C for 5 minutes. The initial aliquot was withdrawn within 30 to 40 seconds; others were withdrawn over a course of 5 to 30 minutes. Resulting compounds were fractionated by electrophoresis as described in the above section. In some instants TLC on HR/DEAE cellulose was used to fractionate assay mixtures.

Assay of Amidase II.

Conditions for assay of amidase II were the same as for amidase I. In this case, however, the substrate was purified ^{32}P-compound V at ∿60 uM. In detecting, amidase II in column fractions, compound V was sometimes generated in situ by incubation of compound I with amidase I for 1 hour and aliquots of column fractions were added afterwards. Assay mixtures were fractionated as done for amidase I.

Assay of Esterases.

Esterase activity was determined at 37 ^{o}C in 24 mM citrate, pH 3.0 with ^{14}C-LPS (330 uM phosphate, 1050 cpm/nmole) in 10 ul reaction mixture. The entire reaction mixture was fractionated by TLC with silica gel plates and non-hydroxylated fatty acids quantitated.

Assay of Phosphatases.

Phosphatase activity was determined primarily from ^{32}P-inorganic phosphate released during assay of either amidase with its radiolabelled substrate. In some instants, it was assayed with p-nitrophenyl phosphate in 50 mM citrate, pH 3.0. Reaction was terminated with an equal volume of 1 M Na_2CO_3 and color was determined spectrophotometrically at 420 mu.

Assay of N-Acetyl-Glucosaminidase.

Glucosaminidase was assayed colorimetrically with p-nitrophenyl-N-acetyl-β-D-glucosamine in 0.1 M acetate, pH 4.5. As with phosphatase, the assays were terminated with equal volumes of carbonate and quantified at 420 mu.

Chapter 3

PURIFICATION OF AMIDASE I

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SUMMARY

An amidase that hydrolyzes β-hydroxymyristoyl amide in derivatives of lipid A, specifically at site A, has been purified >110 fold from crude extract of D. discoideum NC4. Amidase I was purified by a sequence of heat treatment at 60 °C, ion-exchange chromatography on DE-52 cellulose, and gel filtration through Sephacryl S200. Contamination by acid phosphatases was completely removed, but some esterase activity remained. Heat treatment at 60 °C was especially useful in suppressing labile phosphatase activity. The nature of these phosphatases and of LPS-specific esterases is discussed. Affinity purification and hydrophobic chromatography of amidase I were also evaluated

INTRODUCTION

Amidase I of Dictyostelium discoideum hydrolyzes amide linkage A on the diglucosamine backbone of LPS (fig. 3-1); amidase II follows with hydrolysis of the other. D. discoideum possesses many other acid hydrolases able to partially degrade LPS, notably phosphatases and esterases. The object of this chapter is to report satisfactory purification of amidase I from these interfering activities, from extracts of a wild type strain NC4.

Source of Amidase I.

Extracts of vegetative NC4 myxamoebae were found to abound in amidase I, acid phosphatases and esterases; they were, however, comparatively deficient in amidase II (see Chapter 4). NC4 crude extract converts compound I to the monoacylated compound V and also liberates inorganic phosphate (fig.3-2). However, only upon prolonged incubation does fully deacylated compound VI begin to appear. This deficiency offered a special advantage in purification of amidase I and in distinguishing its mode of action from that of its congener.

Acid Phosphatases of D. Discoideum.

The main problem proved to be separation of the acid phosphatases from amidase I. As discussed earlier, Solomon et al. (165) have resolved at least 5 isozymes and demonstrated their sequential appearance during the stages of differentiation. Although such activity is partially suppressed by potassium fluoride, the rate of liberation of inorganic phosphate from compound I by crude extract normally exceeded that for the generation of compound V, the product of amidase I. Resistance of phosphatase activity to complete inhibition by high fluoride concentrations suggests that some isozymes are sensitive while others are not.

FIGURE 3-1

Sequential degradation of compound I by amidases I and II.

Site A indicated the amino group or amide near the reducing end.

Site B indicates the distal amino or amide.

This figure repeated from the introduction (chapter 1).

FIGURE 3-1

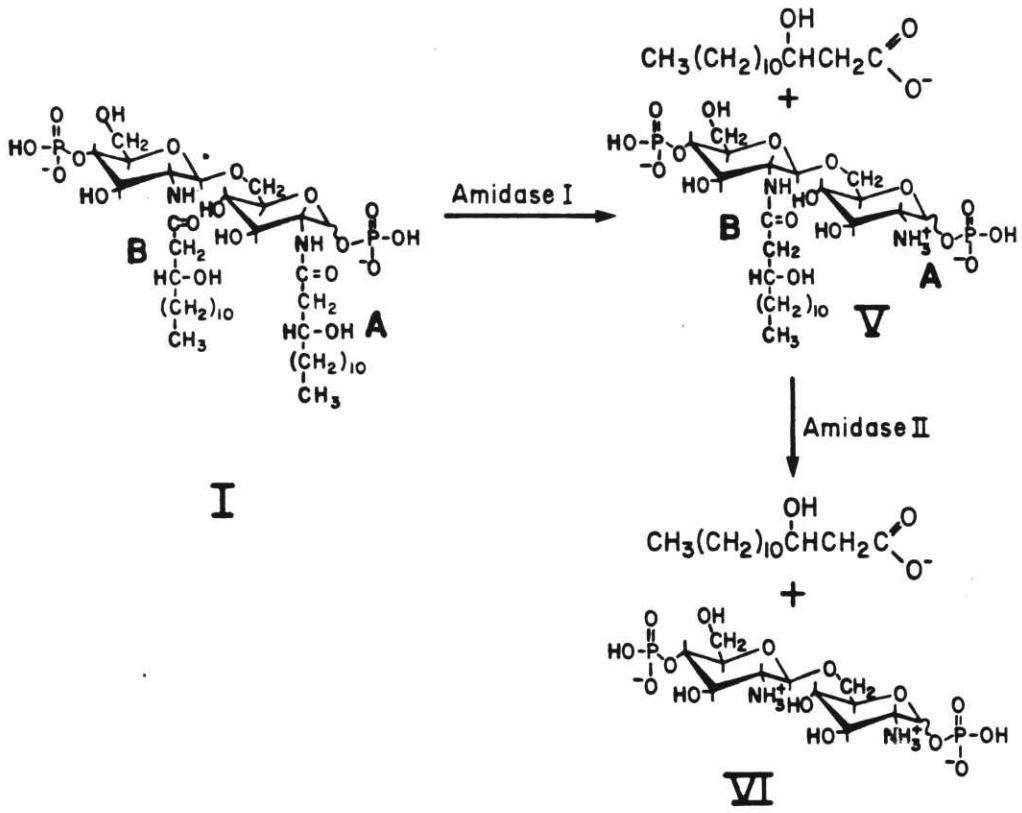


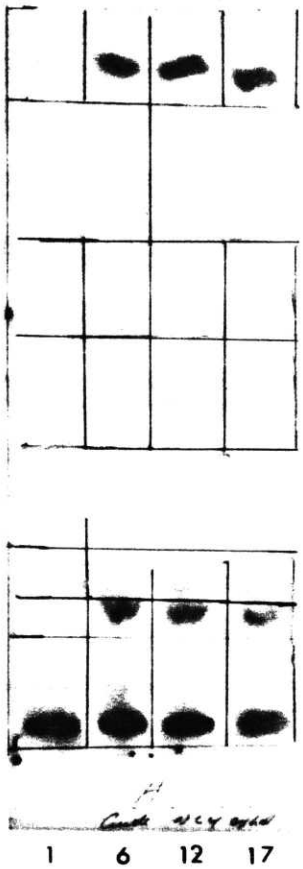
FIGURE 3-2

Degradation of compound I by D. discoideum NC4 crude extract. ^{32}P-compound I (120 µM) was treated with NC4 extract (7.7 mg protein per ml) as described in Materials and Method for assay of amidase I. At the indicated times 14 µl aliquots were removed and spotted on DE-81 paper, then were fractionated by electrophoresis and quatitated as described in Materials and Method.

3 µl	1 M KF
15 µl	0.1 M citrate, pH 3.0
15 µl	0.5 mM compound I
30 µl	NC4 crude extract (16 mg/ml)

Note the faint bands above compound V and above compound VI are the result of phosphatase action whereby one phosphate is removed from the disaccharide .

FIGURE 3-2



CULTURE AND HARVEST

D. discoideum NC4 was cultured on agar with E. coli B/r as described in Materials and Methods. When lysates of cells at various stages of development were assayed, it became clear that amidase I is produced only during the vegetative phase. Its activity persists briefly (∿2 hrs) as cells begin their aggregation, then rapidly declines; culminating cells were devoid of activity. In this respect, amidase I behaves like other developmentally-regulated hydrolases (see Chapter 1, Modulation of Lysosomal Enzymes).

So as to maximize yield, confluent amoebae were harvested prior to aggregation after roughly two days of growth. Cells from 20 steel trays (27x40 cm) were skimmed off the agar surface with a clean microscope slide and immediately suspended in 240 ml of buffer A, kept on ice. The operation was complete within 30 minutes. Finally, the suspension was frozen in a bath of dry-ice and ethanol, and stored at -80 ^{o}C. Estimated with a haemocytometer, the recovery of cells approached 10^{10} cells per tray or $2x10^{11}$ cells for an entire preparation.

PREPARING CELL LYSATE

Amoebae were lysed by thawing the frozen suspension. According to microscopic examination of a dilute aliquot, nearly 60% of cells were lysed. Another cycle of freezing and thawing increased observable lysis beyond 75%. It is very likely that apparently intact cells are indeed quite leaky after two freeze-thaw cycles. E. coli, also harvested with the amoebae, are not broken by this proceedure and were seen suspended in the lysate.

Centrifugation at 30,000xg for 15 minutes served to remove intact E. coli and D. discoideum cell debris; a cloudy supernatant devoid of structure

at 1,000 magnification resulted. Further centrifugation at 100,000xg for 1 hour clarified the supernatant fluid by removing such particulate matter as ribosomes.

Ammonium sulfate crystals were added to the lysate over a period of 1 hour at 4 ^{o}C to 70% of saturation. Precipitated protein was sedimented at 15,000xg for 15 minutes, redissolved in buffer A, then dialyzed against the same buffer. After dialysis,a net 794 mg of protein was recovered in a total 42.5 ml volume. The estimated specific activity (0.71 u/mg) is only a lower limit due to the inordinate level of acid phosphatase (table 3-1).

HEAT LABILITY OF ACID PHOSPHATASES AND AMIDASE I

Acid phosphatases in crude extract prevent accurate determination of amidase I activity with ^{32}P-compound I since radioactivity in both product and substrate is depleted as inorganic phosphate (fig. 3-2). Moreover, very high phosphatase levels, occurring in some preparations are not effectively removed in subsequent chromatography. Thus, the following protocol to reduce phosphatase activity by heating crude extract at 60 ^{o}C was developed.

Studies at 50 ^{o}C.

Stabilities of amidase I and of fluoride-resistant phosphatase were evaluated at 50 ^{o}C. The enzyme preparation studied had been partially purified on DEAE cellulose (see below); it contained both amidase I and a phosphatase co-purifying with the amidase. pH was 7.8 at 4 ^{o}C. Aliquots (20 ul) were incubated at 50 ^{o}C in a water bath, quenched at varying times with four volumes of buffer B and chilled on ice. Both activities, assayed with ^{32}P-compound I in the presence of 200 mM fluoride, remained unaffected even after 15 minutes at 50 ^{o}C.

Studies at 60 ^{o}C.

Further studies were undertaken at 60 ^{o}C (fig. 3-4,3-5; table 3-2). Two

FIGURE 3-3

Flow chart of the purification of amidase I from D. discoideum NC4.

FIGURE 3-3

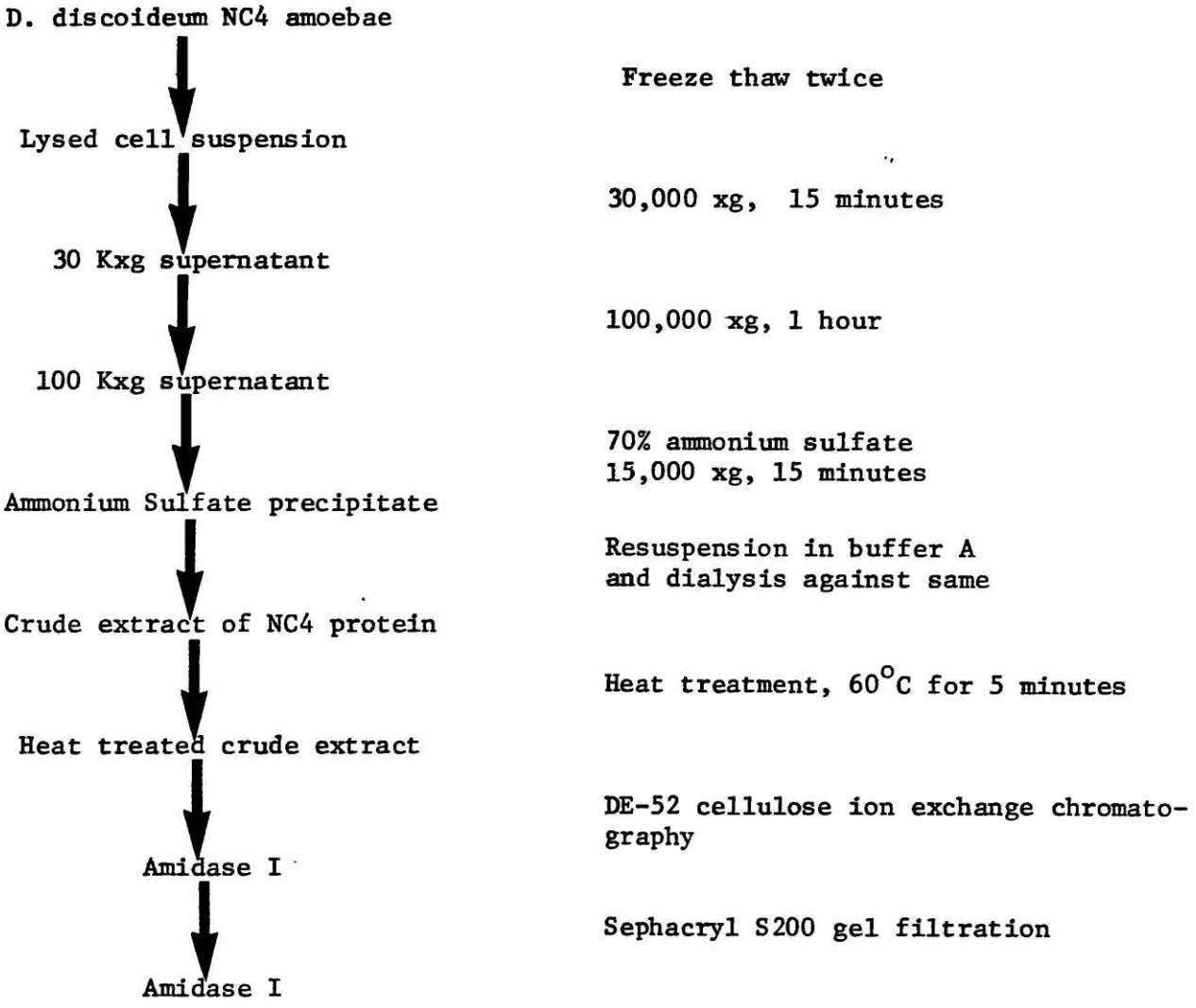

D. discoideum NC4 amoebae
Freeze thaw twice
Lysed cell suspension
30,000 xg, 15 minutes
30 Kxg supernatant
100,000 xg, 1 hour
100 Kxg supernatant
70% ammonium sulfate
15,000 xg, 15 minutes
Ammonium Sulfate precipitate
Resuspension in buffer A
and dialysis against same
Crude extract of NC4 protein
Heat treatment, 60°C for 5 minutes
Heat treated crude extract
DE-52 cellulose ion exchange chromatography
Amidase I
Sephacryl S200 gel filtration
Amidase I

TABLE 3-1

	units[b]/ml	mg/ml	ml	units[b]	mg	unit/mg	Purity	Phosphatase Units[b]	Ratio P-ase/ Amidase[c]	Yield of Step
Crude	11.48	16.25	18.7	214.7	303.9	0.71	1	233	1.08	100%
Heated	10.23	15.82	18.7	191.0	295.0	0.64	0.91	148	0.774	90%
DE-52	8.026	1.01	25.0	200.0	25.2	7.93	11.2	40.7	0.203	104%
S200[a]	18.04	0.23	2.14	38.6 (96.5)[d]	0.49	78.43	110.0	0.85	0.020	48%
									Overall	45%

a only 10 ml of enzyme from the previous step added to the column.

b 1 unit = 1 nanomole product per minute.

c Phosphatase to amidase I ratio was derived from the ratio of P_i to compound V generated by the preparations from ^{32}P-compound I.

d Predicted units of amidase I which would result if the entire preparation from DE-52 were passed through the last step.

FIGURE 3-4

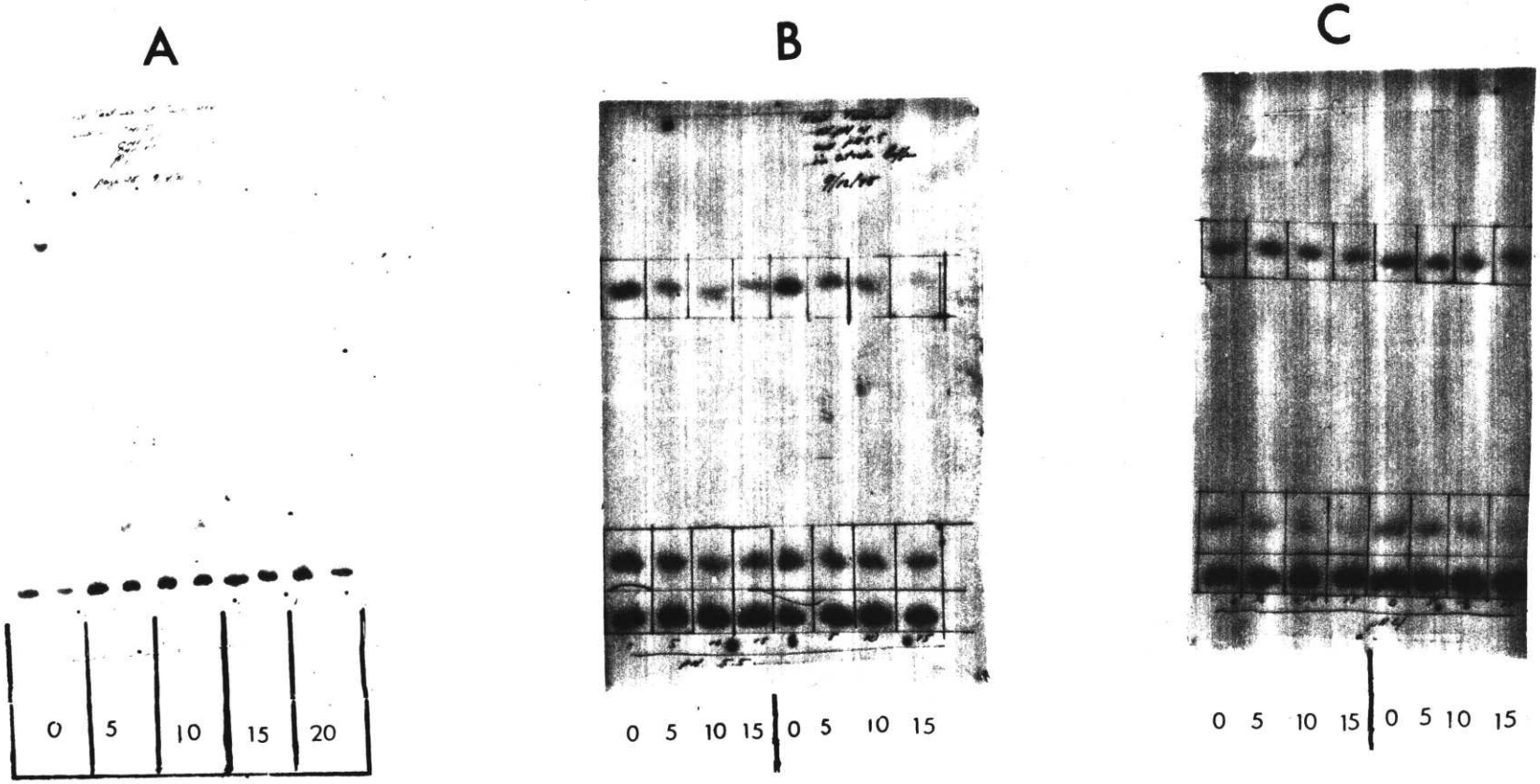
Autoradiograms of electrophoretic fractionation of assay mixtures for heat-treated crude extract.

Ten to twenty microliter volumes of crude extract dissolved in (A) buffer A, pH 7.5, (B) buffer C, pH5.5 and (C) buffer D, pH 4.0 were heated at 60 ^{o}C for various times at the end of which an equal volume of cold buffer was added. Protein concentrations were (A) 16 mg/ml, and (B,C) 8 mg/ml.

Heat-treated solutions were then assayed without further dilution as described in Materials and Method. For A, 2 aliquots from the assay mixture were spotted on paper after 45 seconds and 6 minutes of incubation. For B and C, only one aliquot was spotted after 6 minutes.

The procedure for electrophoresis is described in Materials and Methods.

FIGURE 3-4



Duration of Heat Treatment

FIGURE 3-5

Dependence of amidase I and phosphatase activity on extent of heat treatment.

Samples of amidase I were subjected to heat treatment at 60 °C as outlined in the legend for figure 6.

Amidase I (●——●) and phosphatase (⊡——⊡) activity were determined as described in Materials and Methods , based on the production of compound V and P_i.

A. Crude extract, pH 7.5, buffer A.
B. DE-52 and Sephacryl S200 purified amidase, pH6.5, buffer B.
C. Crude extract, pH 5.5, buffer C.
D. Crude extract, pH 4.0, buffer D.

(See table 3-2, next page, for tabulation of halflives)

FIGURE 3-5

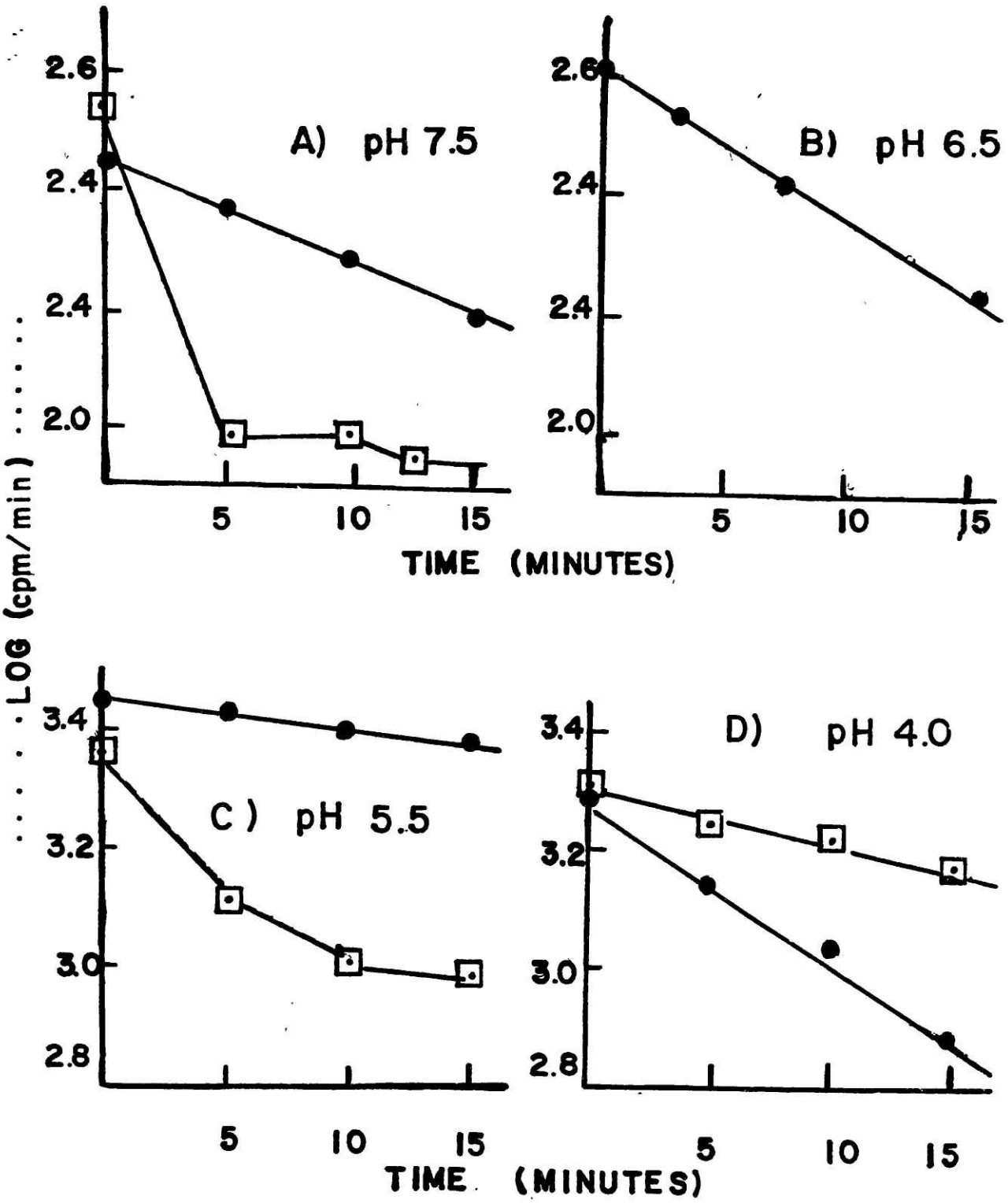

A) pH 7.5
B) pH 6.5
C) pH 5.5
D) pH 4.0
LOG (cpm/min)
TIME (MINUTES)
TIME (MINUTES)
2.6
2.4
2.4
2.0
3.4
3.2
3.0
2.8
5
10
15

TABLE 3-2

HALF LIVES AT 60 °C

	Amidase I	Phosphatases
Crude extract, pH 7.5	19 minutes	<3 minutes
Purified Amidase I †	12 minutes	———
Crude Extract, pH 5.5	35 minutes	≤ 5 minutes
Crude Extract, pH 4.0	11.5 minutes	36 minutes

† Amidase I purified by sequence outlined in figure 4. Phosphatase in such a preparation is too low for a determination of $t_{1/2}$.

* Half lives calculated from slopes of semilogarithmic plots of activity vs. duration of heat treatment (figure 7).

$$t_{1/2} = (\log 2)/ k$$

where k = slope of semilog plot.

amidase I preparations were examined: crude extract of NC4 cells (buffer A, pH 7.5) and enzyme purified by both ion-exchange and gel filtration (buffer B, pH 6.5). Aliquots of crude enzyme (20 ul) were heated at 60 ^{o}C for various times before being quenched with an equal volume of buffer and placed on ice. They were then assayed by the standard protocol. While fluoride-resistant phosphatase declined by 70% within 5 minutes and levelled off, amidase I decayed more slowly with half life of 19 minutes in buffer A (fig. 3-5A). Purified amidase I in buffer B was slightly more labile with a half life of 12 minutes than in the crude extract (fig. 3-5B). This may be an artifact reflecting the effect of phosphatase declining in the crude extract. As phosphatase declines, the apparent amidase I activity must increase. As a result of these studies, crude extracts were routinely heated to 60 ^{o}C for 5 minutes prior to column chromatography.

Effect of pH on Heat Lability.

In the above experiments, enzyme solutions were buffered near pH 7. Heat lability at 60 ^{o}C was examined in crude extract buffered at pH's 5.5 (buffer C) and 4 (buffer D). At pH 5.5, amidase I was more stable than at 7.5 with a remarkable half life of 35 minutes while phosphatase declined by 60% in 10 minutes (fig. 3-5C). Again phosphatase decay was biphasic and levelled off within 7 to 10 minutes, suggesting multiple isozymes. At pH 4, phosphatase was remarkably stable, exhibiting a half life of 36 minutes while amidase I became the more labile with a half life of 11.5 minutes (table 3-2).

Preparative Heat Treatment.

NC4 crude extract (pH 7.5) was heated at 60 ^{o}C in a water bath for 5 minutes, then rapidly chilled in a mixture of ice, water and salt. Heat-treated extract was subjected to centrifugation at 30,000xg for 15 minutes; yet, little protein sedimented. Phosphatase activity was reduced by 40%.

ION EXCHANGE ON DEAE CELLULOSE

Preparative Fractionation of Heat-Treated Extract.

A small portion of extract was set aside for analysis; the rest was applied to a column of DE-52 cellulose (3.5x29cm) equilibrated with buffer A. Protein was eluted with a linear gradient of KCl (50-700mM) in the same buffer (fig. 3.6), and quantified by its absorbance at 280 mu. A large burst observed in early fractions consisted of protein not retained by the ion exchanger and much of the colored material initially present in the extract. Amidase I was not eluted at this point. A set of late fractions #100-120, were rich in nucleic acids as evidenced by relative absorbance at 280 and 260 mu; this obviated the need for other procedures to remove them before chromatography.

Enzymatic Activities.

Three activities were monitored in the eluant: amidase I, phosphatase and esterases. Amidase was eluted as a broad peak at ∿200 mM KCl. Phosphatase consistently emerged as two peaks, when assayed against ^{32}P-compound I. The peak coinciding with amidase I was fortunately the lesser (see Nature of Phosphatases below). Esterase activity was fractionated into two peaks, one of them coincident with the amidase. Though in this profile the higher salt peak appears as a shoulder of the other, the two are more cleary resolved in other runs with extract that was not heat-treated. This evidence argues for at least two esterases present in late vegetative amoebae. In one experiment, N-acetyl glucosaminidase was also monitored and its separation from amidase I was clearly established (fig. 3-8).

Ion Exchange of Non-Heated Extract.

When an aliquot of the same extract, set aside before heat-treatment, was fractionated on DE-52 cellulose, two peaks of phosphatase were also

FIGURE 3-6

Profile of DE-52 column chromatography of D. discoideum NC4 crude extract heated at 60°C for 5 minutes.

Column was equilibrated with at least 10 volumes of buffer A and 18.7 ml of heat treated extract (303 mg of protein) were applied to the column.

Column dimensions: 3.5 x 29cm

Elution: 3 liter linear gradient from 50 to 700 mM KCl

Flow rate: 3.3 ml/minute

Amidase I. phosphatases, and esterases were assayed as described in Materials and Methods. Fractions 45 through 63 were pooled and concentrated to 25 ml.

FIGURE 3-6

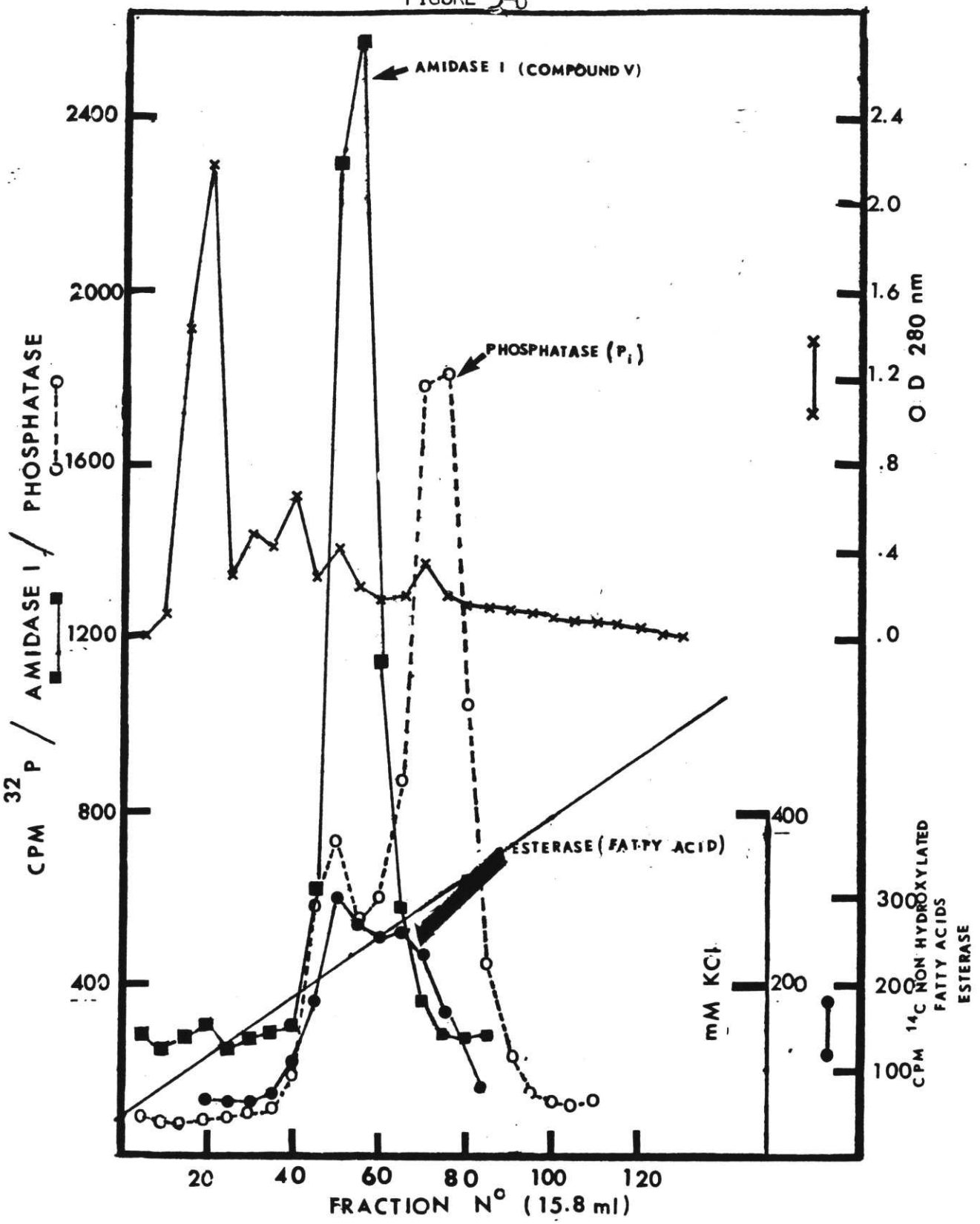


FIGURE 3-6

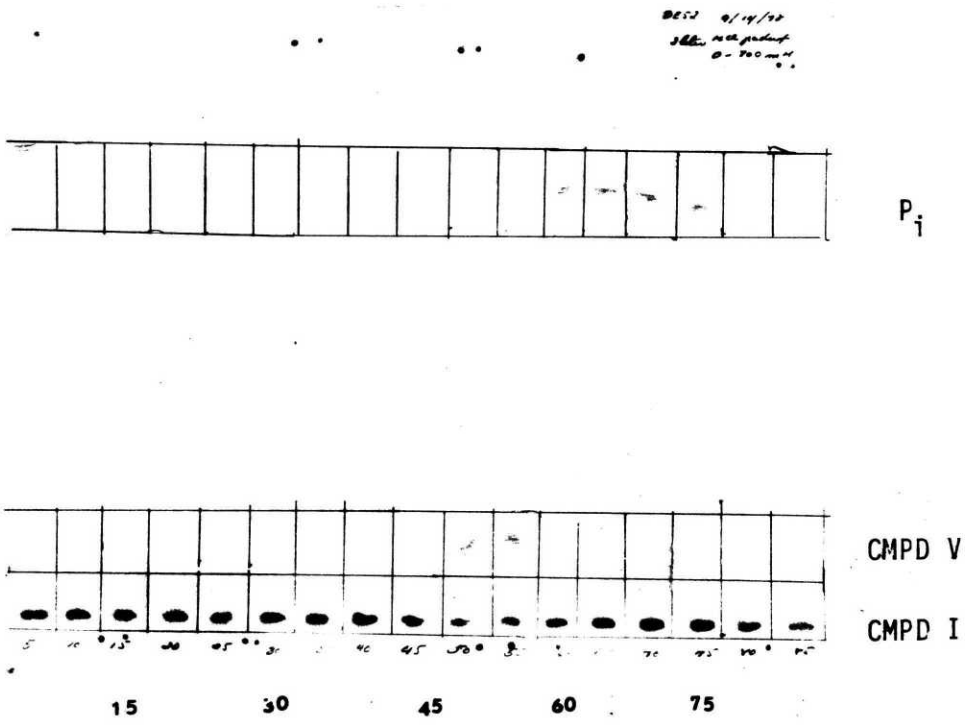
FIGURE 3-7

Autoradiogram of electrophoretic fractionation of amidase I and phosphatase assay mixtures for fractions arising from the DE-52 column presented in figure 3-6.

Every fifth fraction from 5 to 110 were assayed using ^{32}P-compound I as described in Materials and Methods. Only fractions 5 through 85 are depicted here.

For the identity of the spots observed see figure 3-2.

FIGURE 3-7



FRACTIONS 5 – 85

FIGURE 3-8

Profile of DE-52 column chromatography of D. discoideum NC4 extract (not heat treated).

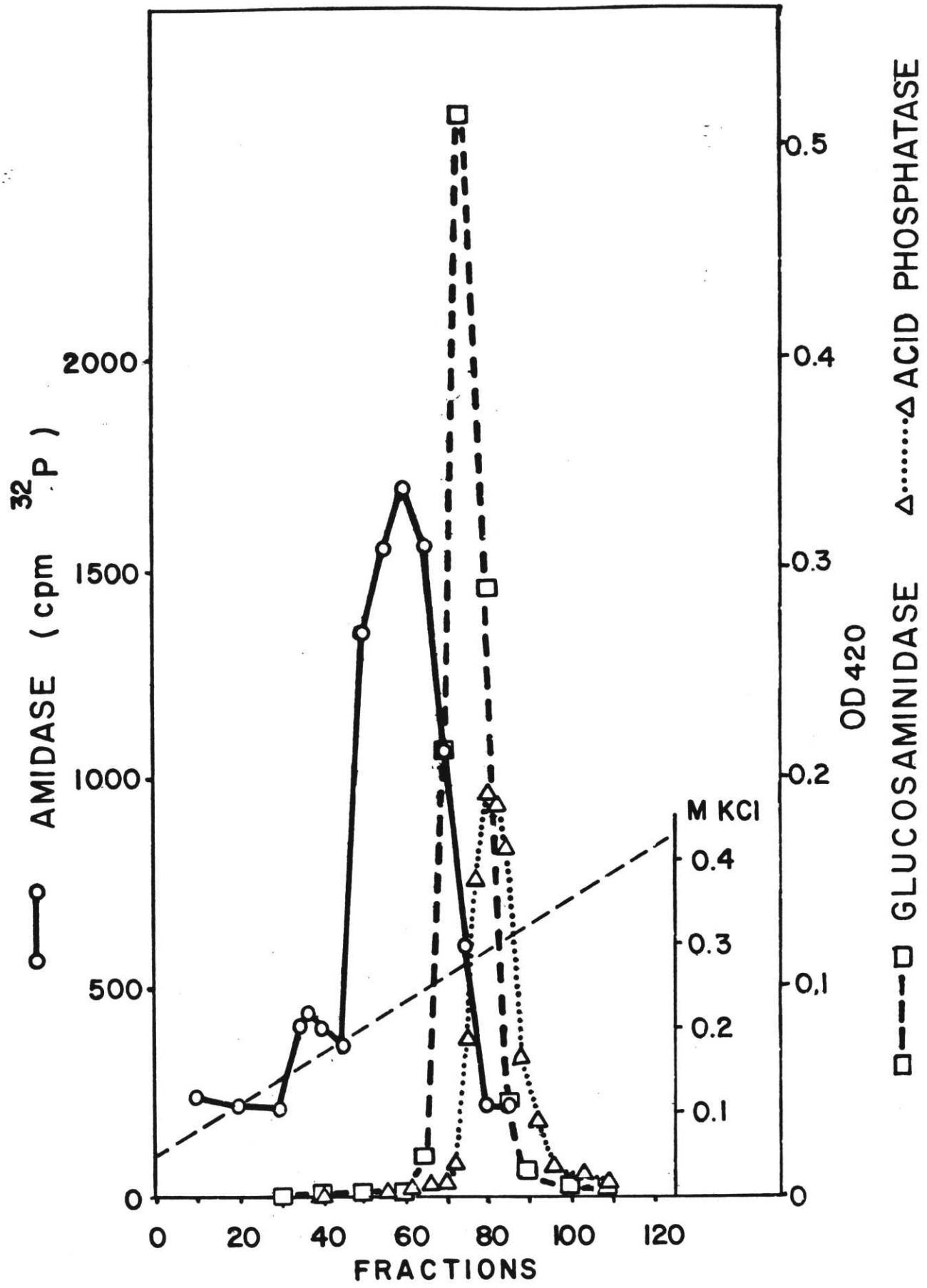
The column was executed as described in figure 3-6 with 690 mg of protein.

Column dimensions: 3.5 x 29 cm

Elution: 3 liter linear gradient from 50 - 700 mM KCl

Flow rate: 1.8 ml/minute

Amidase I was assayed with radioactive substrate; phosphatase with colorimetric substrate, p-nitrophenyl phosphate; glucosaminidase was also assayed as described in Materials and Method.


AMIDASE (cpm ^{32}P)
OD 420
M KCl
FRACTIONS
GLUCOSAMINIDASE
ACID PHOSPHATASE
0
500
1000
1500
2000
0.1
0.2
0.3
0.4
0.5
0
20
40
60
80
100
120

resolved (fig. 3-9). In this case, both peaks were nearly of equal height and overshadowed amidase I. This contrasts with the profile of heated extract (fig. 3-6) where the phosphatase peak coincident with amidase I was only one third the height of the other, and where amidase I was comparatively more active than either phosphatase. Although both phosphatases declined upon heat treatment, the one coincident with amidase I is much more labile. Thus, heat treatment preferentially affects that phosphatase activity most troubling to purification of amidase I.

Nature of Phosphatases.

DE-52 ion-exhange resolved acid phosphatase species in agreement with earlier observations of multiple isozymes in D. discoideum. This also corroborates conclusions reached in the above heat-inactivation studies that infer multiple isozymes in vegetative cells. As noted in the previous section, the peak co-eluted with amidase I is the more heat-labile.

The isozymes may be distinguished by their substrate specificities. While both liberate inorganic phosphate from LPS derivatives only the later eluting peak acts on p-nitrophenyl phosphate. Figure 3-8 clearly demonstrates this where fractions from non-heated extract were assayed with the synthetic substrate. Only the later peak released the chromophore. In chapter 4, this phosphatase is shown to be the one suppressed by potassium fluoride.

Pooling Amidase I Fractions.

From chromatography of the heat-treated extract (fig. 3-6), amidase I was pooled so as to exclude the larger, non-coincident phosphatase. One esterase and the other phosphatase could not be exluded. In this preparation, fractions 45-63 were concentrated by means of an Amicon ultrafiltration cell with PM30 filter to a volume of 25 ml. In the concentrate, more activity was recovered than was apparently applied to the column. Once again

FIGURE 3-9

Profile of DE-52 column chromatography of D. discoideum NC4 crude extract with no prior heat treatment.

Column was equilibrated with at least 10 volumes of buffer A. 0.5 ml of NC4 extract were applied to the column.

Column dimensions: 1.5 x 11 cm

Elution: 260 ml linear gradient from 50 to 550 mM KCl.

Flow rate: 2.1 ml/minutes

Amidase I and phosphatases were assayed as dexcribed in Materials and Methods. Esterases were not followed in this case.

FIGURE 3-9

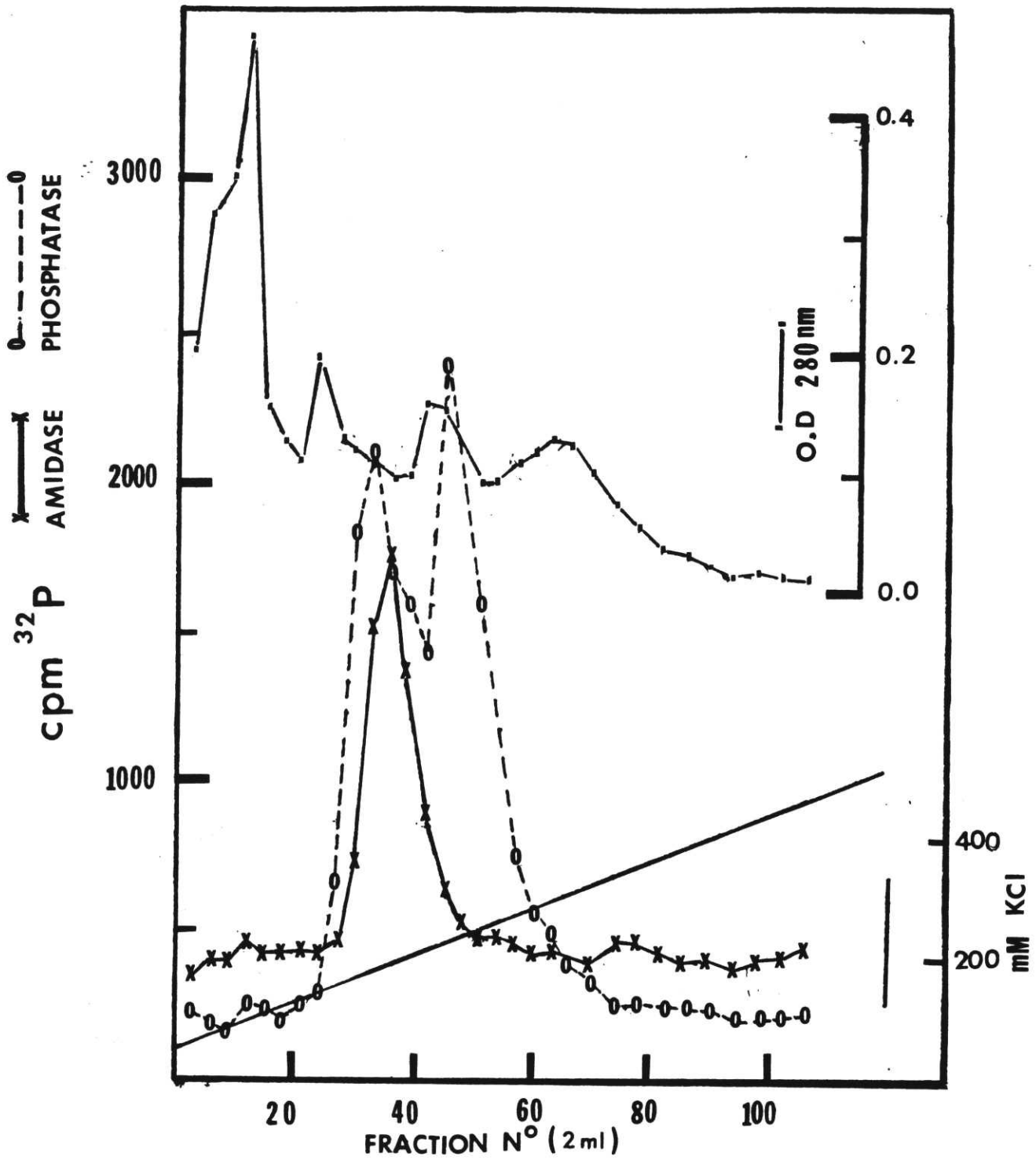


FIGURE 3-9

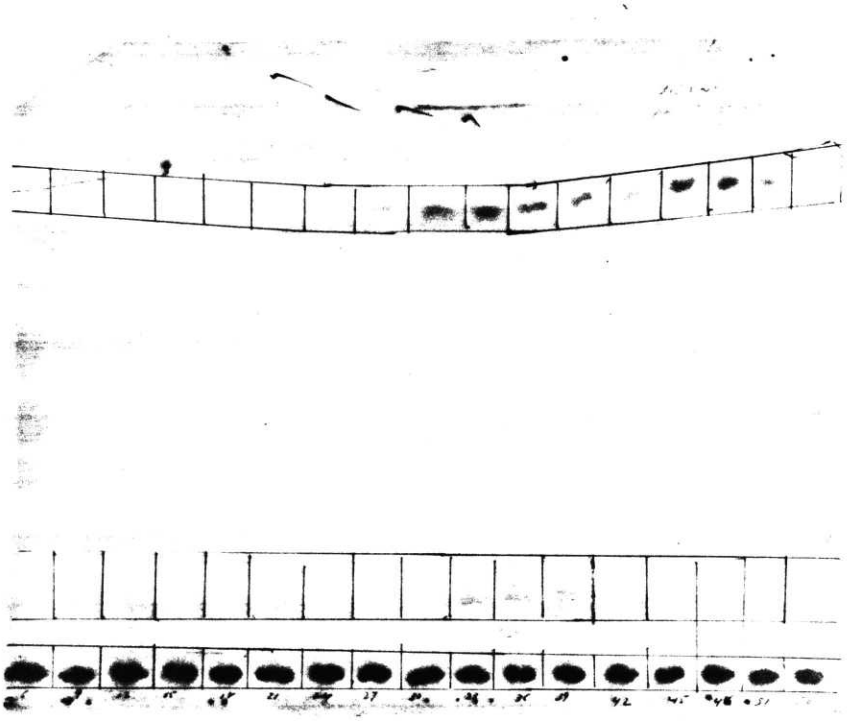
FIGURE 3-10

Autoradiogram of electrophoretic fractionation of amidase I and phosphatase assay mixtures for fractions arising fron the DE-52 column presented in figure 3-6.

Every third fraction up to 120 were assayed using ^{32}P compound I as described in Materials and Methods. Only fractions 6 to 54 are depicted in this figure.

For the identity of the spots on the electrophoretogram see figure 3-2.

FIGURE 3-10



the interference of phosphatase is manifest. Specific activity of the amidase was increased 11-fold as the ratio of amidase I to phosphatase improved 4-fold.

GEL FILTRATION

Preliminary studies showed that amidase I purified as described above is included by Sephacryl S200 (1.5x50cm) but only slightly so by Sephadex G100 (1.5x50cm). Limits of both columns were established with blue dextran (Mwt>200,000) for the void volume and with cytochrome C (Mwt=16,000) for the included volume. It was also evident that the phosphatase contaminant lagged slightly behind amidase I.

Preparative Gel Filtration on Sephacryl S200.

Concentrated enzyme (10ml) from the above ion-exchange procedure was fractionated on Sephacryl S200 (fig. 3-11). The column was eluted with buffer B at 12 ml/hr. Phosphatase appears at the trailing end of a much larger amidaseI peak and can be effectively separated from it. Esterase, however, overlaps significantly with the amidase so as to render resolution impossible. The majority of protein is eluted as a broad peak just after the void volume , well before amidase I. As a result, specific activity is much increased (>10-fold).

When crude extract was not heat treated prior to ion-exchange chromatography, the resulting amidase preparation yields a markedly changed profile (fig. 3-12). Rather than one major protein peak near the void volume, two peaks are observed, one of which contains amidase I. Although amidase I and the phosphatase are resolved, overall purification declined.

Pooling and Concentrating Amidase I.

Fraction 65-75 were combined so as to exclude the phosphatase. They were concentrated in an Amicon ultrafiltration cell with PM30 filter to

FIGURE 3-11

Profile of gel filtration on Sephacryl S200 of amidase I prepared by DE-52 ion exchange chromatography (figure 3-6).

Sephacryl S200 column was equilibrated with buffer B and 10 ml of concentrated amidase I (10 mg) from prior DE-52 column was eluted with the same buffer.

Column dimension: 2 x 90 cm

Flow rate: 12 ml/ hour

Amidase I, phosphatases, and esterases were assayed as described in Materials and Methods. Fractions 67 through 75 were pooled and concentrated to 2.14 ml.

See table 3-1.

FIGURE 3-11

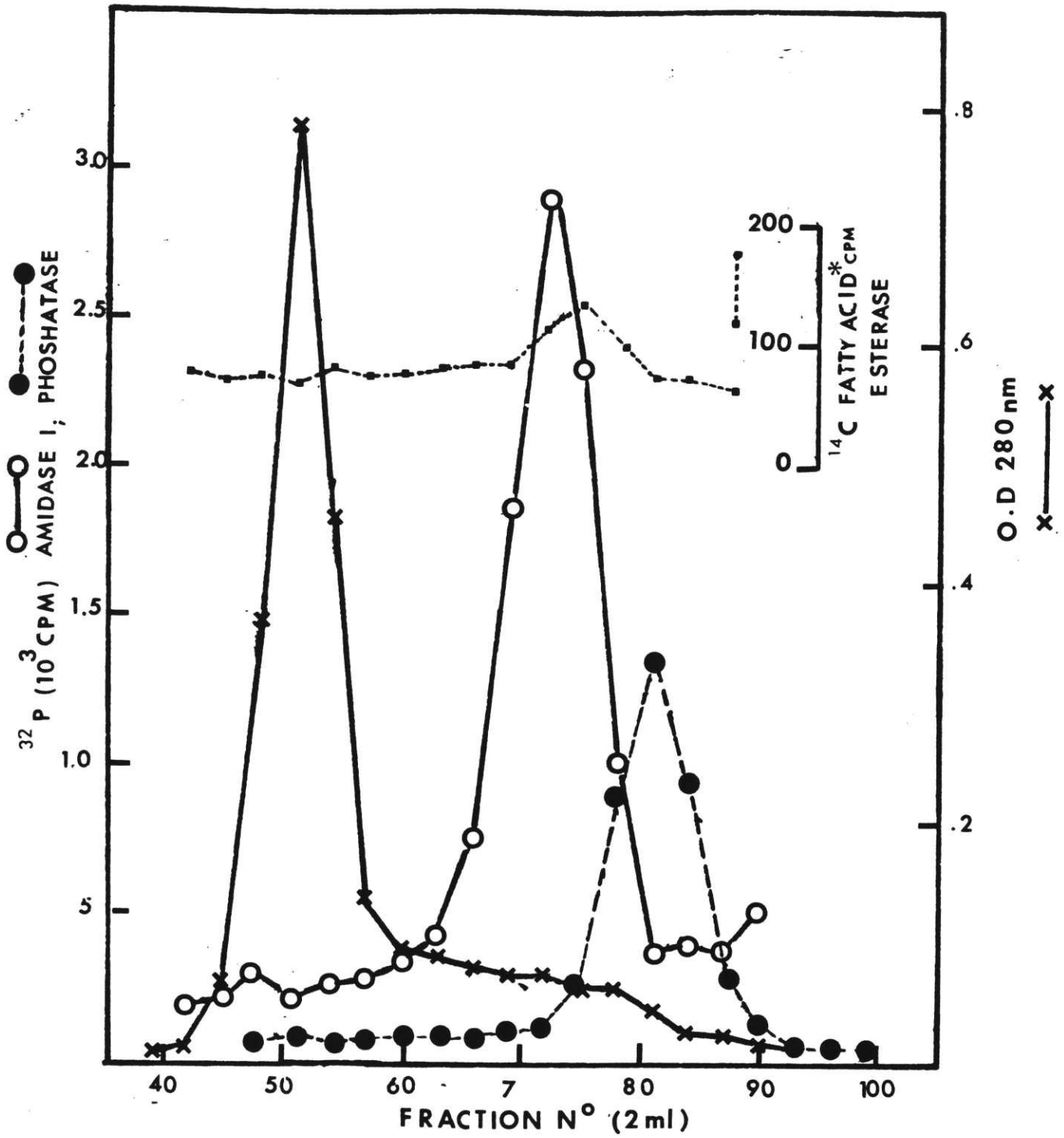

AMIDASE I; PHOSHATASE
^{32}P (10^3CPM)
3.0
2.5
2.0
1.5
1.0
5
200
100
0
^{14}C FATTY ACID* CPM
ESTERASE
.8
.6
.4
.2
O.D 280nm
40
50
60
7
80
90
100
FRACTION N° (2ml)

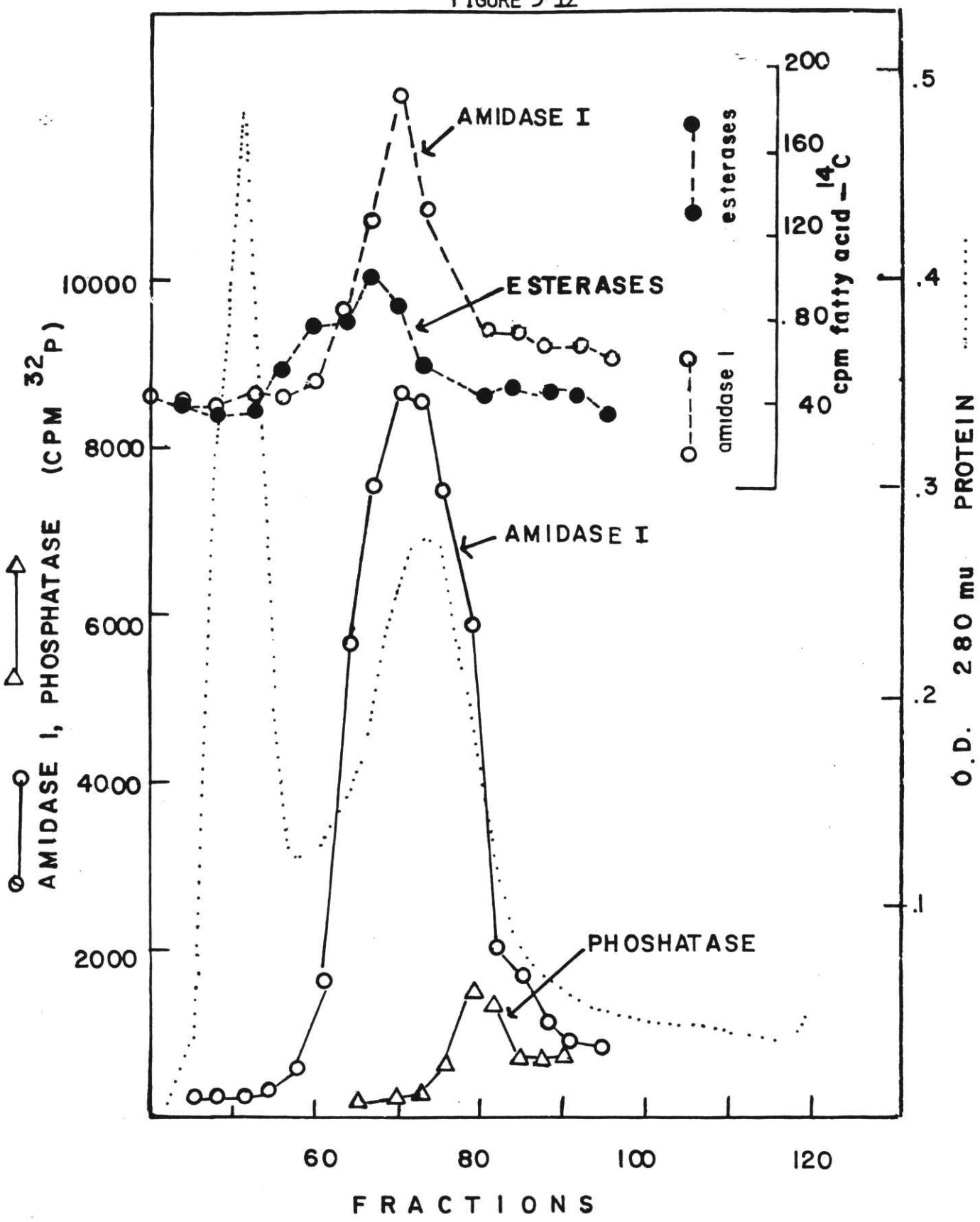
FIGURE 3-12

Profile of gel filtration on Sephacryl S200 of amidase I prepared by DE-52 ion exchange chromatography (see fig. 3-8)

The column was equilibrated and executed as described in the legend of figure 3-11.

Amidase I and phosphatase activity were determined in the routine fashion with ^{32}P-compound I.
Amidase I was also determined from release of β-hydroxymyristate from ^{14}C-LPS; esterase activity was also measured in the same reaction by release on non-hydroxylated fatty acid.

FIGURE 3-12


AMIDASE I
ESTERASES
AMIDASE I
PHOSHATASE
esterases
amidase I
cpm fatty acid ^{14}C
AMIDASE I, PHOSPHATASE (CPM ^{32}P)
O.D. 280 mu PROTEIN
FRACTIONS
10000
8000
6000
4000
2000
200
160
120
80
40
.5
.4
.3
.2
.1
60
80
100
120

2.14 ml. At this point, the specific activity reached 110 units/mg.

Molecular Weight Estimate for Amidase I.

The Sephacryl S200 column were calibrated with blue dextran (>200,000 daltons), bovine serum albumin (60,000) and cytochrome (16,000). It was observed that amidase I chromatographed only slightly ahead of BSA (fig.3-13). Peak height of BSA is much lower than predicted from the amount applied because much of this protein binds to the cibacron dye on blue dextran and is therefore voided. The small peak centered at fraction 92 represents BSA uncomplexed to dextran. From these observations, the molecular weight of amidase I was estimated in the range of 60-80,000 daltons. Upon rechromatography, S200-purified amidase I was eluted in the same fractions centered at #87.

Electrophoretic Analysis of Purified Protein.

Samples from all stages of purification were examined by SDS-PAGE (fig. 3-14). As indicated by table 3-1, little protein was lost as a result of heat treatment; profiles both before and after heat treatment were nearly identical. Ion-exchange chromatography greatly simplifies the protein pattern. After gel filtration, the amidase I preparation consists of 8-10 polypeptides on SDS-PAGE.

AFFINITY PURIFICATION

Affinity purification of the amidases was attempted with N-acetyl glucosamine and di-N-acetyl chitobiose coupled to epoxide-activated Sepharose 6B. Experiments described in chapter 6 show that amidase I is inhibited by both of these compounds. A control gel also prepared by quenching activated Sepharose in the absence of ligand (Materials and Methods). The preparation of the affinity resins by bis-diglycidil-ether activation introduces

FIGURE 3-13

Comparaison of amidase I to bovine serum albumin and cytochrome C by gel filtration on Sephacryl S200. DE-52 purified Amidase I was chromatographed; BSA and cytochrome C were chromatographed through the same column under identical conditions.

Column dimensions: 3.1 x 115 cm.

Flow rate: 32 ml/hour.

Elution profile of BSA and Cyt. C is not included, only the center of their peaks is depicted.

FIGURE 3-13

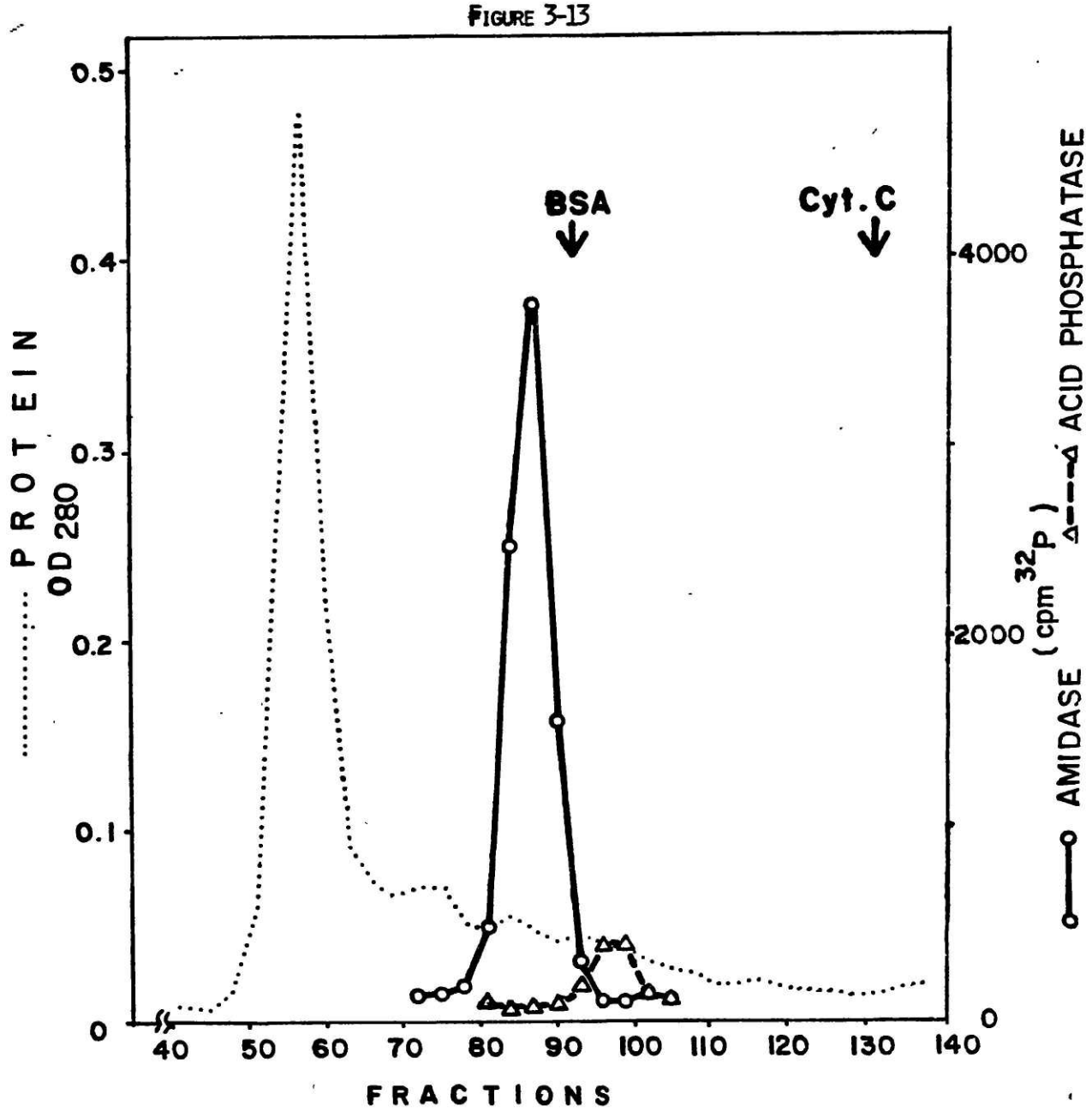

PROTEIN
OD 280
0.5
0.4
0.3
0.2
0.1
0
BSA
Cyt.C
4000
2000
0
AMIDASE (cpm ^{32}P) ACID PHOSPHATASE
40
50
60
70
80
90
100
110
120
130
140
FRACTIONS

FIGURE 3-14

Polyacrylamide-gel electrophoresis of amidase I preparation, executed as described in Materials and Methods.

A. Crude extract.

B. Heat-treated crude extract.

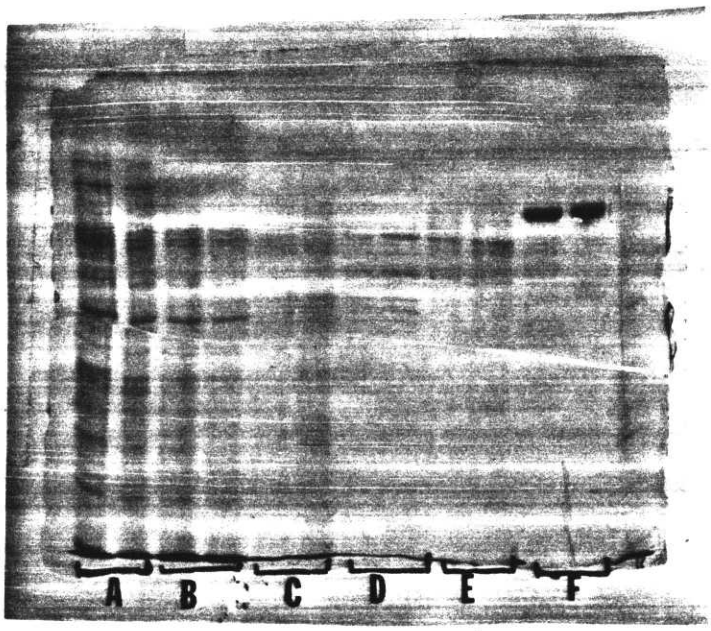
C. DE-52-purified amidase I preparation.

D. Sephacryl-S200-purified amidase I preparation.

E. Same as D except that protein was concentrated by lyophilization.

F. Bovine Serum Albumin.

FIGURE 3-14



a chain of 10 carbons and oxygens between ligand and resin surface. This avoids steric interference by the resin surface with the interaction of enzyme and ligand.

Amidase I purified up to the DE-52 cellulose stage (2.5 mg protein/ml) was applied in 180 ul aliquots to affinity columns (0.5x1.6 cm) equilibrated at pH 6.5, 4.5 or 3.0. They were then eluted with the equilibration buffers, and five fractions (0.6 ml) were collected at 0.1 ml/minute. In all cases, amidase I was voided in the first three fractions; even at pH 3. the amidases' optimum, no retention was observed (table 3-3).

To verify the effectiveness of the columns, wheat germ agglutinin, an N-acetyl-glucosamine binding lectin, was chromatographed a pH 4.5. Retention was demonstrated and specific elution of the lectin effected with 0.1 M N-acetyl glucosamine.

HYDROPHOBIC CHROMATOGRAPHY

The utility of hydrophobic chromatography was evaluated with one milliliter columns of alkylated agarose (Myles) with alkyl chains of 2 to 10 carbons , also an unsubstituted control. Before each trial, resins were rinsed with dioxane/water (1/1,v/v) and then equilibrated with buffer B, 100 mM KCl.

Amidase I (DE-52 purified, 2.5mg/ml) was applied to the column and eluted with the above buffer. When emerging activity was plotted against alkyl chain length (fig. 3-15), it is clear that chains of 6 carbons or more retained all of the applied activity. With 4 carbons, activity was partially retained.

As discussed in chapter 7, both amidases are active in buffers containing ethylene glycol. Sephacryl S200 purified amidase I (200 ul) was applied to

TABLE 3-3

	Fraction	Control	N-acetyl-glucosamine	di-N-acetyl-chitobiose
A				
	1	802	875	778.5
pH 6.5	2	508	979	638
5 mM phosphate	3	196	361	160
50 mM KCl	4	135	275	119
0.1 mM EDTA	5	168	255	106
B				
	1	267	391	343
pH 4.5	2	556	307	305
10 mM acetate	3	213	132	120
50 mM KCl	4	187	127	102
0.1 mM EDTA	5	154	122	94
C				
	1	2989	7343	6043
pH 3.0	2	5181	6657	5216
10 mM citrate	3	1080	5804	922
50 mM KCl	4	524	2556	658
	5	490	664	461

^{32}P Sp. Act. A
B
C

Five fractions from 1 ml affinity columns, activity of amidase I here presented as counts per minute of compound V generated.

FIGURE 3-15

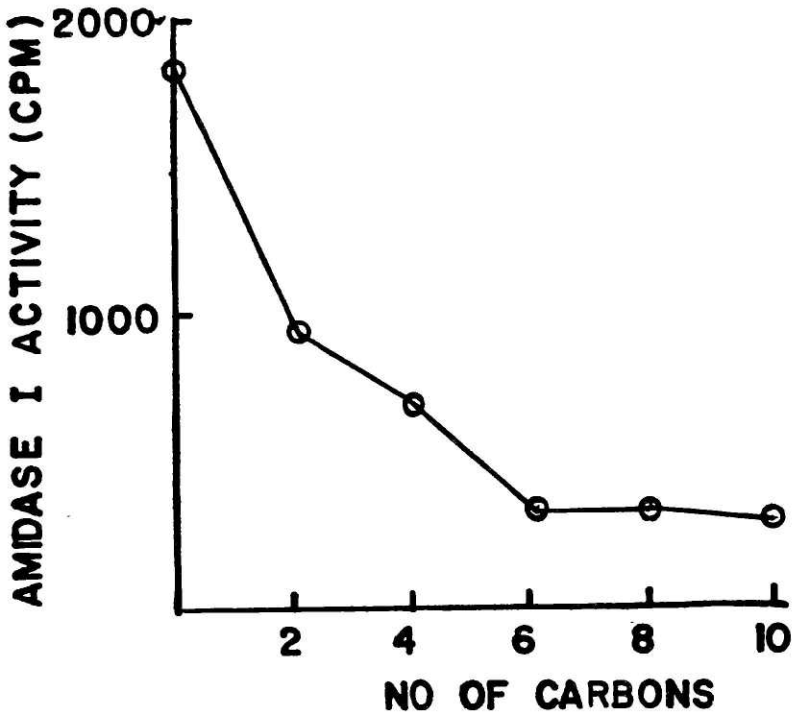
Amiase I activity retained by alkyl agarose columns of varying chain lengths.

Column dimensions: 0.5 x 5 cm

Buffer B, 100 mM KCl.

Details in text.

FIGURE 3-15



the the C_4-alkylated column, washed with buffer B, 100mM KCl, and eluted with buffer B containing 40% ethylene glycol. Figure 3-16 shows an autoradiogram of the TLC-fractionated assay of each fraction from this column. Amidase I activity, reflected in the appearance of compound V, is eluted only upon application of the ethylene glycol. Thus, this column is of potential utility in further purification of amidase I.

STORAGE OF AMIDASE I

At -80 ^{o}C, purified preparations lost more than 80% of their activity within one month, although crude extract remained active when frozen at that temperature for extended periods. Pure amidase I was stable when stored at 0 ^{o}C in an ice-filled dewar. It has been preserved thus for nearly two years with little loss of activity.

LPS-CLEAVING FATTY-ACYL ESTERASES

By DE-52 cellulose chromatography, LPS-specific fatty-acyl esterases were resolved into two species, one of which co-purified with amidase I even on Sephacryl S200. Yet, since the overlap is not exact, amidase and esterase activity most likely belong to distinct proteins. Many amidases, notably proteases, also hydrolyze ester-analogs of their natural substrates; in this instant, that is not the case.

After gel filtration on Sephacryl S200, fractions were assayed with ^{14}C-LPS labelled predominantly on its fatty-acyl chains (Materials and Methods). Amidase I was determined from ^{14}C-β-hydroxymyristic acid liberated, while esterases were quantitated in terms of non-hydroxy fatty acids. These two classes of fatty acids are well resolved by TLC on silica plates.

FIGURE 3-16

Assay of amidase I in fractions eluted from C_4 alkyl agarose column (0.5x5cm) with buffer B, 100 mM KCl.
After fraction 5, 40% ethylene glycol was included into the elution buffer.

Assays were analyzed by TLC on HR/DEAE cellulose (Materials and Methods)

FIGURE 3-16



1 2 3 4 5 6 7 8 9 10 11 M

FRACTIONS

Note that only one of the four ester-linked fatty acids is a hydroxyacid. For corroboration, amidase I was also assayed in the standard fashion with ^{32}P-compound I by monitoring ^{32}P-compound V. The profile of amidase I in terms of either assay coincided perfectly (fig. 3-12). On the other hand, the esterase profile, defined by non-hydroxy acids, was somewhat shifted with respect to amidase I. This observation suggests that distinct molecules are involved.

Esterase activity was found to dissappear in a 6 month old preparation of amidase I purified by DE=52 ion-exchange. This preparation was active with respect to amidase I as evident in release of β-hydroxymyristic acid from ^{14}C-LPS (fig. 3-17) and degradation of compound I (not shown); however, it lacked esterase activity as shown by the failure to release non-hydroxylated fatty acid (fig. 3-17). The characteristics of this enzyme preparation reinforces the suggestion that amidase I and esterase are separate enzymes. Furthermore, the restricted substrate specificity of amidase I (chapter 6) makes it unlikely that it would hydrolyze other sites on LPS.

FIGURE 17

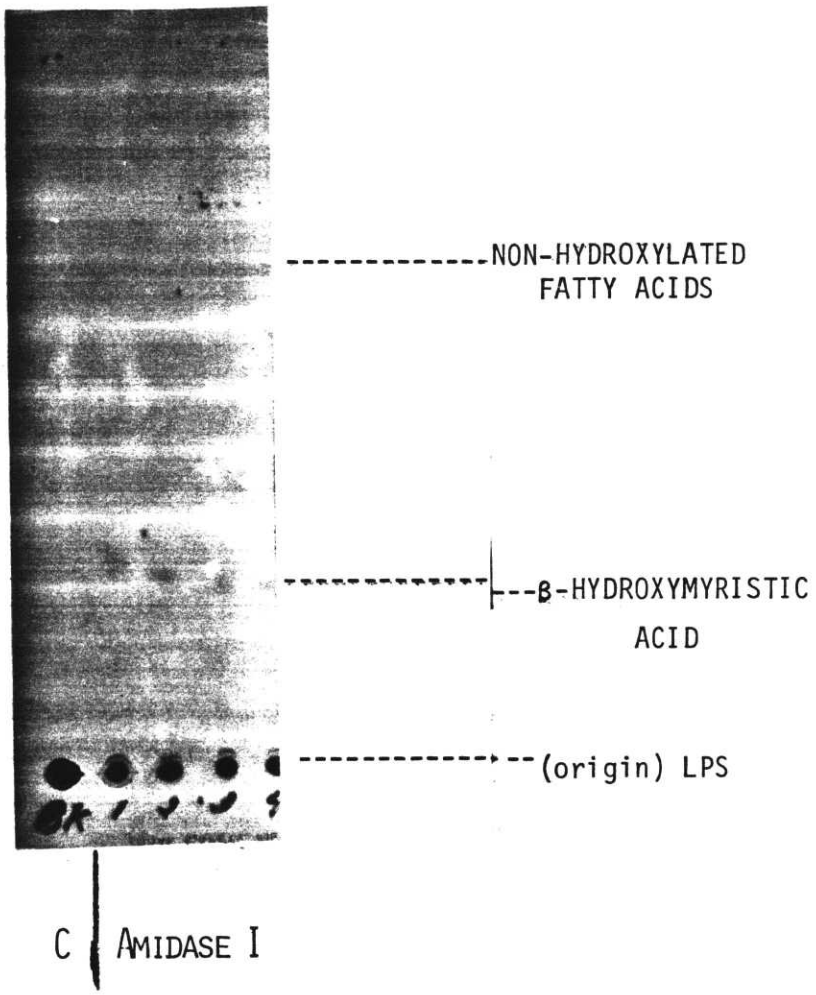
Deacylation of ^{14}C-LPS by an amidase I preparation purified by ion-exchange on DE-52 cellulose. This preparation lacks esterase activity.

Amidase I is reflected by release of β-hydroxymyristic acid
Esterases should be manifested in release of non-hydroxylated fatty acid.

C = Control assay mixture with no enzyme added

AMIDASE I = active amidase I preparation lacking esterase.

FIGURE 3-17


NON-HYDROXYLATED
FATTY ACIDS
β-HYDROXYMYRISTIC
ACID
(origin) LPS
C
AMIDASE I

Chapter 4

PURIFICATION OF AMIDASE II

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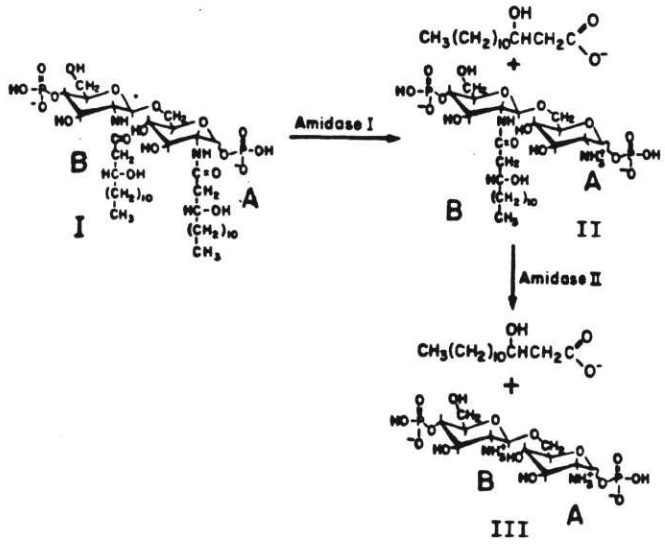
STORAGE OF AMIDASE II. 151

AMIDASE I IN D. DISCOIDEUM AX-3. 153

SUMMARY

An amidase, hydrolyzing the fatty-acyl amide at site B of lipid A derivatives subsequent to cleavage at site A by amidase I (see Chapter 3), was purified >12 fold from protein secreted by an axenic D. discoideum mutant AX-3. Ion-exchange chromatography on DE-52 and gel filtration through Sephacryl S200 were applied. The enzyme, amidase II, is free of contaminating phosphatase and has no amidase I activity. Chromatography of amidase II on affinity columns and on hydroxylapatite is also discussed.

Amidase I was absent among the secreted hydrolases of AX-3 and could not be detected in lysate of axenically grown cells. In contrast, cells grown on the gram-negative bacteria Aerobacter aerogenes did contain amidase I. Differences in the enzymic repertoire, amidases and phosphatases, were compared among AX-3 grown axenically and NC4 or AX-3 grown on bacteria.



Successive degradation of Compound 1 by Amidase 1 and Amidase 11

INTRODUCTION

Amidase II, hydrolyzing the distal amide linkage of LPS derivatives, occurs in low levels in crude extracts of vegetative D. discoideum NC4. On the other hand, high activity is found among secreted protein of AX-3 when grown axenically; in fact, it was first isolated as contaminant of purified N-acetyl-glucosaminidase purified from such secretions (20). Purification of amidase II is outlined in figure 4-1 and table 4-1.

Extracellular Hydrolase of AX-3.

When cultured axenically, AX-3 cells remain in the vegetative phase and secrete large quantities of acid hydrolases (173). When washed free of nutrients and placed on buffered filters, they proceed through the final stages of development, aggregation and culmination (161). While amidase II abounds among the secreted hydrolases, we could detect no amidase I activity. Also only one of two phosphatases observed in NC4 lysate was found among these hydrolases and it was fully inhibited by KF (fig. 4-2).

Origin of the Extracellular Hydrolases.

It is clear that the secreted enzymes are of lysosomal origin. Firstly, glycosidases isolated from extracellular fluid by Watts and Ashworth (185) among others are also concentrated in the lysosomal fraction of amoebae. Secondly, their pH optima, ~3, suggests that they must be sequestered in an organelle capable of generating such acidity. Thirdly, some possess a common antigen prevalent among lysosomal hydrolases of D. discoideum (170).

Secretion of lysosomal enzymes into the extracellular fluid (pH 6.5) serves no apparent catabolic role and may reflect commonalities between axenically grown cells and those undergoing the early stages of development as shown by Dimond et al. (168,169). The absence of amidase I and of one phosphatase isozyme may indicate heterogeneity of lysosomal particles and

TABLE 4-1

		Volume	Units/ml	Units		Mg/Ml	Mg	Units/Mg	Efficiency
1)	0.5 M	24 ml	5.59	134.2	273	9.0	216	0.62	100%
2)	1 M NaCl	40	3.49	139.6		5.3	212	0.66	
3)	DE-52 Fract. 16-27	183.6	0.764	140.3		~ 0.1	~ 18	~ 7	51%
4)	Conc[a] DE-52 Fract.	3.4	9.66	32.8 *120.44		1.7	5.8	5.67	85%
5)	Conc[b] DE-52 fract.	5.79	9.91	57.3 *78.94		2.7	15.63 *21.5	3.67	49%
6)	S-200[c]	2.22	6.96	15.45 *43.7		0.9	1.99	7.73	62%
								Overall	16%

[a] From 50 mls of 3

[b] From 133 ml of 3

[c] From 2.5 ml of 5

* Expected quantity if all recovered activity from previous step was utilized.

FIGURE 4-1

Flow chart of the purification of amidase II from the extracellular fluid of D. discoideum AX-3 cultured in axenic medium.

FIGURE 4-1

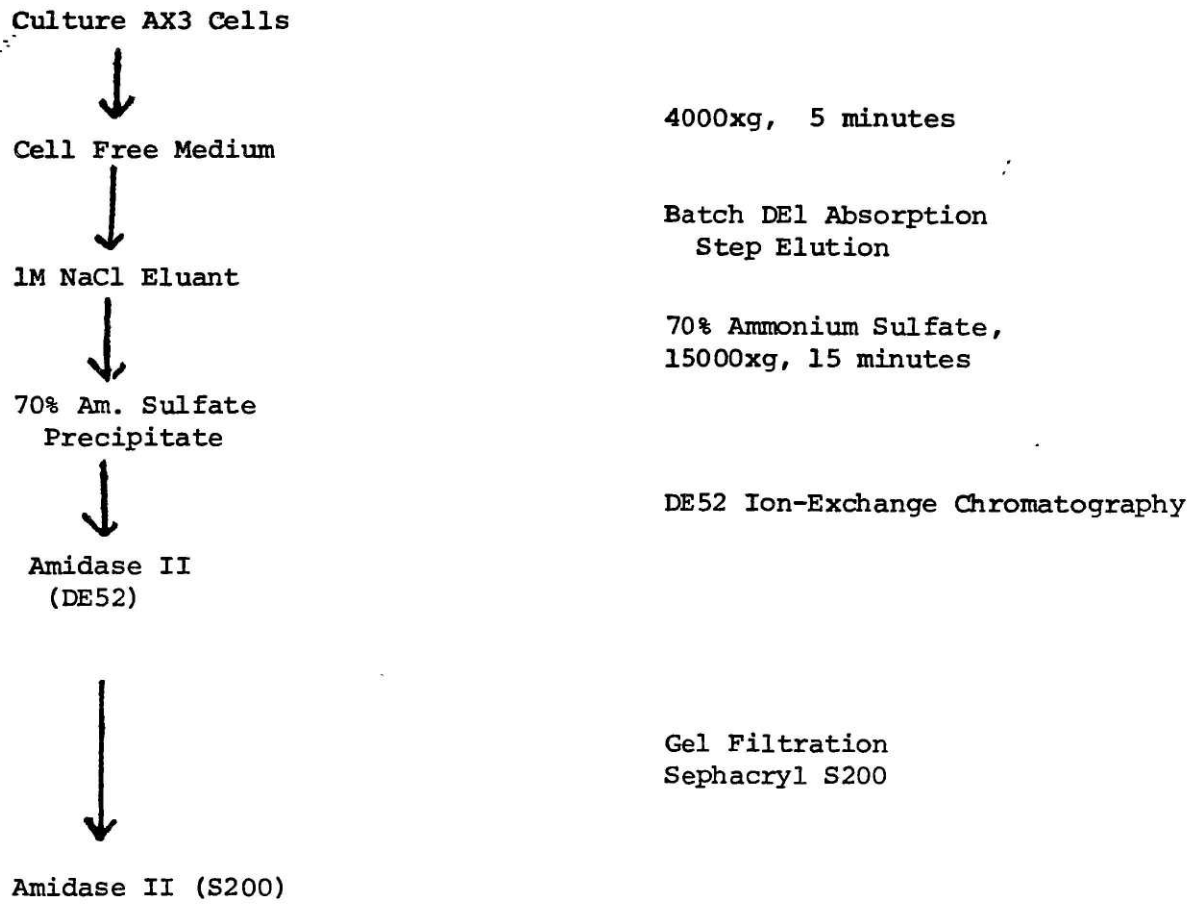
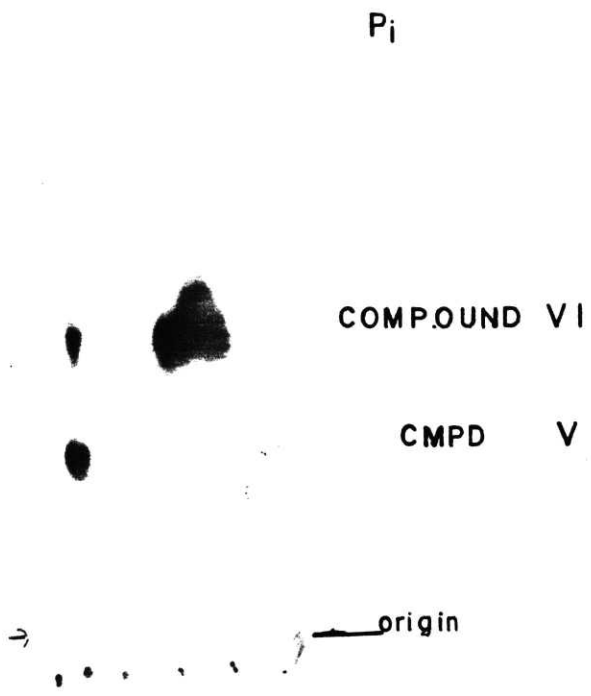

Culture AX3 Cells
4000xg, 5 minutes
Cell Free Medium
Batch DE1 Absorption
Step Elution
1M NaCl Eluant
70% Ammonium Sulfate,
15000xg, 15 minutes
70% Am. Sulfate
Precipitate
DE52 Ion-Exchange Chromatography
Amidase II
(DE52)
Gel Filtration
Sephacryl S200
Amidase II (S200)

FIGURE 4-2

Degradation of compound V by D. discoideum AX-3 crude extracellular hydrolases. Standard assay protocol described in Materials and Methods, reactions anlyzed by electrophoresis.

FIGURE 4-2



that axenic cells maintain only a subset of these. Lysosomal heterogeneity has been demonstrated with respect to certain hydrolases (169). Weiner and Ashworth (166) have established clear differences in lysosomal content and density between cells grown axenically or on bacteria. Thus axenic conditions suppress some while promoting other hydrolases.

PREPARING CRUDE EXTRACELLULAR HYDROLASES

The protocol to concentrate the extracellular enzymes was first described by Watts and Ashworth (185) and followed with slight modification. In our case, 9.6 liter cultures of D. discoideum AX-3 in HL5 medium, subdivided into 800 ml portions, were grown to 0.6 to $1x10^7$ cells/ml. Small aliquots were sampled at various times and counted with a haemocytometer. The doubling time was ~11 hours. Cells were removed by centrifugation at 4000xg for 15 minutes and resuspended in buffer A as described for NC4 myxamoebae. Throughout, these operations were carried out at 4 ^{o}C or vessels were kept on ice.

Floc-grade DEAE cellulose (1 kg, Whatman) was washed as prescribed with 1 N HCl and 1 N NaOH and equilibrated with buffer B. It was stirred into the cell-free supernatant at 4 ^{o}C for 30 minutes; then, protein was eluted in three successive 1 liter washes of 0.1M, 0.5M and 1.0M NaCl in buffer B. All three fractions were concentrated by ammonium sulfate precipitation at 70% of saturation, and protein was recovered by centrifugation at 15,000xg for 15 minutes. These pellets were redissolved in small volumes of buffer B and dialyzed against the same.

Amidase I was equally distributed between the two higher fractions (table 4-1) while insignificant activity was eluted into the 0.1M fraction. In subsequent preparations, the intermediate elution, 0.5M, was omitted and

compensated for by increasing the 1M NaCl elution to 1.5 liters. AmidaseII (273 units) was recovered from this procedure at a specific activity of ∿0.63 units /mg.

Acid phosphatase was observed in the absence of fluoride whether by release of inorganic phosphate from ^{32}P-compound V or determined colorimetrically p-nitrophenyl phosphate. Unlike that activity in NC4 lysate, this phosphatase was fully inhibited at high concentrations of fluoride.

ION EXCHANGE ON DEAE CELLULOSE

The two amidase II containing fractions were combined and applied onto a DE-52 cellulose column (3.5x29cm) pre-equilibrated with buffer B at 50 mM KCl. Protein were fractionated with a 2 liter gradient of KCl (50-500mM) (fig. 4-3). Two major protein peaks dominate the protein profile.

Enzymatic Activities.

Amidase II activity straddles the two protein peaks. When the column was equilibrated without KCl, unabsorbed protein could be eluted in a pre-wash while amidase II and the later protein peak are retained until the gradient is applied. In this case, amidsae II emerges on the leading shoulder of the second peak near 75 mM KCl. N-acetyl-glucosaminidase was well resolved as it emerges near 250 mM, as did this activity derived from NC4 lysate (fig. 3-7), and must represent the same protein. Unlike the NC4 lysate, the AX-3 protein produce only one of the two phosphatase peaks eluting after glucosaminidase at 300 mM KCl.

Nature of Phosphatase Activity.

As discussed in the previous chapter, two phosphatase species were observed in NC4 lysate and eluted on both sides of glucosaminidase. Moreover, only the late peak hydrolyzes p-nitrophenyl phosphate while the other is

FIGURE 4-3

Profile of DE 52 column chromatography of D. discoideum AX3 crude extracellular protein.

Column was equilibrated with 10 volumes of buffer B.

Both the 0.5 and 1 M NaCl fraction were applied onto the same column.

Column dimensions: 3.5x29 cm

Elution: 2 liter linear fradient, 50-500 mM KCl

Flow rate: 7.4 ml/min.

Amidase II, phosphatases, and glucosaminidase were assayed.

FIGURE 4-3

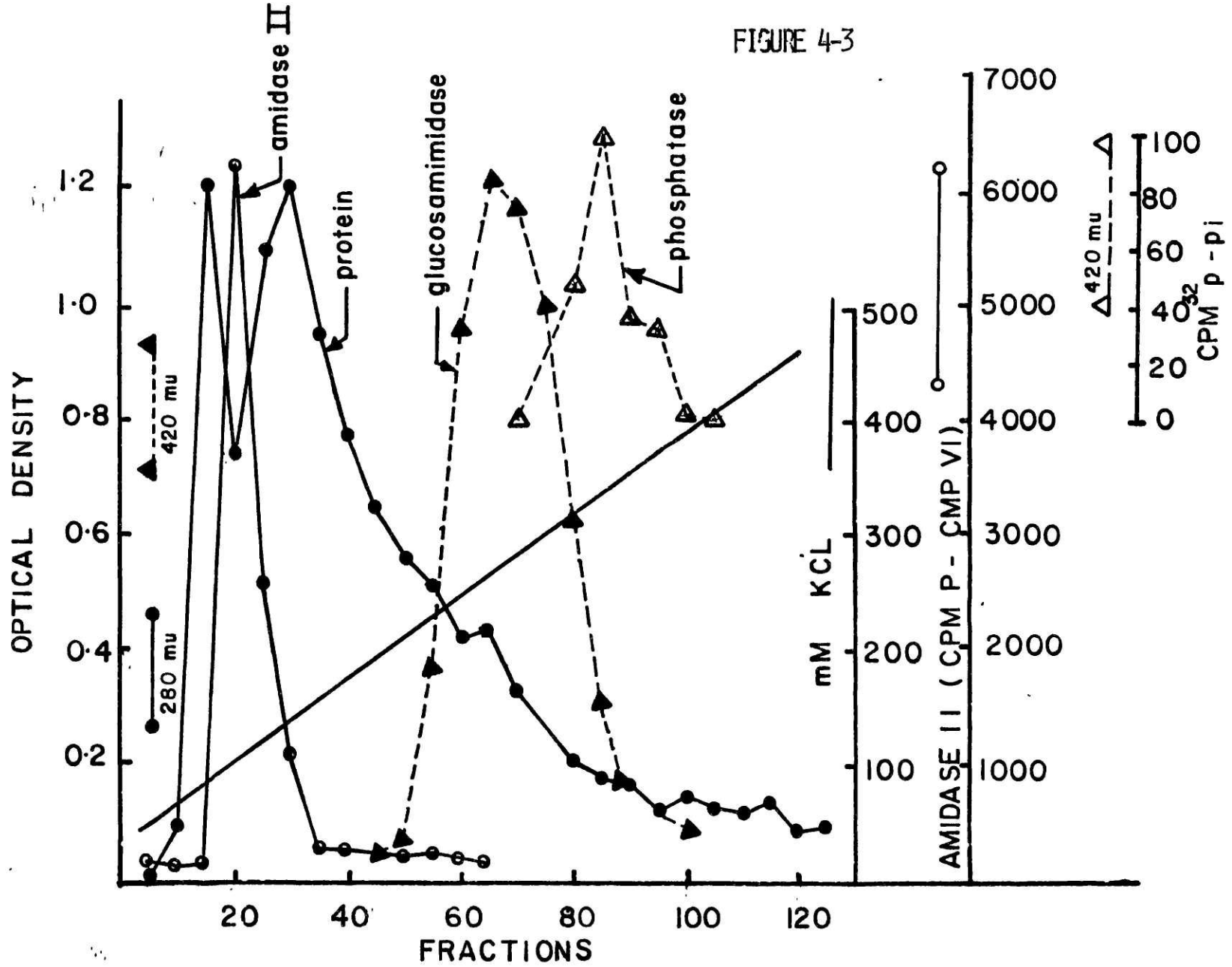
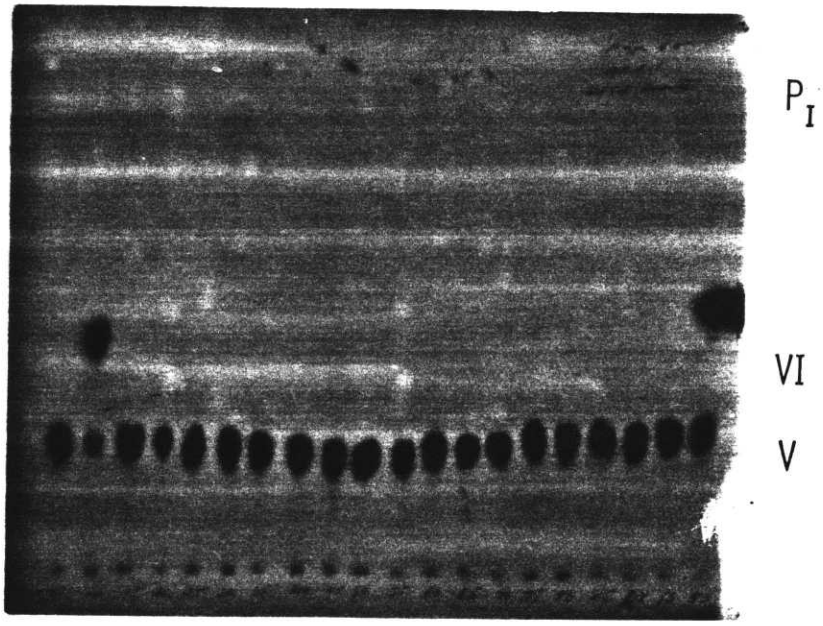

amidase II
protein
glucosamimidase
phosphatase
OPTICAL DENSITY
1·2
1·0
0·8
0·6
0·4
0·2
420 mu
280 mu
mM KCL
500
400
300
200
100
AMIDASE II (CPM P- CMP VI)
7000
6000
5000
4000
3000
2000
1000
Δ420 mu
100
80
60
40
20
0
CPM p32 - pi
20
40
60
80
100
120
FRACTIONS

FIGURE 4-4

Electrophoretic fractionation of amidase II and phosphatase assay mixtures for fractions arising from the DE-52 column depicted in figure 4-4

Assay of every 5th fraction

FIGURE 4-4



the more heat-labile. In the extracellular fluid of AX-3, only the late phosphatase peak is observed whether with ^{32}P-compound V or p-nitrophenyl phosphate (fig. 4-3)

The AX3-derived extracellular phosphatase, partially purified on DE-52 cellulose,was examined for its susceptibility to fluoride. The enzyme was assayed with p-nitropheny phosphate in varying concentrations of potassium fluoride. As shown in figure 4-5, this phosphatase was highly sensitive and is inhibited almost completely above 200 uM fluoride.

Pooling and Concentrating Amidase II.

Amidase II containing fractions, 16-27, were combined and found to contain 51% of the applied activity. Immediate precipitation with ammonium sulfate (70% of saturation) recovered 85% of the pooled activity with >10-fold increase in specific activity over the crude mixture. The enzyme was unstable in dilute solution. Delay in precipitation by 1 week caused 35% inactivation; compare lines 4 and 5 in table 4-1.

GEL FILTRATION

Concentrated enzyme from the previous column was subjected to gel filtration on Sephacryl S200 (fig. 4-6) under the same conditions describe for amidase I in chapter 3. Protein was eluted as two major protein peaks. Amidase II emerged as a component of the second one; therefore, specific activity was not much increased. Active fractions, 60-67, were concentrated in an Amicon ultrafiltration cell with PM30 filter. Yield was slight (16%) as a result of the amidases instability in dilute solution.

Molecular Weight Estimate for Amidase II.

When compared to blue dextran, BSA and cytochrome C, both amidases are eluted slight;y ahead of BSA. Their molecular weights must be comparable

FIGURE 4-5

Inhibition of D. discoideum AX-3 extracellular phosphatase partially purified by DE-52 ion-exchange of protein from axenic culture.

Standard Assay reaction with ^{32}P-compound V, with varying concentrations of potassium fluoride [KF].

Plot of %inhibition vs.KF with reference to control without KF.

FIGURE 4-5

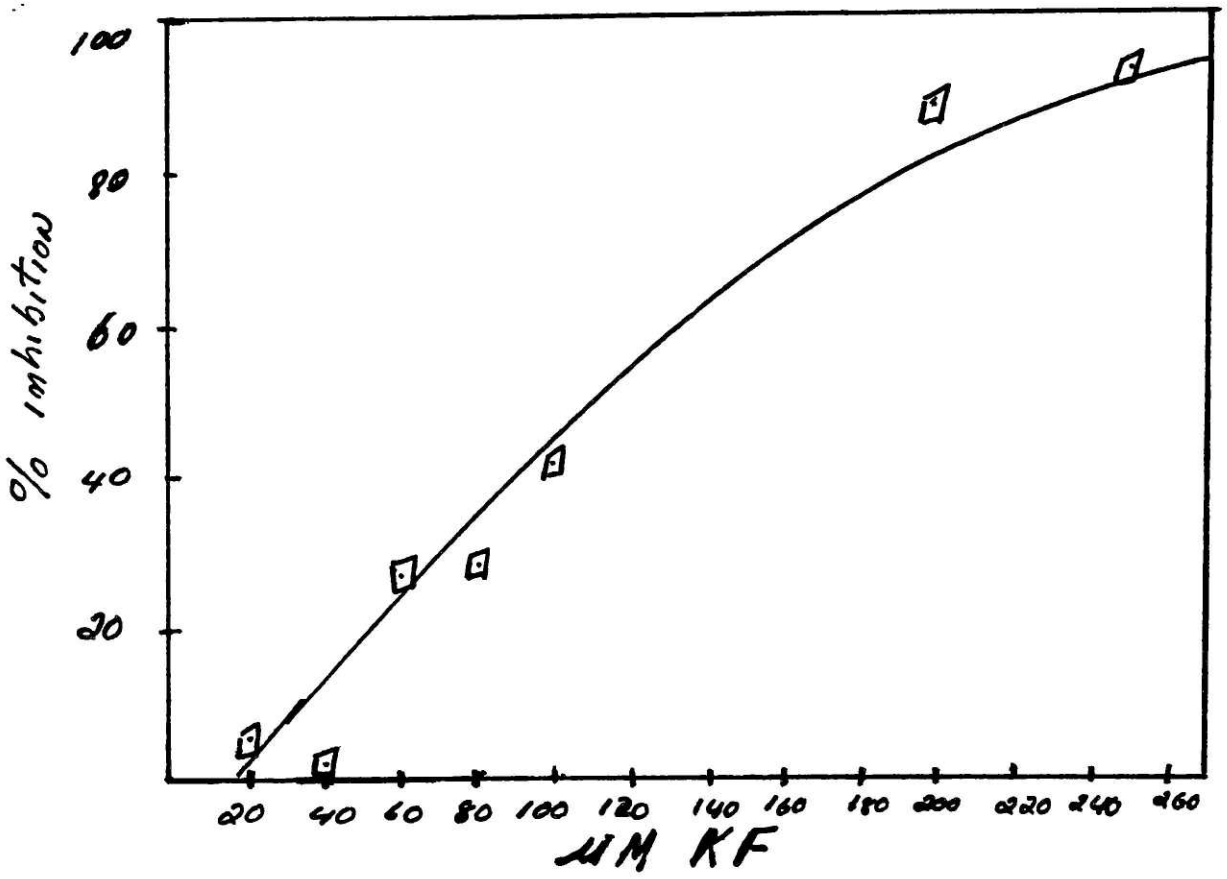

100
80
60
40
20
% inhibition
20
40
60
80
100
120
140
160
180
200
220
240
260
μM KF

FIGURE 4-6

Profile Sephacryl S200 gel filtration of Amidase II (DE 52 purified, 2.5 ml, table 3).

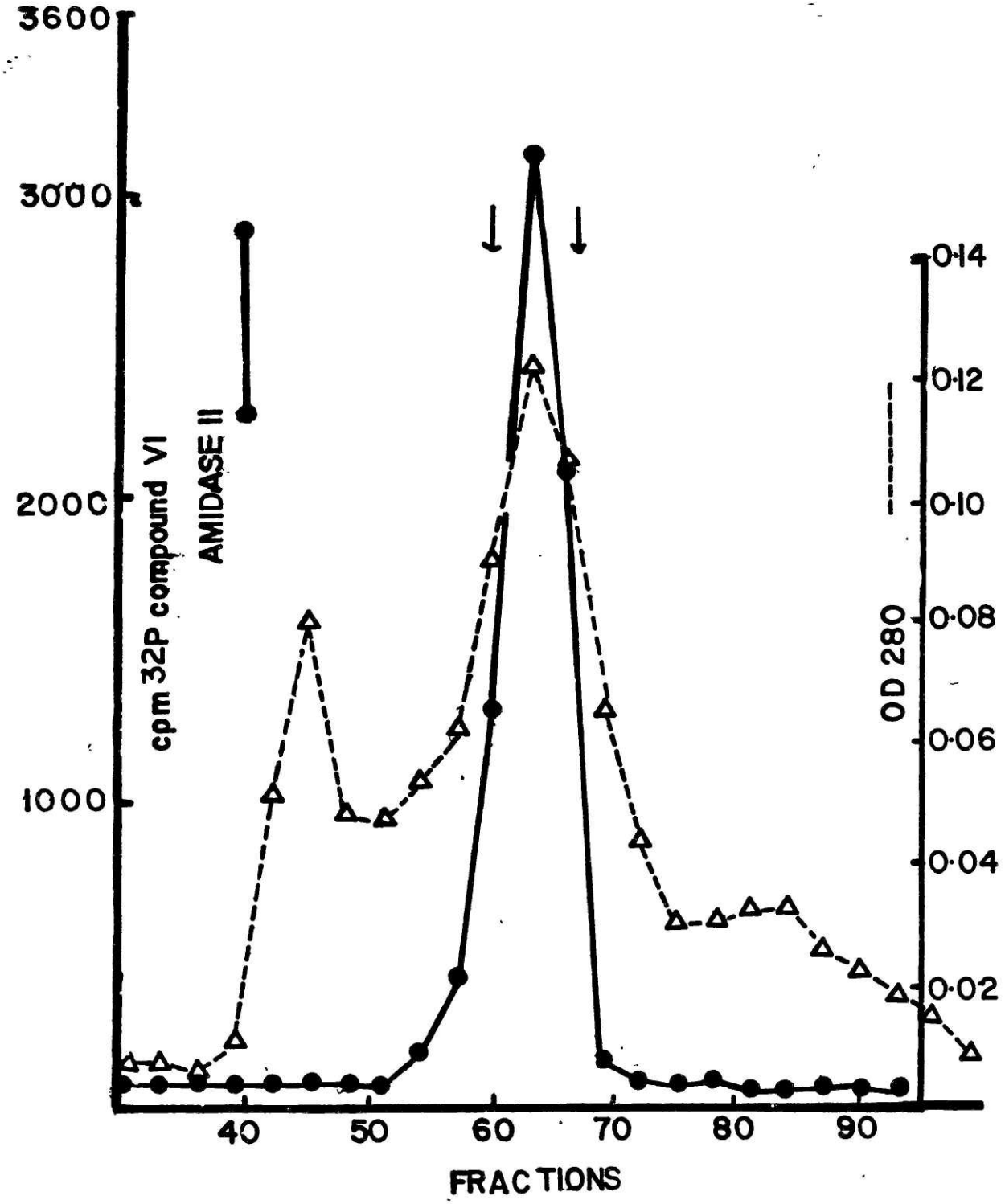
Column was run in buffer B, 100 mM KCl.

Column dimension: 2x90 cm

Flow rate: 0.26 ml/min

Amidase II weas assayed. No phosphatase, as Pi, was observed.

FIGURE 4-6


3600
3000
2000
1000
cpm 32P compound VI
AMIDASE II
0·14
0·12
0·10
0·08
0·06
0·04
0·02
OD 280
40
50
60
70
80
90
FRACTIONS

to albumin and, therfore, in the range of 60-80,000 daltons.

CHROMATOGRAPHY ON HYDROXYLAPATITE

In an analytical test, small amounts of amidase II from DE-52 chromatography were fractionated on hydroxylapatite (1.5x3.8cm) with a linear gradient of ammonium sulfate (0-500mM) in buffer B. While the majority of protein was voided as a sharp peak, amidase II was eluted as a broad peak between 75 and 250 mM salt. Utility of this column was undermined by considerable loss of activity. Note that, both amidases are not inactivated by ammonium sulfate.

AFFINITY CHROMATOGRAPHY

As with amidase I, affinity purification was attempted on columns of Sepharose 6B modified with di-N-acetyl chitobiose and N-acetyl glucosamine (see chapter 3). With this enzyme, the columns were run at pH 3, the optimal pH of the amidases. DE-52 purified enzyme (2.5 mg/ml) were buffered with buffer D before being applied in an 80ul aliquot. Five fractions of 0.6 ml were collected and assayed (table 4-2). No retention was found.

STORAGE OF AMIDASE II

Crude protein from the 1 M NaCl elution off floc cellulose retains its amidase II activity for extended periods when frozen at -80 ^{o}C. The activity tolerates freezing less as it is purified. Amidase II was stored at 0 ^{o}C in an ice-filled dewar. Under these conditions, DE-52 purified enzyme remained active for a year or more; S200-purified enzyme,however, lost activity within weeks.

TABLE 4-2

Fraction #	CPM compound VI		
	C_0	C_2	C_4
1	1752	1836	7318
2	18700	11097	11335
3	4351	1692	1625
4	2684	2086	1263
5	1530	1620	1048

First five fractions (0.6 ml) eluted from alkyl agarose solumn on which amidase II had been applied. Activity expressed in counts per minute of compound VI liberated by amidase II.

AMIDASE I IN D. DISCOIDEUM AX-3

As mentioned above, amidase I activity was totally lacking among excreted hydrolases of axenically grown AX-3. To determine the intracellular abundance of amidase I, $\sim 8 \times 10^{10}$ myxamoebae from 9.6 liters of axenic culture were lysed by freezing and thawing and concentrated crude lysate was prepared by ammonium sulfate precipitation, as described in chapter 3 for NC4. Though amidase I activity abounds in lysate of NC4, none was detected in the identically prepared lysate of axenically grown AX-3.

To test whether the absence of amidase I depended on culture condition, AX-3 cells grown on gram-negative bacteria were examined. Cells were isolated from a 250 ml saturated culture of AX-3 growing on Aerobacter aerogenes (gift of Gail Burnett). Lysate was prepared as prevously described and assayed (fig. 4-7). Amidase I activity was observed in this case.

Clearly, growth on bacteria is required for synthesis of Amidase I. In this respect, axenic cells resemble NC4 myxamoebae growing on bacteria, that lose amidase I as they begin to differentiate.

FIGURE 4-7

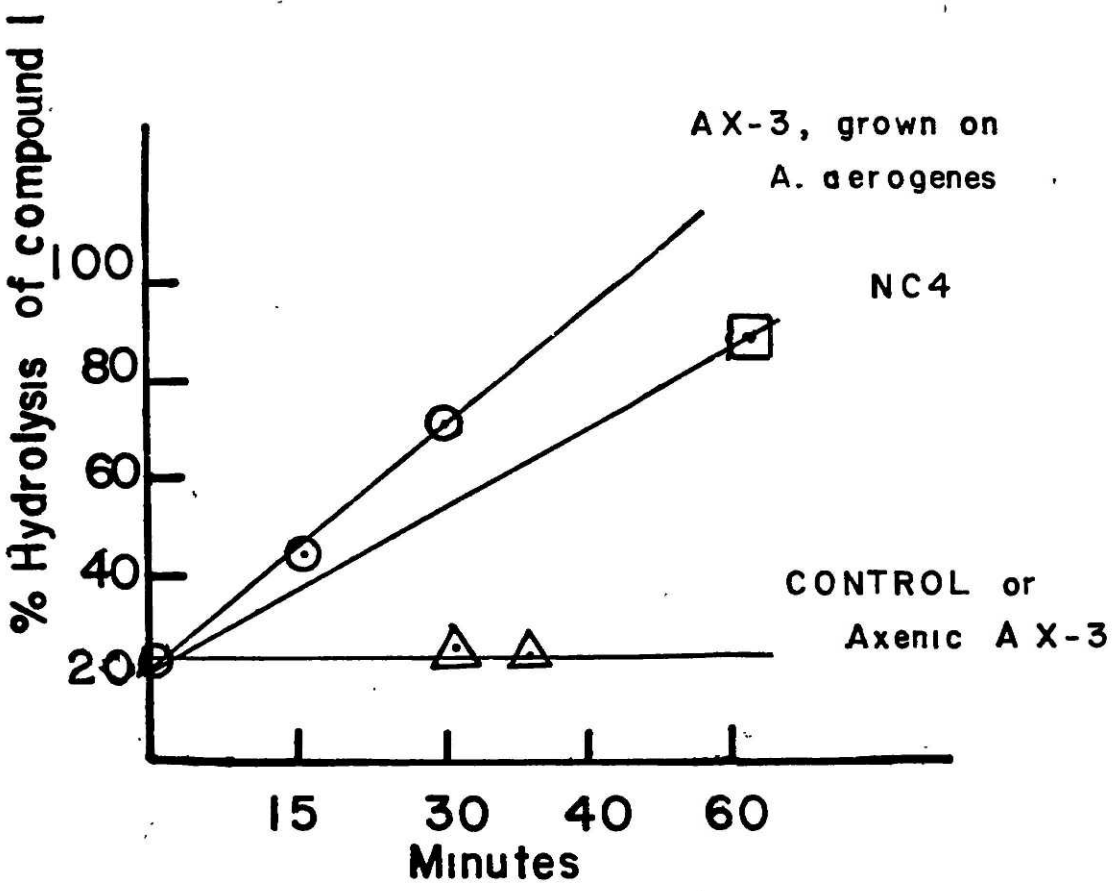
Comparaison of amidase I activity in lysates of D. discoideum strains grown bacteria (NC4 on E. coli and AX-3 on A. aerogenes) and AX-3 cultured axenically.

Lysates prepared as described in chapter 3.

Assay as in Materials and Methods.

Control was without enzyme, buffer was added instead.

FIGURE 4-7


% Hydrolysis of compound I
100
80
60
40
20
15
30
40
60
Minutes
AX-3, grown on
A. aerogenes
NC4
CONTROL or
Axenic AX-3

Chapter 5

KINETIC ANALYSIS

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CRITICAL MICELLE CONCENTRATION OF COMPOUND I 182

SUMMARY

This chapter establishes that amidases I and II follow Michaelis-Menten kinetics. Dependence of the rate of substrate hydrolysis on enzyme concentration and on substrate concentration was evaluated and is discussed within. Michaelis constants were determined at pH 3 for amidase I with compund I and for amidase II with compound V: they were respectively 3 uM and 20 uM.

Amidases I and II are active in non-ionic detergents, nonidet, Brij 58 and Tween 20; they were inactive in SDS, an anionic surfactant. Both amidases remained active when incubated in as much as 40% ethylene glycol.

The cmc of compound I was estimated at 13 uM in dye binding studies.

INTRODUCTION

Enzymes Dependent on Hydrophobic Enviroments.

Many enzymes require the hydrophobic eviroment provided by lipid bilayers or detergent micelles in order to function. Transport enzymes and ion pumps comprise an important category (195). Some enzymes require a specific lipid as cofactor. Mitochondrial β-hydroxybutyrate dehydrogenase not only must insert into a hydrophobic phase but also requires specifically phosphatidylcholine as an activator (196). Certain lipases, including the D. discoideum amidases, define another category whose substrate is normally membrane bound, while they themselves are water-soluble.

While membrane bound enzymes exhibit an all-or-none reliance on a hydrophobic phase, many enzymes of the latter category, such as pancreatic phospholipase A_2 (197), undergo the phenomena known as interfacial activation. By this is meant that their activity is enhanced, sometimes up to 1000 fold, when substrates are inserted into membranes, detergent micelles or an ether-water interface, compared to much lower activity towards monomeric substrates. When the dependence of catalytic rate on substrate concentration is determined for such enzymes, a marked increase in activity is observed above the cmc of their substrates.

Determining Critical Micelle Concentrations (cmc).

The critical micelle concentration of compound I was determined through absorbance changes of the probe rhodamine 6G. Many techniques have been adapted to determining cmc's ranging from measurement of surface tension to various spectroscopic techniques (193,194). Dye binding studies are especially useful since they are amenable to routine biochemical instrumentation under ordinary laboratory conditions.

Detergents Evaluated in this Chapter.

The effect of various detergents on the amidases was examined. These were sodium dodecyl sulfate (cmc=0.015-0.23%), nonidet (290 uM), Brij 58 (77 uM) and Tween 20 (60 uM) (ref.#192). These inquiries were important for two principal reasons. Firstly, preparation of compound III by selective hydrolysis of C_4 phosphate with alkaline phosphatase requires 0.5% SDS to inhibit cleavage of the glycosidic phosphate (18). Initially, it was unclear whether SDS might inhibit the amidases acting on compound III prepared in this manner. Secondly, detergents were needed to solubilize certain hydrophobic substrate analogs in order to present them to the enzymes.

METHODOLOGY FOR KINETIC ANALYSIS

Establishing Initial Substrate Concentrations $[S_o]$.

Compound I (fig. 5-1) and the more hydrophobic derivatives of LPS bind to walls of most reaction vessels, thus rendering substrate concentration uncertain. Coatings of polyethylene glycol or silanization with dichlorodimethyl silane were applied to 1.5 ml polypropylene microcentrifuge tubes. Ten to 20% of compound I was found to dissappear from solution despite the coatings. Teflon tubes and parafilm sheets did no better. Compounds V and VI (fig. 5-1), being more hydrophilic, did not bind significantly to their containers.

It is assumed that the enzymes do not interact to any significant extent with substrates adsorbed to vessel walls. Thus, for kinetic analyses, initial concentration $[S_o]$ was taken to be the portion free in solution. This was calculated from the total radioactivity recovered following paper electrophoresis or TLC.

Estimating K_m from Average Rates and Substrate Concentration.

In measuring Michaelis constants, initial rates, V_o, at varying initial

FIGURE 5-1

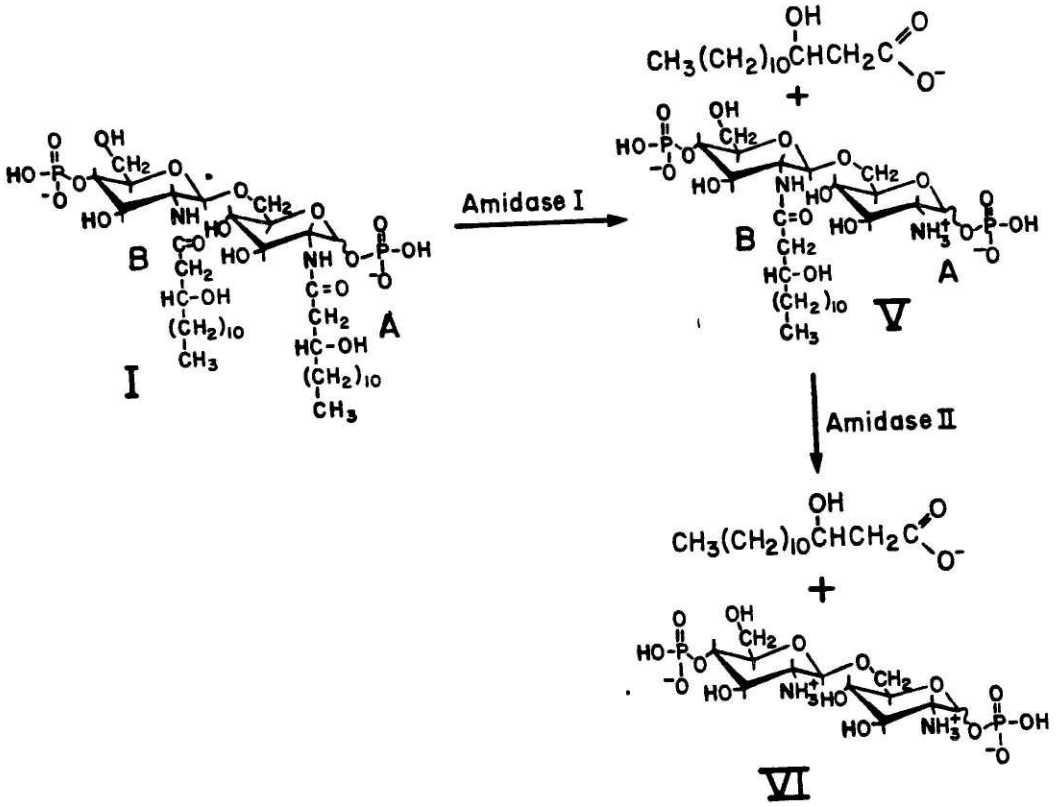
Sequential degradation of compound I by amidases I and II

Site A indicates the reducing end amino group or amide

Site B indicates the distal position

Note amidase II does not hydrolyze compound I

FIGURE 5-1



substrate concentrations $[S_o]$ are normally compared. V_o at low substrate concentrations could not be precisely measured. Therefore, reactions were allowed to proceed to significant extents; average rates, $\bar{V}$, were measured and compared to average substrate concentrations $[\bar{S}]$. K_m was calculated by linear regression analysis of reciprocal plots with the Texas Instrument linear regression program.

According to the mean value theorem, any average rate must correspond to a mean substrate concentration. Theoretically, analyzing mean values of [S] and average rates must reveal the same dependence of V vs. [S] applicable under initial conditions. Lee and Wilson (186,187) have shown that average substrate concentration closely approximates the mean value and that a systematic error of no more than 5% arises in estimates of K_m when that substitution is made, even when reaction has proceeded to 50% conversion.

Product inhibition limits the accuracy of the above method of analysis when K_m is determined from average rates and substrate concentrations. The effect of such inhibition is normally apparent in deviations from linearity of the reciprocal plot (Lineweaver-Burke).

Calculating $[\bar{S}]$

Average substrate concentration was calculated with the following expression:

$$[\bar{S}] = (f_1 + f_2)[S_o] / 2$$

where $[S_o]$ represents the initial substrate concentration and where f_1 and f_2 correspond to the fraction of substrate remaining at each of two time points between which average rate is measured.

KINETIC ANALYSIS OF AMIDASE I

Product versus Time.

The time course for deacylation of compound I (30 uM) by amidase I was evaluated with Sephacryl-purified amidase I (preparation A). The reaction mixture was as described in Materials and Methods. The rate of product formation was constant, even up to 40% hydrolysis (fig. 5-2). From this, it is apparent that product inhibition was not significant.

When reaction was initiated by adding cold enzyme solution to substrate dissolved in buffer (room temperature), the solution temperature drops to ∿15 ^{o}C before warming to the assay temperature. As a result, a lag of ∿5 minutes is observed (fig. 5-3). Therefore, assays of amidases were normally begun by adding substrate to enzyme dissolved in buffer that was preincubated at 37 ^{o}C for 5 minutes.

Rate versus Substrate Concentration, ($\overline{V}$ vs. $[\overline{S}]$).

Amidase I (prep A) was diluted with buffer B so as to give an activity of 2 units/ml in assay mixtures (84 ul). Five minute preincubation of enzyme was maintained throughout. An aliquot (19 ul) was spotted on DE-81 paper within 30-40 seconds of substrate addition; three further aliquots were with drawn at 5 minute intervals. Although some loss of the substrate occured initially by adsorption to vessel walls, the substrate concentration in solution remained constant at all time points. In fact, a slight upward shift in $[S_o]$ was discerned, and is attributed to condensation onto the lids of the containers and evaporation as the tubes are opened for sampling.

The radioactivity of compound V was plotted against time. Average rates were determined between the first two time points, and $[\overline{S}]$ calculated as described above. The graph of $\overline{V}$ vs. $[\overline{S}]$ (fig. 5-3) is a rectangular hyperbola, characteristic of Michaelis-Menten kinetics. The substrate compound

FIGURE 5-2

Formation of compound V by amidase I as a function of time

Extent of hydrolysis of compound I by amidase I

$[S_o]$ = 30 uM compound I; specific activity 5700 cpn/nmole.

Ten microliter aliquots of the assay mixture were spotted at varying times on DE-52 paper for elextrophoretic analysis.

FIGURE 5-2

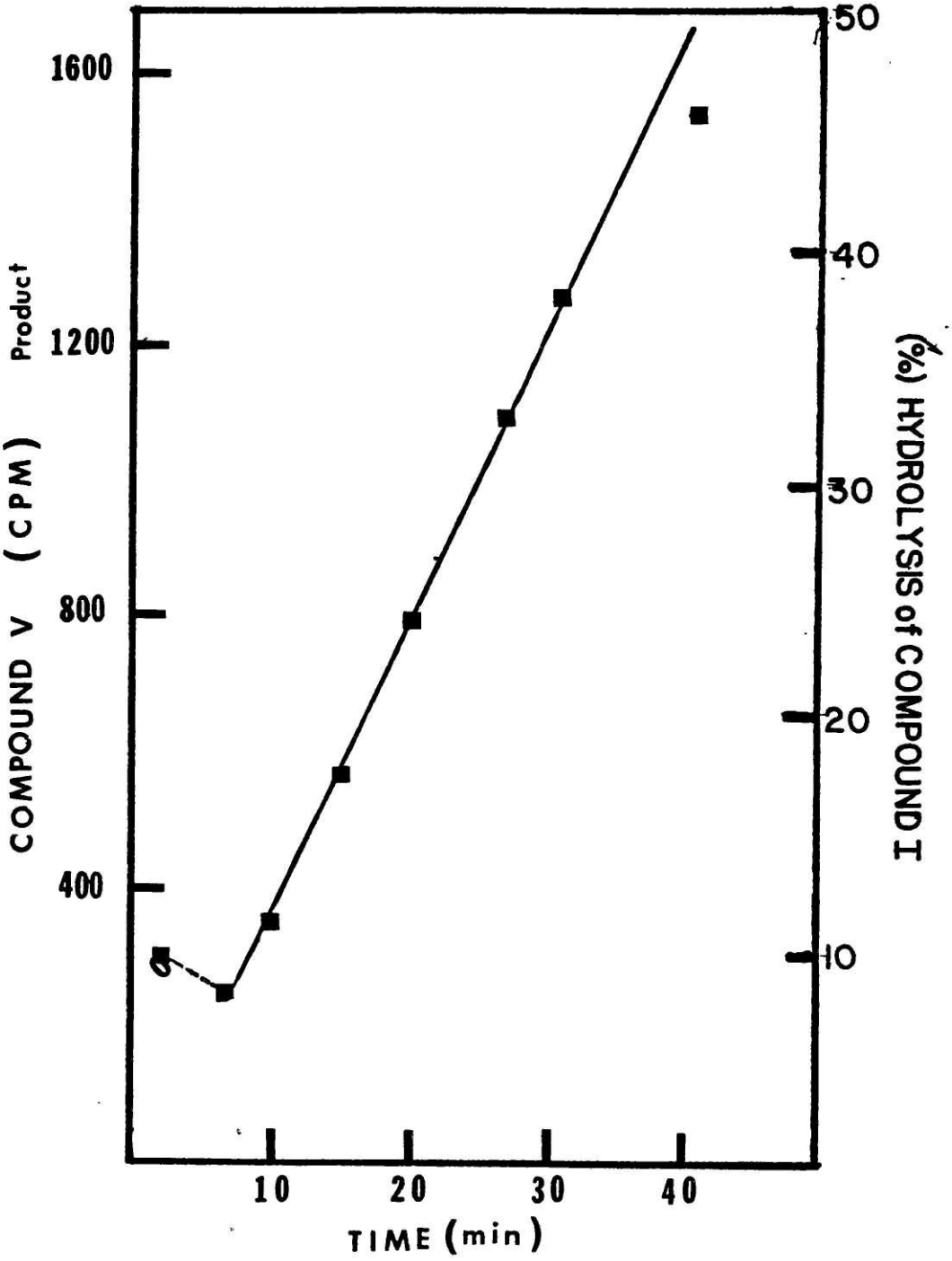

1600
1200
800
400
COMPOUND V (CPM) Product
50
40
30
20
10
(%) HYDROLYSIS of COMPOUND I
10
20
30
40
TIME (min)

FIGURE 5-3

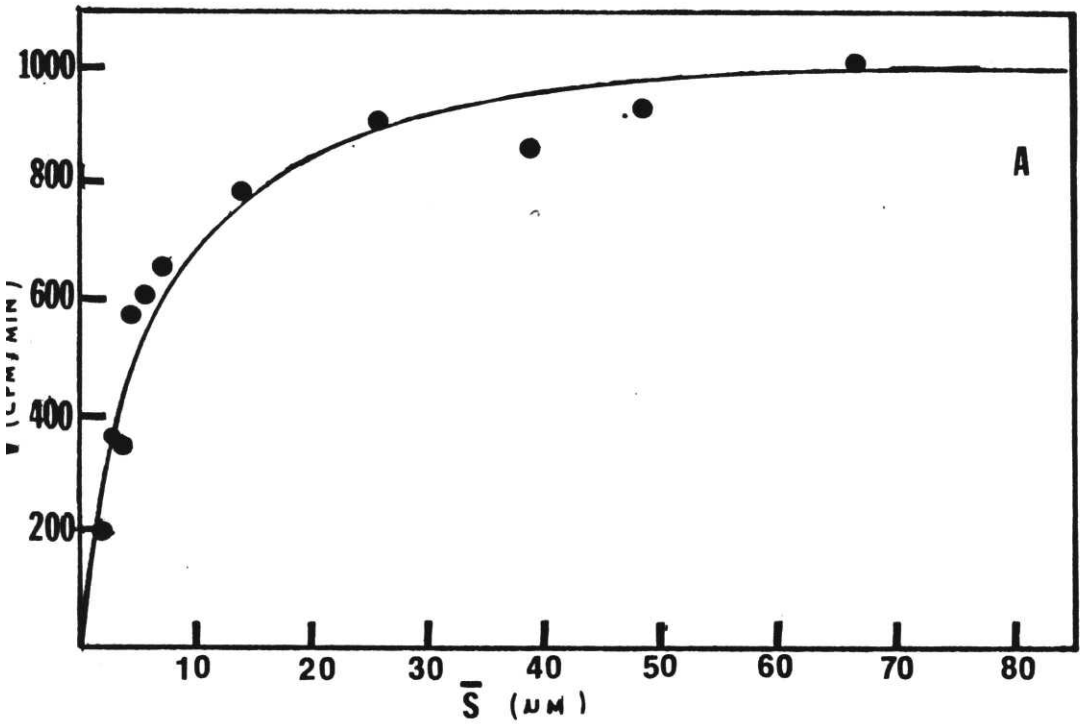
Dependence of velocity on substrate (compound I) concentration.

(A) $\overline{V}$ vs $\overline{S}$ and (B) $1/\overline{V}$ vs $1/\overline{S}$.

Assay mixture.

4 µl	1 M KF
20 µl	0.1 M citrate, pH 3.0
20 -x µl	distilled water
40 µl	amidase I (purified as outlined in figure 4, 3.6 units/ml).
x µl	^{32}P-compound I (28,744 CPM/nmole).

19 µl aliquots of reaction mixtures were spotted on DE-81 paper and analyzed electrophoretically as described in Materials and Methods.


1000
800
600
400
200
A
10
20
30
40
50
60
70
80
$\bar{S}$ (μM)

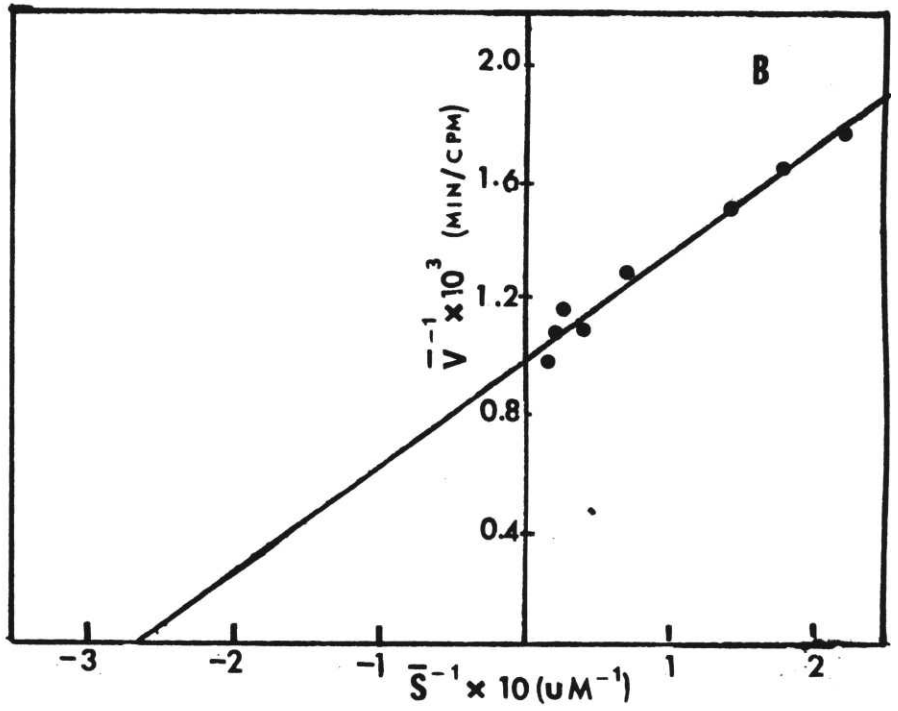

B
2.0
1.6
1.2
0.8
0.4
$\bar{V}^{-1} \times 10^3$ (MIN/CPM)
-3
-2
-1
1
2
$\bar{S}^{-1} \times 10$ (μM^{-1})

FIGURE 5-3

I is amphipathic, but no break or sigmoidality occurs that might indicate a monomer-to-micelle transition in this concentration range. The Lineweaver-Burke plot is linear (fig. 5-3), and Km was estimated to be 3.1 uM. In another determination with one half the enzyme activity, Km was estimated to be 2.8 uM; in this experiment there was more scatter.

Effect of Dilution on Amidase I Activity.

Amidase I (prep A) was assayed at various dilutions so as to demonstrate correlation of the rate of hydrolysis and enzyme concentration. As shown in figure 5-4, low dilutions of enzyme were inactive. Similar behavior was observed with acid phosphatase activity, quantitated by release of inorganic phosphate from ^{32}P-compound I. The reason for this behavior is unclear.

KINETIC ANALYSIS OF AMIDASE II

[P] versus Time.

Deacylation of compound VI (50uM) by purified amidase II was followed with time (fig. 5-5). The rate of product formation remained relatively constant even up to 30-40% substrate conversion. Curvature evident in plot results the lower $[S_o]/K_m$ ratio, where first order kinetics begin to play a role. No lag is observed since enzyme was preincubated in buffer at 37 ^{o}C.

$\overline{V}$ versus [S]

Dependence of rate on substrate concentration was examined in the range 3-100 uM with Sephacryl purified enzyme. Ten microliter aliquots were withdrawn from 42 ul reaction mixtures at 5 minute intervals. The first sample was taken within 30-40 seconds of substrate addition. As with amidase I, average rates were determined from the first two time points. No significant adsorption of compound V to vessel walls was observed. The graph of

FIGURE 5-4

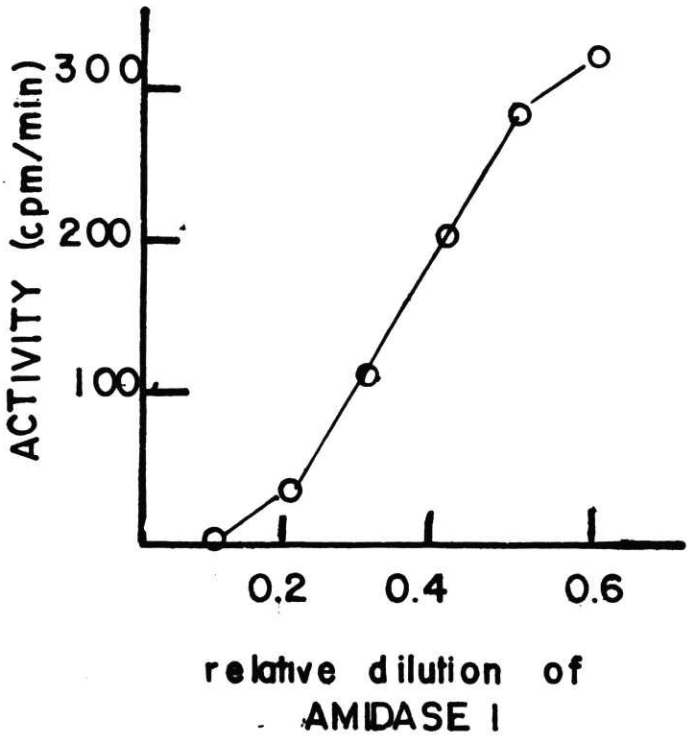
A. Dependence of the rate of hydrolysis (V) of compound I on the relative concentration of amidase I [E].
Amidase I was purified as depicted in figure 3-3, and assayed at varying dilution as described in Materials and Methods.

$[S_o]$ = 189 uM compound I (6195 cpm/nmole)
Enzyme activity (at dilution = 1) = 15 units/ml in assay mixture.

B. Autoradiogram of assay reactions of varying dilutions of amidase I
$[S_o]$ = 48 uM compound I
Enzyme activity at full strength = 15 units/ml in assay mixture.

FIGURE 5-4

A.


ACTIVITY (cpm/min)
300
200
100
0.2
0.4
0.6
relative dilution of
AMIDASE I

B.

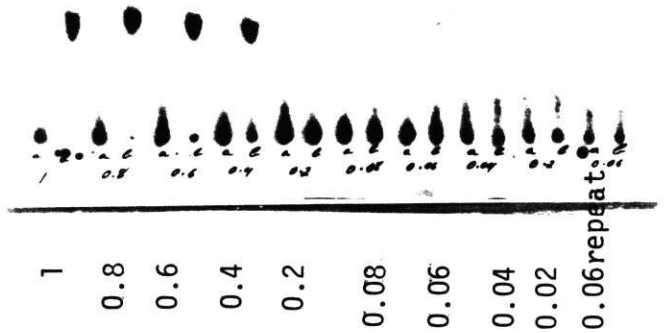

1
0.8
0.6
0.4
0.2
0.08
0.06
0.04
0.02
0.06repeat

FIGURE 5-5

Formation of compound VI by amidase II as a function of time.

Extent of hydrolysis of compound V by amidase II.

$[S_o]$ = 50 uM compound V (16716 cpm/nmole)

Ten microliter amiquots were spotted at varying times onto DE-52 paper for electrophoretic analysis.

FIGURE 5-5

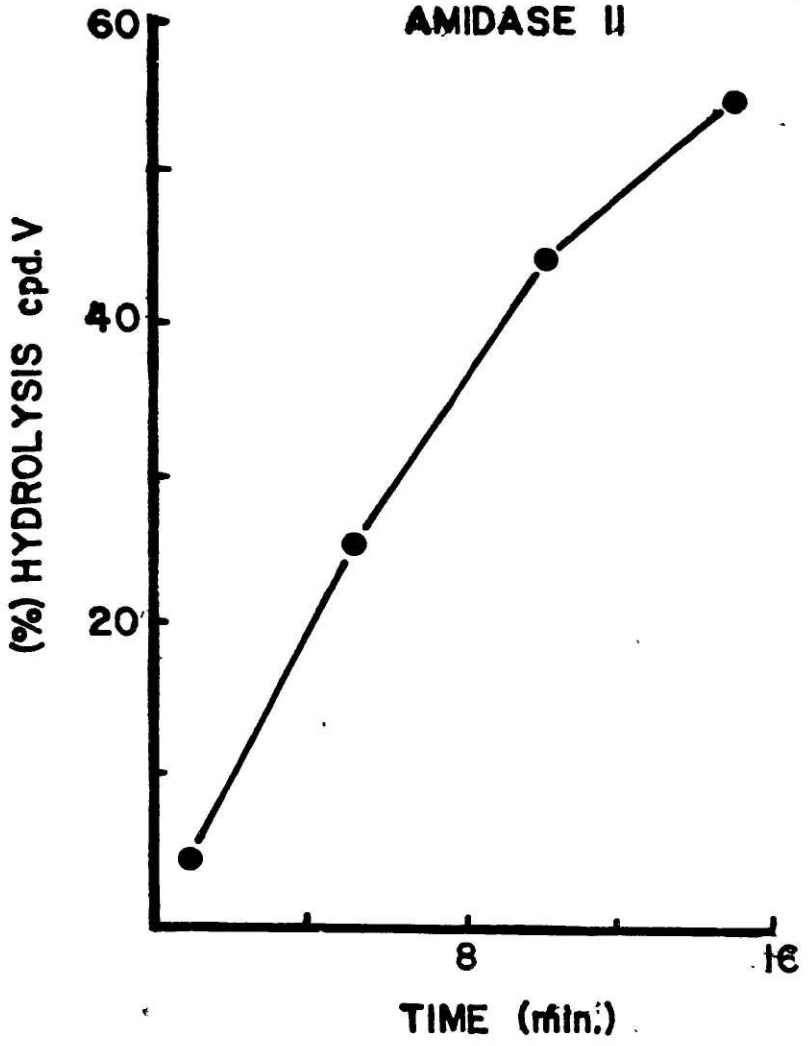


FIGURE 5-6

Dependence of velocity on substrate concentration with Amidase II (S200 purified)

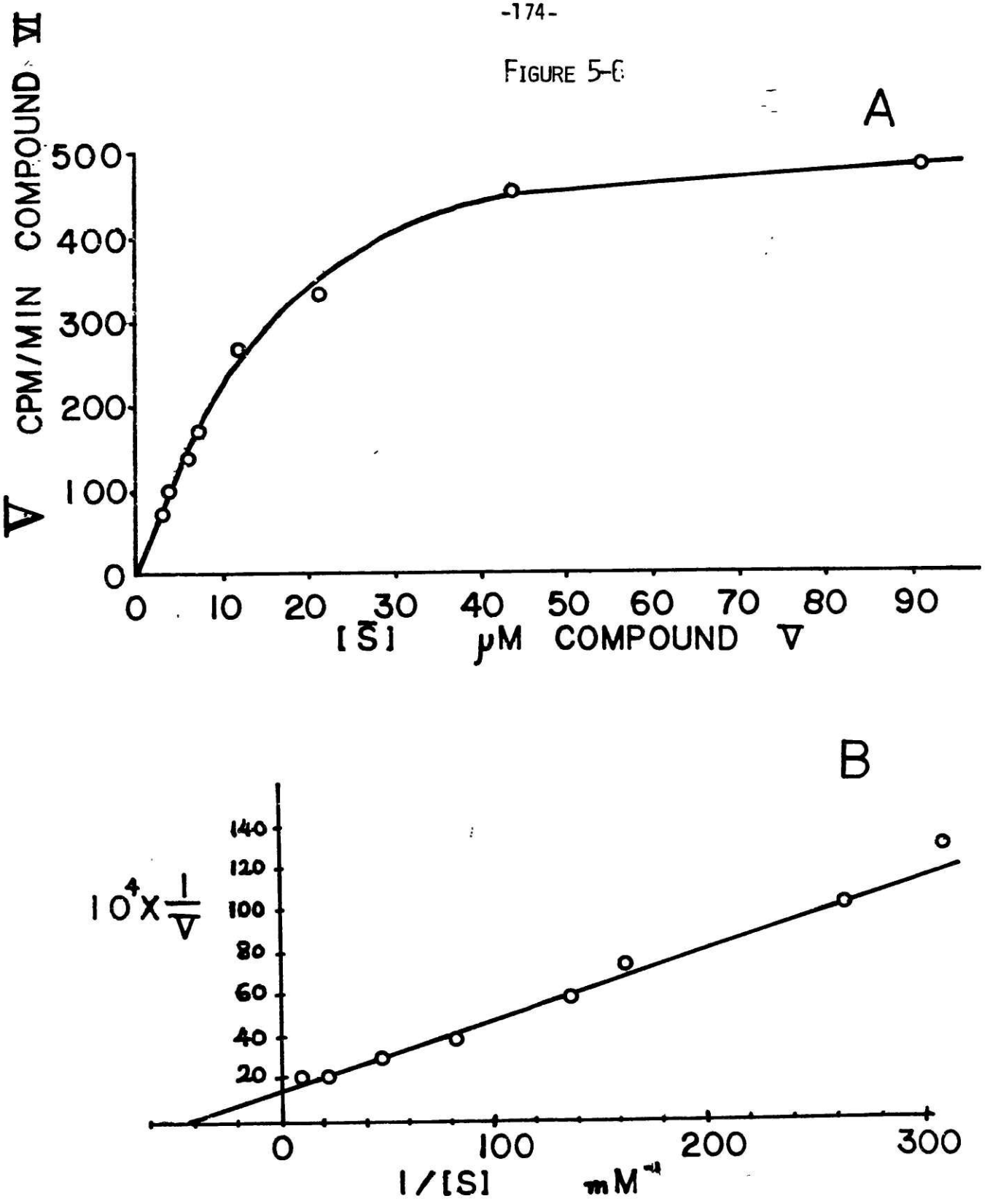
A) $\overline{V}$ vs $\overline{S}$ B. $1/\overline{V}$ vs $1/S$

Assay mixture

10 ul amidase II
12 ul 83 mM citrate, 167 mM KF, pH 3.5
x ul water
20 - x ul compound V (16716 cpm/nmole)

10 ul aliquots were spotted on DE81 paper at 0.6, 5, 10, 15 minutes

FIGURE 5-6


A
V CPM/MIN COMPOUND VI
500
400
300
200
100
0
0 10 20 30 40 50 60 70 80 90
[S] μM COMPOUND V
B
$10^4 \times \frac{1}{V}$
140
120
100
80
60
40
20
0 100 200 300
1/[S] mM^{-1}

$\overline{V}$ versus $[\overline{S}]$ (fig. 5-6a) was a rectangular hyperbola. No singularity occurs, as anticipated with the more hydrophylic compound V. The K_m was estimated at 20 uM from linear regression analysis of reciprocal plots (fig. 5-6b).

EFFECT OF DETERGENTS ON THE AMIDASES

Sodium Dodecyl Sulfate (SDS).

The amidases were assayed in the presence of 0.12% SDS. Parallel control assays were performed without detergent. As depicted in figure 5-7, both enzymes were inactivated by this concentration of SDS, in the vicinity of its salt-dependent cmc.

Non-Ionic Detergents.

Due to lack of charge on the surface of their micelles, non-ionic detergents tend to be less denaturing to enzymes than ionic detergents such as SDS. Three non-ionic detergents were evaluated at 6 times their cmc's: nonidet, a monodisperse form of Triton X-100; Brij 58 and Tween 20 (table 5-1). Tween 20 markedly suppressed amidase I; nonidet somewhat less. However, Brij 58 increased this enzyme's activity with respect to the control. As for amidase II, both Brij 58 and nonidet stimulated its activity while Tween 20 inhibited by 30%. Apparently, some detergents either present substrate in a more favorable conformation for cleavage or allosterically influence the enzymes.

Effect of Ethylene Glycol.

In order to define appropriate eluting conditions for hydrophobic column chramatography of the amidases, their activity was assayed in varying concentrations of ethylene glycol (fig. 5-8). Enzymes in buffer B were incubated on ice for 30 minutes in increasing concentrations of glycol prior to assay in the normal way. In the assay mixtures, glycol concentrations were reduced 2-fold by dilution. Amidase I activity remained constant in up to 30% glycol while 76% of the activity persisted in 48% glycol. With amidase II, results were evaluated only quantitatively due to high substrate conver-

FIGURE 5-7

The Effect of 0.12% sodium dodecyl sulfate on activity of amidases I and II. SDS solution was added directly to the standard assay mixtures.

Three time points were spotred on DE-52 paper for electrophoretic analysis: 0.5, 5 and 15 minutes.

FIGURE 5-7

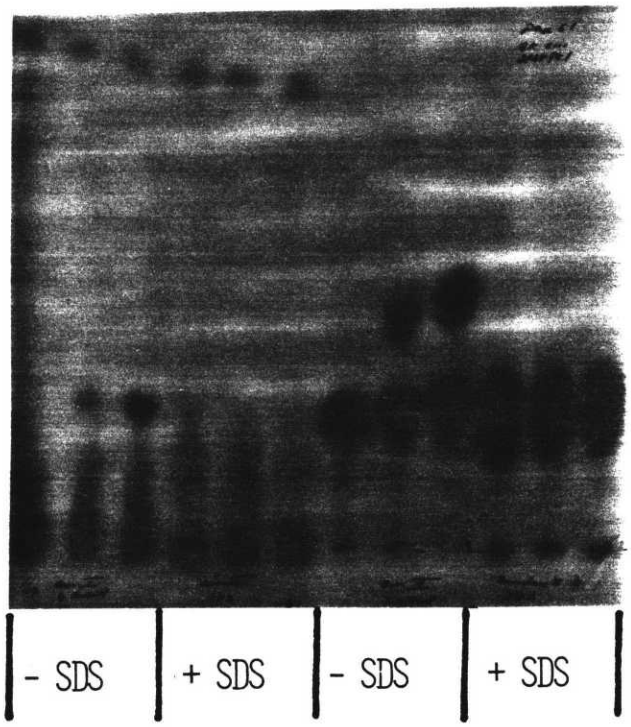


TABLE 5-1

	% HYDROLYSIS OF SUBSTRATE	
	AMIDASE I*	AMIDASE II†
CONTROL	14%	53%
NONIDET (1.7 mM)	8%	69%
BRIJ 58 (0.46 mM)	18%	64%
TWEEN 20 (0.36 mM)	2%	35%

The effect of various detergents on the activity of amidases I and II.

$[S_o]$ = 41 uM compound I for amidase I and
37 uM compound V for amidase II

* percent hydrolysis determined to a precision of ± 2

† percent hydrolysis determined to a precision of ± 5

FIGURE 5-8

The effect of incubation in ethylene glycol on the activity of amidases I and II.

The amidases were incubated at 0 °C in buffer B containing varying concentrations of ethylene glycol.
After 30 minutes of incubation, they were assayed by the protocol described in Materials and Methods.

Initial time points at 30 seconds and final at 20 minutes were analyzed electrophoretically.

FIGURE 5-8

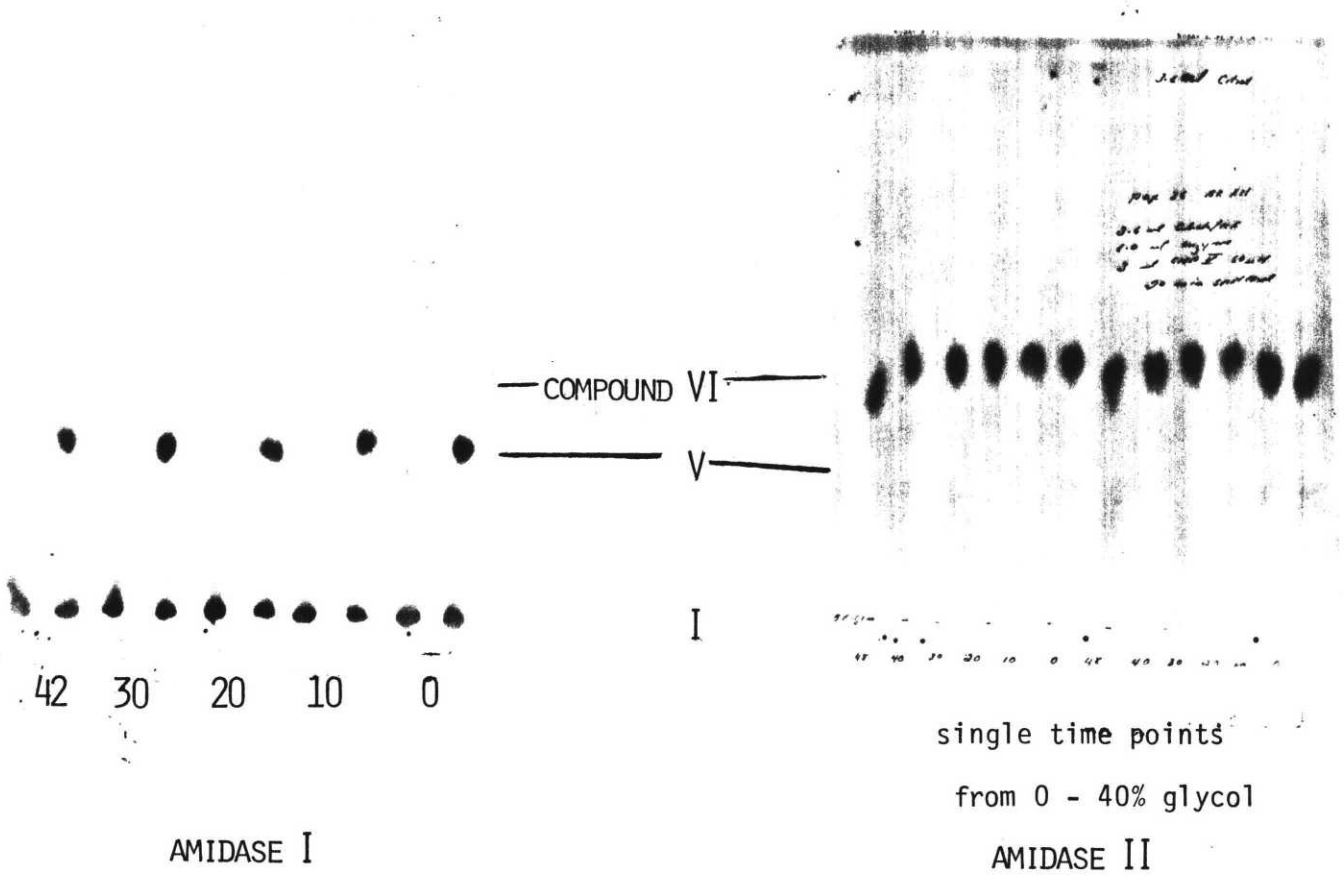


FIGURE 5-8

sions. No significant inactivation of the enzyme could be discerned even up to 42% ethylene glycol. Thus both enzymes could easily be eluted and detected from columns run with glycol-containing buffers.

CMC OF COMPOUND I

To establish whether amidase I interacts with substrate monomers or with micellar aggregates, the critical transition of compound I was examined with rhodamine 6G . As solvent polarity is decreased, this chromophore exhibits a bathochromic shift and increased extinction (fig. 5-9). As described by Carey and Small (194), plotting λ-max of the dye against the logarithmic concentration of the amphiphile reveals a sharp transition in λ-max from 523.4 to ∿531 mu in the vicinity of the cmc.

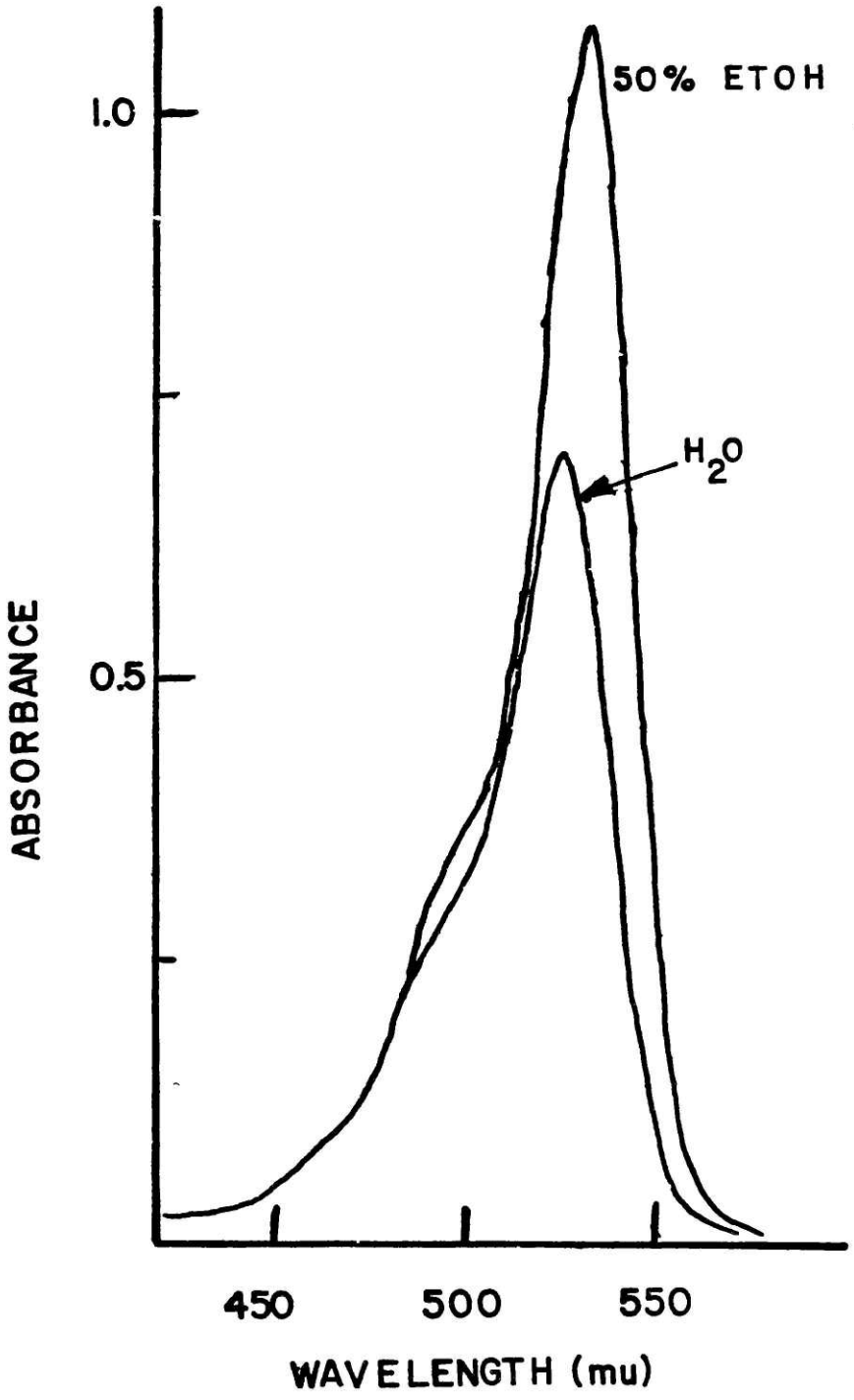
In these studies, the absorbance at one wavelength (539 mu) was compared to the amphiphile concentration rather than determining λ-max. This was valid since OD_{539} must increase dramatically as the dye is inserted into the hydrophobic enviroment of a micelle. Precision in determining λ-max on our Cary-15 spectrophotometer was inadequate for the analytical method of Carey and Small; therefore, the alternate approach was chosen. Reliability of this technique was verified by determinations on deoxycholate.

A stock solution of ^{32}P-compound I, diluted with unlabelled compound, was prepared to 460 uM concentration and 629 cpm/nmole specific activity. It was diluted, then, to various concentrations in 400 ul of 10 mM citrate, pH 3.0, and 2.5 uM rhodamine 6G. Visible spectra were taken on the Cary 15 from 430-590 mu. By adding 5 ul of 1 N NaOH directly to the 400 ul samples in the cuvette, the pH was raised to 6.7, and spectra were repeated at that pH. After spectra were recorded at each pH, samples (20-200 ul) were withdrawn and counted in a liquid scintillation counter so as to establish the

FIGURE 5-9

Spectra of 10 uM Rhodamine 6G dissolved either in deionized water or 1:1 ethanol-water.

FIGURE 5-9



concentrations of compound I.

Absorbance at 539 mμ was calculated from measurements made directly on the recorded spectra with a ruler. In figure 5-10, the reciprocal plot of OD_{539} versus logarithmic concentration reveals a broad transition originating near 13 uM compound I. Transition is complete by 65 uM. No pH dependence was evident as the profiles at pH's 3 and 6.7 were identical within the precision of this method. At pH's 3 and 6.7 charge on the compound I molecule is expected to remain the same. The cmc is taken to be the beginning of transition, 13 uM.

One caveat must be noted. While deoxycholate or fusidate (190) have cmc's in the millimolar range, well above the probe concentration (2.5 uM), compound I begins it transition some 6-fold above the dye concentration. Therefore, the dye might significantly affect the observed transition in its earlier phase.

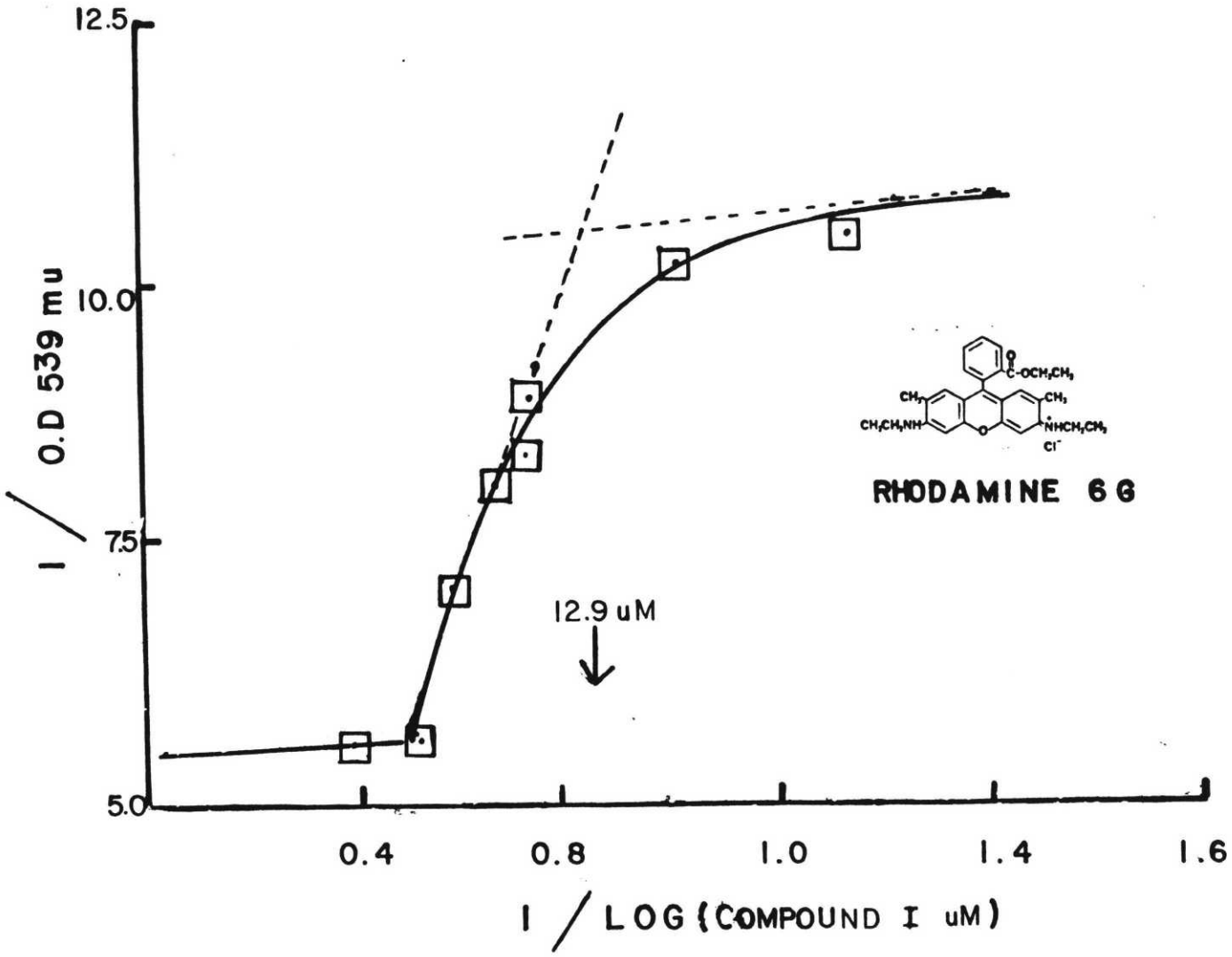
In kinetic studies of amidase I, no singularity was observed in plots correlating $\overline{V}$ and $[\overline{S}]$ although, as shown above, a monomer-to -micelle transition occurs in the concentration range studied. Thus, amidase I does not undergo interfacial activation as does phospholipase A_2.

FIGURE 5-10

Absorbance of Rhodamine 6G in solutions containing varying concentrations of compound I.

Plot of $(\text{O.D. 539 mu})^{-1}$ vs. $(\log[\text{cmpd I, uM}])^{-1}$

FIGURE 5-10


12.5
10.0
7.5
5.0
1 / O.D 539 mu
12.9 uM
0.4
0.8
1.0
1.4
1.6
1 / LOG (COMPOUND I uM)
RHODAMINE 6G

Chapter 6

SUBSTRATE SPECIFICITY OF THE AMIDASES

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SUMMARY

Substrate specificity and sequential action of the amidases were examined in terms of various structural determinants on LPS. When the C_1 and C_4' phosphates of compound I were selectively removed, the absence of either one was not found to influence recognition by the amidases or to disrupt sequential specificity. β-hydroxyl groups on fatty acids were not important to action of the amidases since non-hydroxylated fatty-acyl amides were hydrolyzed by both enzymes. Alkali-treated LPS (OH-LPS) with a trisaccharide substituent at C_3' was sequentially deacylated by the amidases. Neutralizing the cationic ammonium at site A by formylating the amino did not deter amidase II acting on the fatty amide at site B, but the formyl amide at site A was not hydrolyzed by amidase I.

Amidase II also deacylates monosaccharide substrates. It hydrolyzed N-myristoyl-α-glucosamine-1-phosphate but not N-myristoyl-glucosamine-6-phosphate. Amidase I could deacylate neither of these.

Amidase I was inhibited by N-acetyl-α-glucosamine-1-phosphate and by di-N-acetyl-chitobiose but not by unacetylated glucosamine α-1-phosphate or 6-phosphate. It was also inhibited by compound V, the product of amidase-I-catalized deacylation of compound I. Amidase II was not inhibited by di-N-acetyl-chitobiose, N-acetyl-α-glucosamine-1-phosphate or by α-glucosamine-1-phosphate. Its activity was markedly suppressed, however, by compound I, not one of its substrates.

Activity of amidase I was unaffected by even as much as 10 mM $CaCl_2$ or $MgCl_2$. Its activity was also resilient to increasing ionic strength with KCl.

INTRODUCTION

The strict sequence and selectivity whereby the amidases recognize and degrade their substrates represent their more useful and interesting properties. As first demonstrated with the fully de-esterified lipid A derivative, compound I, amidase I will cleave only amide linkage A near the reducing end; then, amidase II hydrolyzes the distal amide, site B (174) (fig. 6-1). Amidase II cannot violate this obligatory sequence, and neither enzyme cleaves at the site designated for the other.

In some respects, this sequential specificity mimicks the behavior of pancreatic phospholipases: phospholipase A_2 and 1-acyl-lysophospholipase. Phospholipase A_2, also an important component of reptilean and insectile venoms, hydrolyzes the Sn-2 fatty-acyl chain of phospholipids (188). The resulting 1-acyl lysophophatide can be subsequently degraded by the second enzyme (189). In this system, strict sequential rules are observed. A different order prevails in mamalian lysosomes. Such preparations convert phospholipids to both 1-acyl and 2-acyl lysophosphatides (190) implying the independent and non-sequential action of lipases A_1 and A_2. Gatt has purified ∿50-fold a phospholipase A_1, apparently of lysosomal origin, from rat and calf brain (191); this activity absolutely discriminates between the two ester positions on phospholipids.

Various factors must be considered as possible contributors to the specificity and sequential action of the amidases. One must examine the importance of the disaccharide backbone. Amidase I may conceivably bind both sugar moieties while only the reducing end amide is within reach of the active site nucleophile. Presumably, the phosphates (C_1 and C_4') might contribute to the positioning of vunerable sites for either amidase.

Restriction of amidase II to monoacylated disaccharides may be explained by either of the following models. On a doubly acylated disaccharide, the

FIGURE 6-1

A. Sequential degradation of compound I by amidase I and II

Site A refers to the amino group or amide near the reducing end.

Site B refers to the distal amino or amide.

This figure repeated from chapter 1.

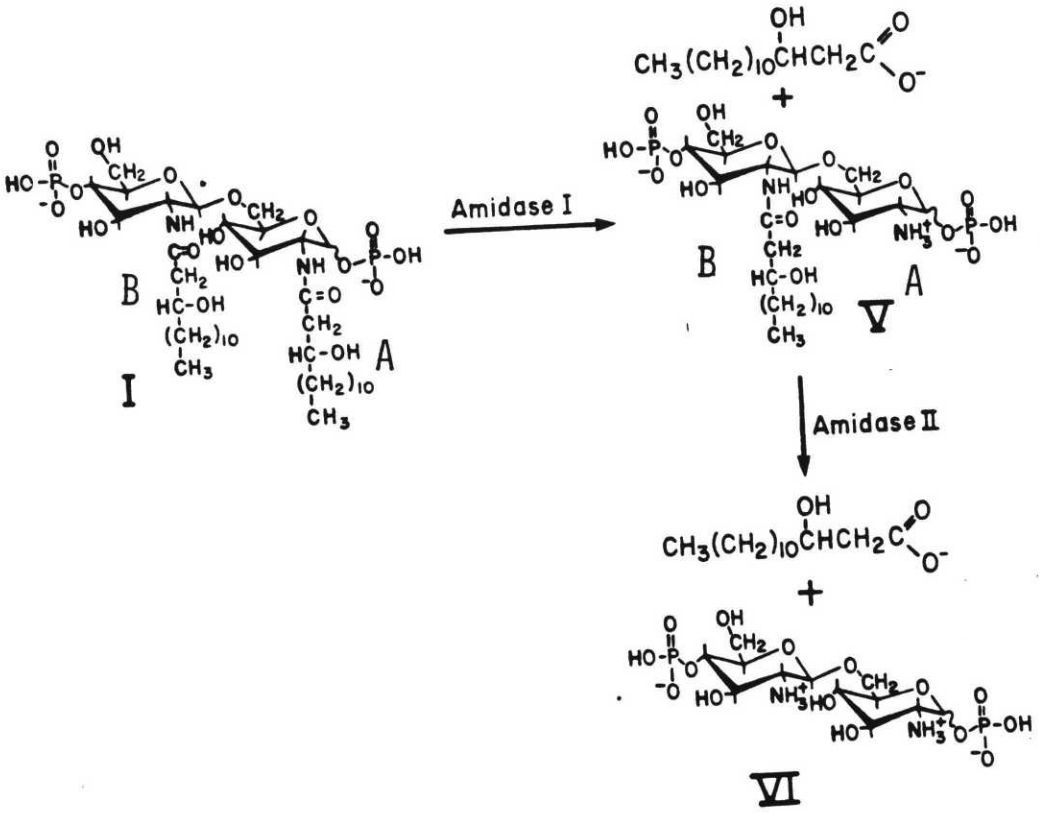
B. Excizing the phosphates of compound I.

a) Acid hydrolysis (pH4.5, 30 min., 100 ^{o}C) of compound I yields compound II, lacking glycosidic phosphate.

b) Alkaline phosphatase excizes the C_4 phosphate of compound I and yields compound III

A AND B ARE ON SEPARATE PAGES.

FIGURE 6-1 (A)



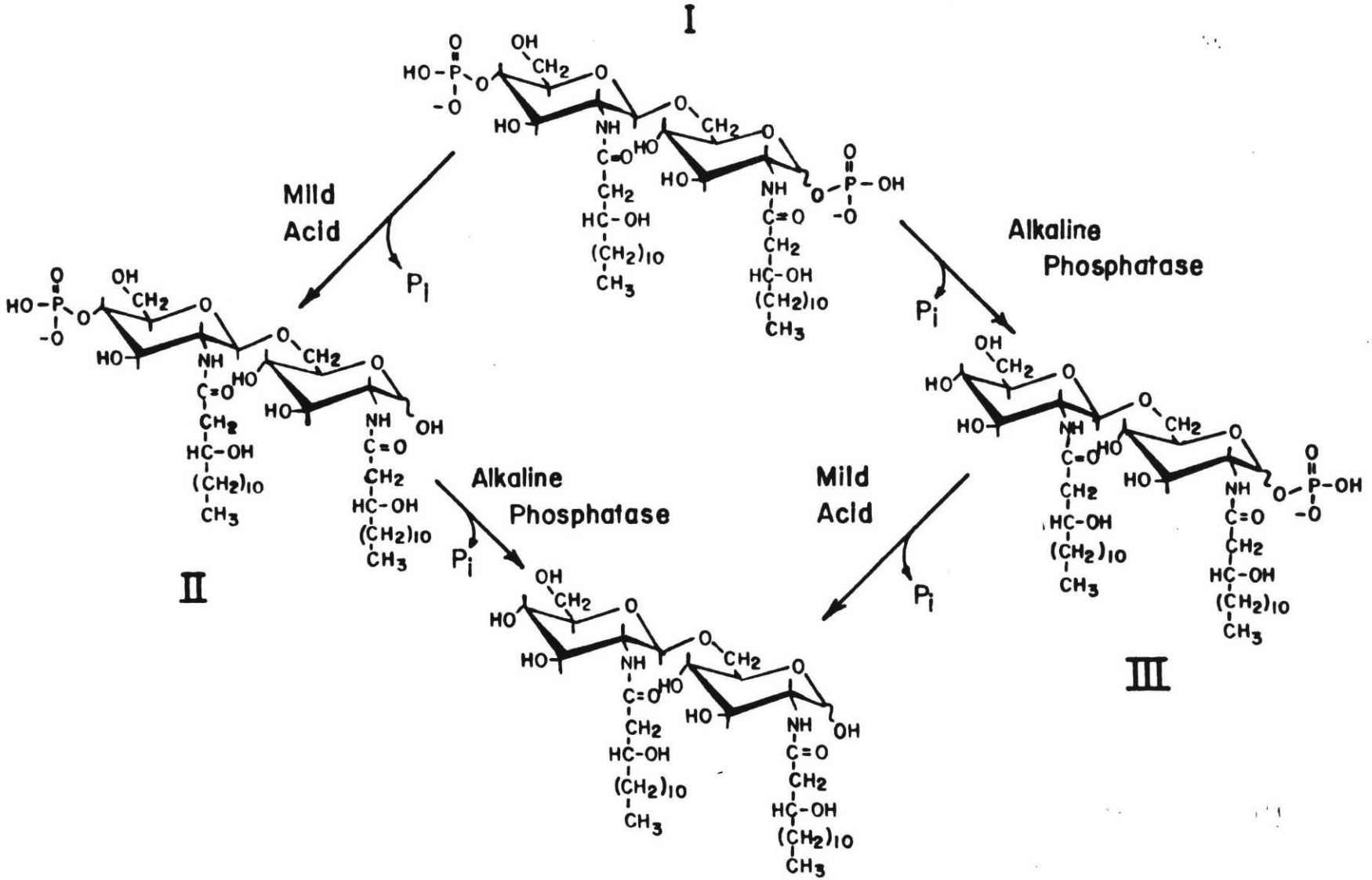


FIGURE 6-1 (B)

-193-

enzyme might bind preferentially to site A but be improperly poised to hydrolyze the amide, a sort of intramolecular competitive inhibition where the hydrophobic site of amidase II is more easily satisfied by site A. Alternatively, the positively charged ammonium, remaining after amidase I action, becomes an important determinant for proper positioning of the substrate on the enzyme.

Substrate determinants important for recognition by the amidases are examined below and their relevance to the issue of sequential specificity considered. Defined modifications of LPS are studied. These include selective removal of each phosphate and of KDO's, also substitution of non-hydroxylated fatty acids at the amides. The free amino of compound V was formylated so as to neutralize the charged ammonium without introducing a large hydrophobic group. To clarify the influence of the disaccharide backbone, monosaccharide substrates based on glucosamine α-1-phosphate and 6-phosphate were prepared and tested as analogs of either end of compound I. Another useful approach was to study inhibition of the amidases with various LPS derivatives and modified glucosamines so as to understand what molecular shapes can successfully occupy the active sites.

THE ROLE OF PHOSPHATE

Preparing Compounds II and III.

Specific removal of either phosphate from compound I has been described by Rosner (18). Compound II, lacking the C_1 phosphate, can be obtained by 0.1N HCl hydrolysis of compound I at 100 ^{o}C. Otherwise, when OH-LPS is hydrolyzed at pH 4.5, compound II, arising from simultaneous excision of KDO and of glycosidic pyrophosphate, can be isolated in the course of purifying compound I on LH-20.

Rosner's procedure for preparing compound III by hydrolyzing the C_4'

phosphate of compound I with alkaline phospatase requires 0.5% SDS to inhibit cleavage of the glycosidic phosphate. SDS also strongly inhibits the amidases; therefore, replacing it with nonidet (a monodisperse form of Triton X-100) or completely omitting detergent were examined (fig. 6-2). Nonidet inhibits cleavage of both phosphates; only partial degradation was apparent even after 2.5 hours of treatment. With SDS, almost stoichiometric conversion to compound III was observed with slight damage at C_1 as shown by the ratio of compound III to phosphate ($\sim$0.9) at the end of reaction. (i.e. complete degradation of compound I). In the absence of detergent, further cleavage at C_1 does occur, albeit more slowly than at C_4'. At the end of reaction, the ratio of compound III to phosphate approaches 0.6. Since the fully dephosphorylated disaccharide bears no label and cannot be detected during autoradiography, its presence should not confuse observation of amidase activity on compound III.

That BAP-catalyzed dephosphorylation without detergent yields compound III and not its isomer compound II was verified by acid hydrolysis and also by reducing sugar analysis. Compound III generated without benefit of SDS was fully dephosphorylated within 30 minutes in 0.1N HCl at 100 ^{o}C, while compound II withstood this treatment. Note that the glycosidic phosphate is the more acid labile (18). Reducing sugar analysis of the unfractionated BAP reactions after 2.5 hours revealed the extent of dephosphorylation at C_1. Without SDS only 11% of the product had free reducing end, in agreement with the ratio of compound III and inorganic phosphate measured; without detergent, 25% of the product was dephosphorylated at the reducing end.

Deacylation of Compound II by the Amidases.

Compound II was subjected to either amidase independently or to both in sequence. Reaction mixtures were analyzed on two TLC systems, HR/DEAE cellulose and silica gel (fig 6-3). Clearly, amidase I deacylates compound II as

FIGURE 6-2

Degradation of compound I with bacterial alkaline phosphatase (BAP).

A) with 0.5% SDS
B) withoutdetergent
C with 4.8 mM nonidet (a monodisperse form of Triton X100).

All reactions contained 250 uM compound I and 1.64 units of BAP/ml.
Total volume: 30 ul
Five aliquots of 3.5 ul were withdrawn at 2, 15, 30, 60, and 145 minutes.

Reactions were carried out at 58 ^{o}C.

FIGURE 6-2

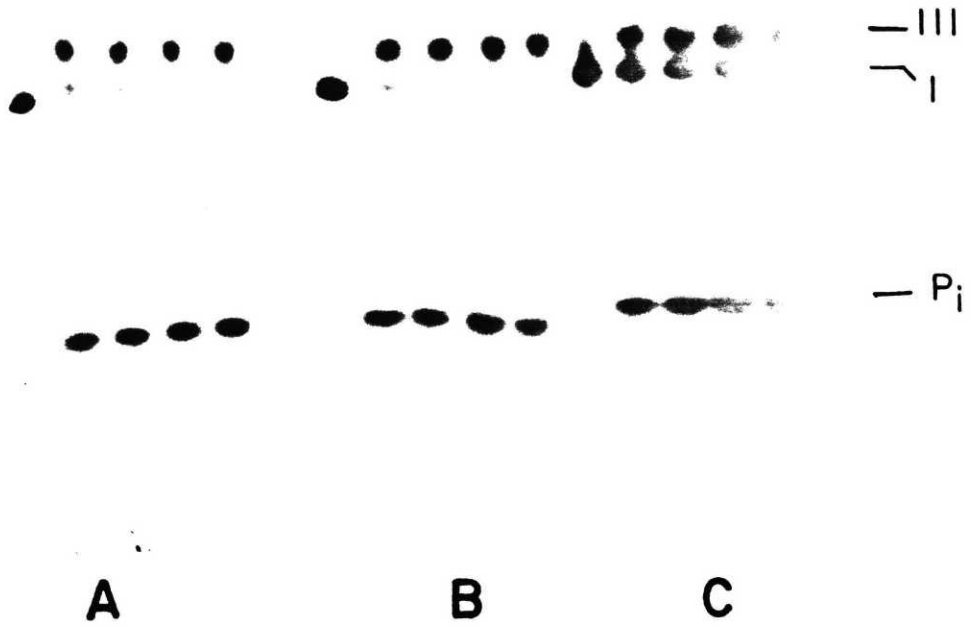


FIGURE 6-3

Sequential deacylation of compound II by amidases I and II.

Reaction mixtures containing 48 uM compound II (or compound for controls) were analyzed by TLC on either HR/DEAE cellulose (A) or on silica gel (B)

For all except sample 3 two time points were taken at 0.5 and 30 minutes. For 3 only the final time point is indicated.

1. Compound II treated with amidase I alone.
2. Compound II treated with amidase II alone.
3. Compound II treated for 30 minutes with amidase I, then with amidase II for 30 minutes more.
4. Marker containing compounds I and II and P_i.

FIGURE 6-3

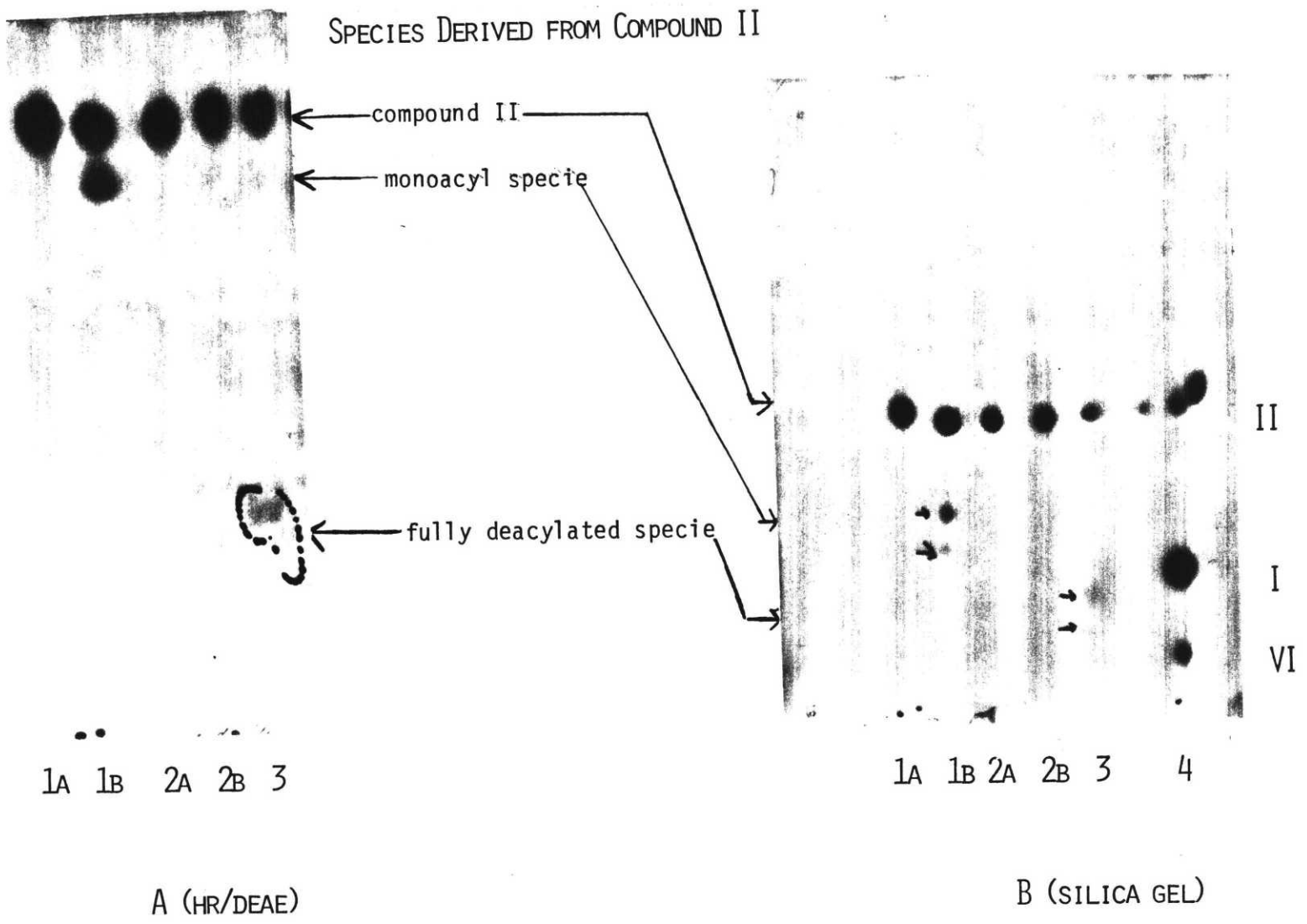


FIGURE 6-3

evidenced by the slower migrating monoacyl compound. On silica gel, a fainter spot trails behind the monoacyl derivative. Its intensity is less than one tenth that of the authentic product; its identity is unknown.

Amidase II leaves compound II unchanged. however, if amidase II is introduced after 30 minutes of amidase I action, the fully deacylated disaccharide (β-1→6-diglucosamine-4'-phosphate) results. On DEAE cellulose, this product migrates much like compound V, the 1-4'diphosphate). Again a faint trailing spot appears on silica plates indicating that its origin is from some compound-I-like molecule that can be sequentially deacylated by the amidases.

In summary, these results indicate that the C_1 phosphate is not an important determinant by itself for recognition by the amidases or for their sequential specificity.

Compounds I and II were treated side by side with amidase I for 30 minutes. An aliquot of each reaction was spotted on DE-81 paper while the remaining reaction was treated with amidase II for another 30 minutes. After electrophoretic fractionation, relative rates were compared. Although more than 90% of compound I was degraded, only 70% of compound II was hydrolyzed. Loss of the glycosidic phosphate did not drastically reduce the rate of hydrolysis (table 6-1). Compound II forms a rather cloudy solution when isolated by LH-20 chromatography; this aggregation prevents precise mesurement of kinetic parameters.

Deacylation of Compound III by the Amidases.

Compound III, prepared without detergent, was immediately acidified to pH 3 in assay buffer to inactivate BAP, and then subjected to either amidase without fractionation to remove inactive BAP or inorganic ^{32}P-phosphate (fig. 6-4). Aliquots were spotted at 0 and 30 minutes on DE-81 paper for electrophoresis. The remaining amidase I reaction mixture was subjected to secondary

TABLE 6-1

	Initial Substrate	Activity (pmol/min)	Relative rate (%)
AMIDASE I	cmpd I	6.7	100 %
	cmpd II	4.5	67
AMIDASE II	cmpd I	5.1	100
	cmpd II	4.1	80

RELATIVE ACTIVITY OF THE AMIDASES TOWARD SUBSTRATES BEARING OR LACKING THE C_1 GLYCOSIDIC PHOSPHATE.

Compounds I and II were treated with amidase I; then the reaction mixture was treated with amidase II. Aliquots were spotted throughout.

FIGURE 6-4

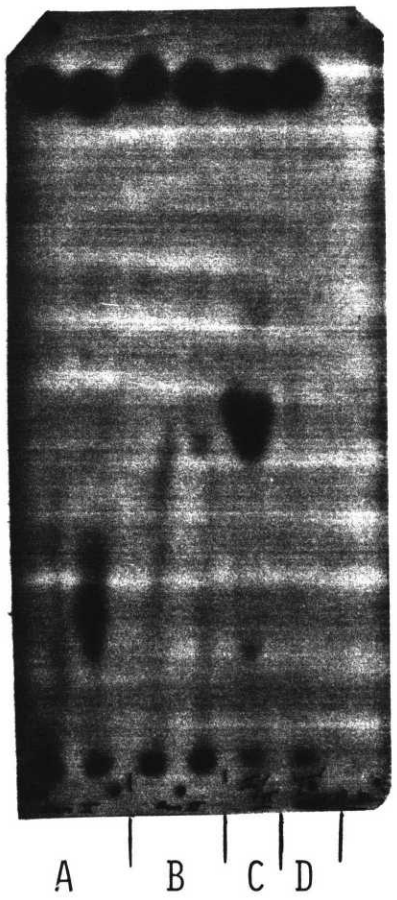
Sequential deacylation of compound III by amidases I and II

Reaction mixtures were composed as described for assay of the amidases in Materials and Methods.

Reaction mixtures initially contained ~30 uM substrate.

A) Compound III treated with amidase I alone.
B) Compound III treated with amidase II alone
C) Compound III treated with amidase I then with one volume of of amidase II solution.

FIGURE 6-4


P_I
FULLY DEACYLATED DERIVATIVE
MONOACYL DERIVATIVE
DIACYL SUBSTRATES
A
B
C
D

treatment with amidase II amd also spotted on paper after 30 minutes.

Amidase I removes an acyl chain; the product migrates somewhat more slowly than compound V, just as does the product of amidase I acting on compound III. Amidase II alone does not degrade compound II, but succeeds amidase I and removes the second chain; this product migrates close to compound VI. Compound II was completely degraded to the fully deacylated derivative, and the amidases acted sequentially.

INFLUENCE OF THE KDO TRISACCHARIDE

Deacylation of OH-LPS.

Saponified LPS from E. coli D31m4 retains the KDO trisaccharide at C_3'. Despite this substituent, amidase I singly deacylates OH-LPS into two products differing by glycosidic orthophosphate or pyrophosphate (fig.6-5). Fractionating OH-LPS mixture on DEAE cellulose (Materials and Methods) separates the two species. Individually, they are also sensitive to amidase I (fig. 6-6). A moderately turbid contaminant arising from the column packing, probably microcrystalline DEAE cellulose, interfered with amidase I hydrolysis of purified OH-LPS II. Despite this contaminant, an upper limit to the K_m of amidase I acting on OH-LPS I was established from the half maximal rate at 8 uM.

In another experiment, DEAE-cellulose purified OH-LPS I was subjected to amidases I and II. After initial treatment with amidase I, it was also treated with amidase II to establish sequential specificity (fig. 6-6). A control of amidases acting on compound I is also represented in the figure. Though amidase I removes the first acyl chain, amidase II alone did not modify OH-LPS I, but it did remove the second chain from the monoacyl product of amidase I. Thus sequential specificity is preserved when the KDO trisaccharide occupies the C_3' hydroxyl

FIGURE 6-5

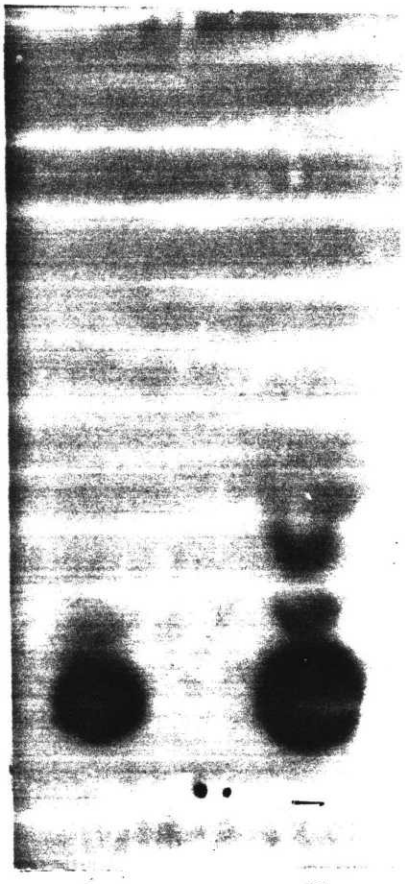
Electrophoretic analysis of the deacylation of alkali-treated LPS (OH-LPS) with amidase I.

The reaction mixture was as described in Materials and Methods and contained ~100 uM OH-LPS mixture.

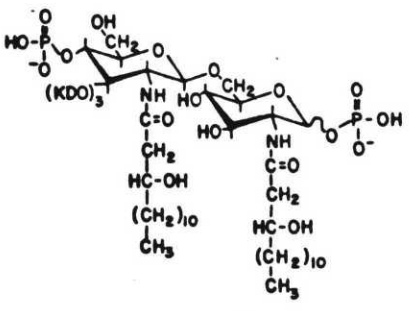
A) Initial time point at 30 seconds

B) Final time point at 30 minutes.

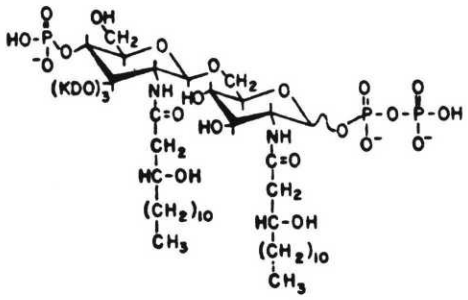
FIGURE 6-5



A B



[ŌH-LPS]-I



[ŌH-LPS]-II

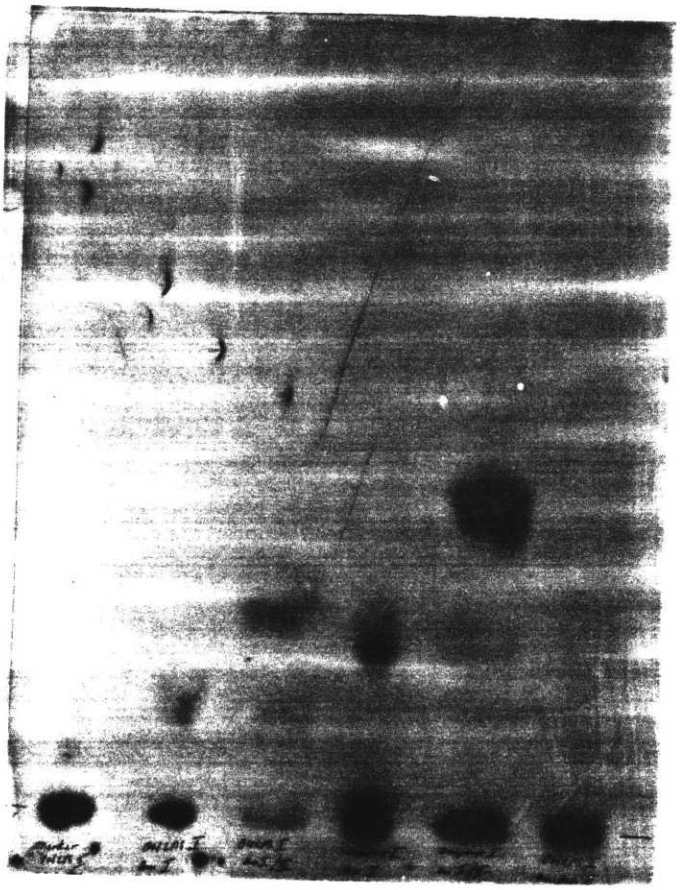
FIGURE 6-6

Sequential deacylation of OH-LPS I by amidases I and II

Reaction mixtures were constituted as described in Materials and Methods with ∿100 uM substrates. They were analyzed by electrophoresis.

A) OH-LPS I without enzyme.
B) OH-LPS I with amidase I alone.
C) OH-LPS I first treated with amidase I, then with amidase II.
D Compound I with amidase I alone.
E. Compound I first treated with amidase I, then with amidase II.
F. OH-LPS I with amidase II alone.

FIGURE 6-6



A B C D E F

ROLE OF HYDROXYLATED FATTY ACIDS

Although it occurs as an intermediate in the synthesis and degradation of fatty acids, β-hydroxymyristic acid is rarely found as a structural component of biological membranes. Its role in the integrity and function of the outer membrane remains unclear. Potential influence of this fatty acid on the amidases was examined by replacing it with it nonβ-hydroxylated analogue.

Deacylation of N-Myristoyl Compound V.

Compound V was myristoylated, as described in Materials and Methods, with the anhydride, and free fatty acid removed by repeated extraction with petroleum ether under acidic conditions. Amidase I did not act on this preparation. It seemed likely that traces of fatty acid remaining might be inactivating the amidase, as both enzymes were inactivated by an anionic detergent SDS (see chapter 7). Thus, N-myristoyl compound V was purified by gel filtration on Biogel P4.

When, in preliminary tests of the column, ^{32}P-compound I and ^{14}C-palmitic acid were fractionated, a curious set of double peaks was observed (fig.6-7a). Apparently, the early doublet represents slowly dissociating mixed micelles, whose components partially separate as they proceed through the column. The later doublet represents monomers; in this region, resolution of compound I and palmitate is optimal. ^{32}P-N-myristoyl-compound V was fractionated on this column (fig. 6-7b). Previous petroleum ether extraction had significantly reduced the fatty acid content; therefore, peaks B and C were much reduced with respect to A. Each peak was pooled and doped with nonidet so as to result in 7.2 mM detergent in B and C and 3.6 mM in A when they were redissolved in water after lyophilization. The detergent prevented loss of the specimen on tube walls after lyophilization.

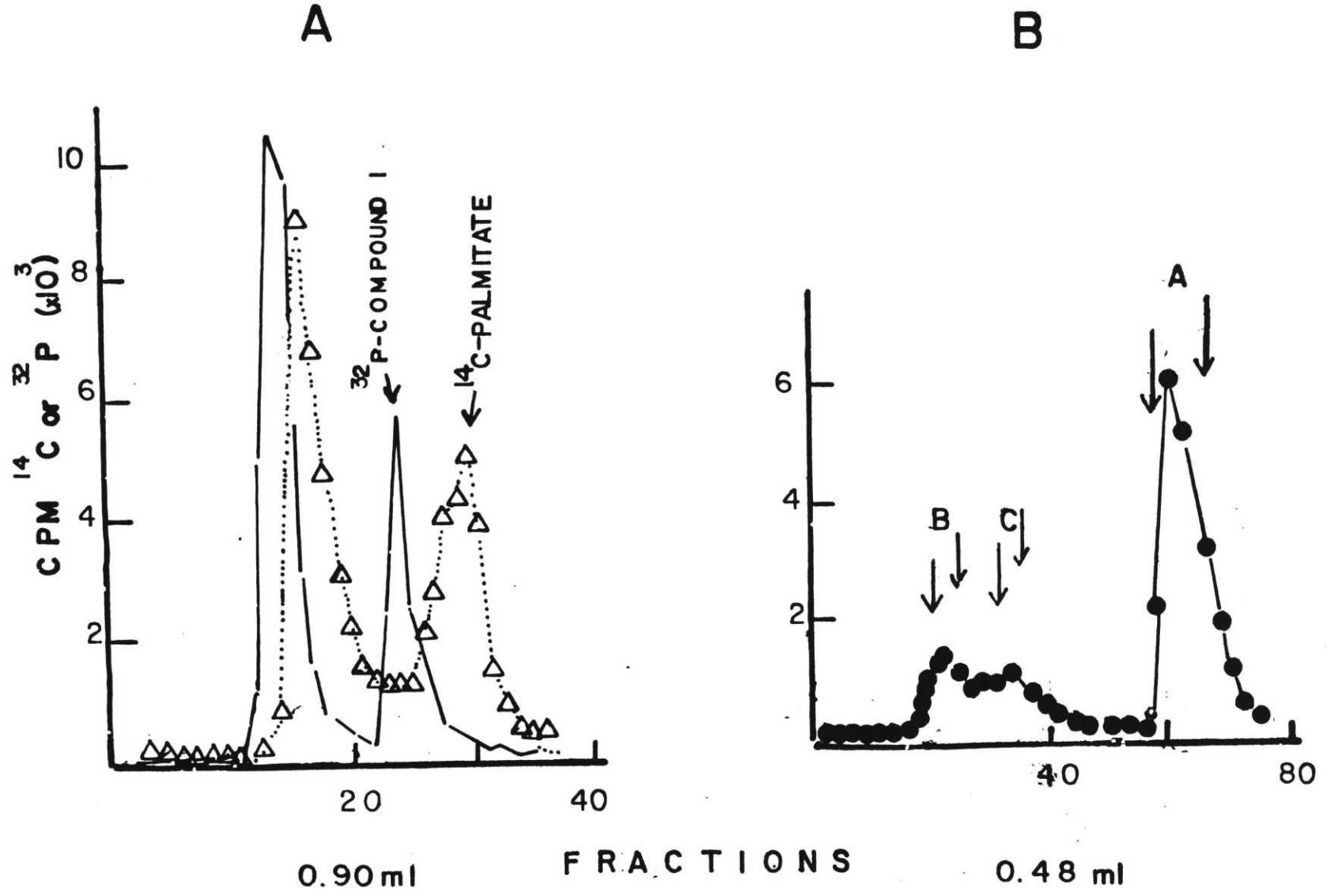
FIGURE 6-7

Purification of N-myristoyl compound V on Biogel P4.

A) Gel filtration of mixture of ^{32}P-compound I and ^{14}C-palmitate on Biogel P4, 200 to 400 mesh, (1x50 cm).
Elution with 0.1M TEAB in water at a flow rate of 4.6 ml/hour.

B) Purificatio of N-myristoyl-V by gel filtration as above.

FIGURE 6-7



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Each peak was assayed against amidase I (fig. 6-8). Peak B was contaminated with compound V. Both peaks A and C contained only N-myristoyl compound V as the only labelled component. It is clear that amidase I cleaves the non-hydroxylated amide and regenerates compound V. Note that the low level of phosphatase generated a monophosphoryl derivative that is also deacylated by amidase I; thus one sees a faster doublet migrating with the characteristics of compounds II an V[H^+].

N-FORMYL COMPOUND V

Verifying Formylation of Compound V.

Compound V was specifically formylated at the free amino with ethyl formate, as described in Materials and Methods. Formylation at site A could not be verified by disappearance of ninhydrin reactivity. The site A amino was unreactive to ninhydrin due to interaction of the α-phosphate with the C_2-ammonium. Similarly, α-glucosamine-1-phosphate did not react with ninhydrin, but acid hydrolysis of the glycosidic linkage restored reactivity.

Evidence for successful formylation was obtained in the greater electrophoretic mobility of N-formyl compound V on neutral Whatman-1 at pH 7.1 as compared to compound V (fig. 6-9). The two compounds, however, are not resolved by electrophoresis on DE-81 paper and are separated only slightly by TLC on silica or DEAE cellulose.

Susceptibility of N-Formyl Compound V to Amidase I.

When treated with amidase I, N-formyl compound V did not revert to its unformylated precursor, nor did it loose the fatty acyl chain at site B that would produce N-formyl compound VI; it remains unchanged (fig. 6-10). Loosing a fatty acyl chain would cause a noticeable increase in electrophoretic mobility on DE-81 or Whatman-1 paper, as shown below. Failure of amidase to

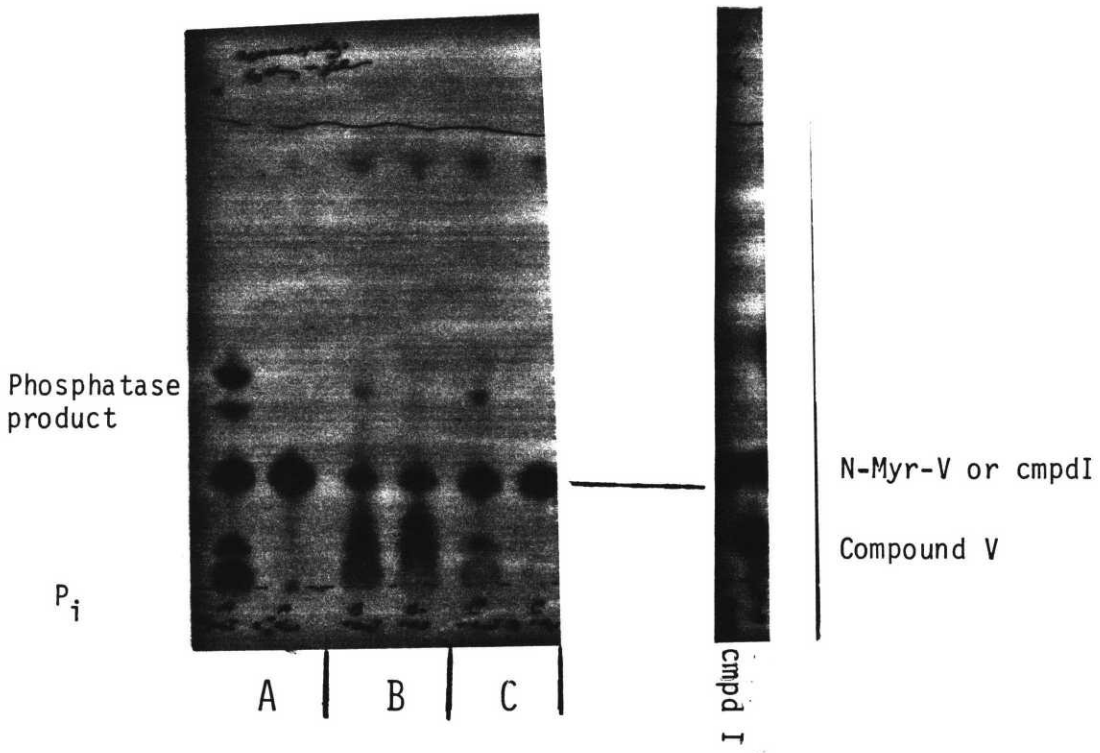
FIGURE 6-8

Deacylation of N-myristoyl compound V by amidase I.

Samples A,B and C from the column presented in figure 6-7 were sujected to amidase I. A second track alongside shows the same sample when incubated in assay buffer without enzyme.

The final lane (cmpd I) shows the deacylation of compound I as a control.

FIGURE 6-8


Phosphatase product
P_i
A
B
C
cmpd I
N-Myr-V or cmpdI
Compound V

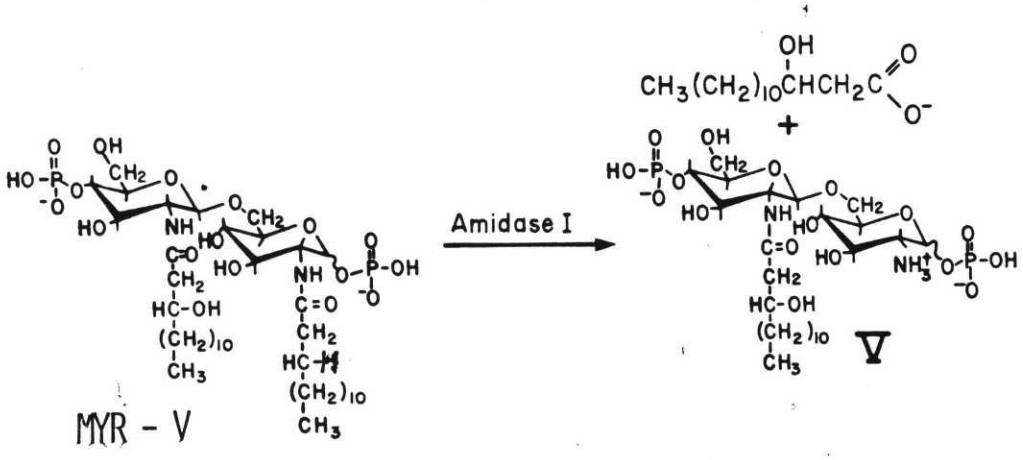

MYR - V
Amidase I
V

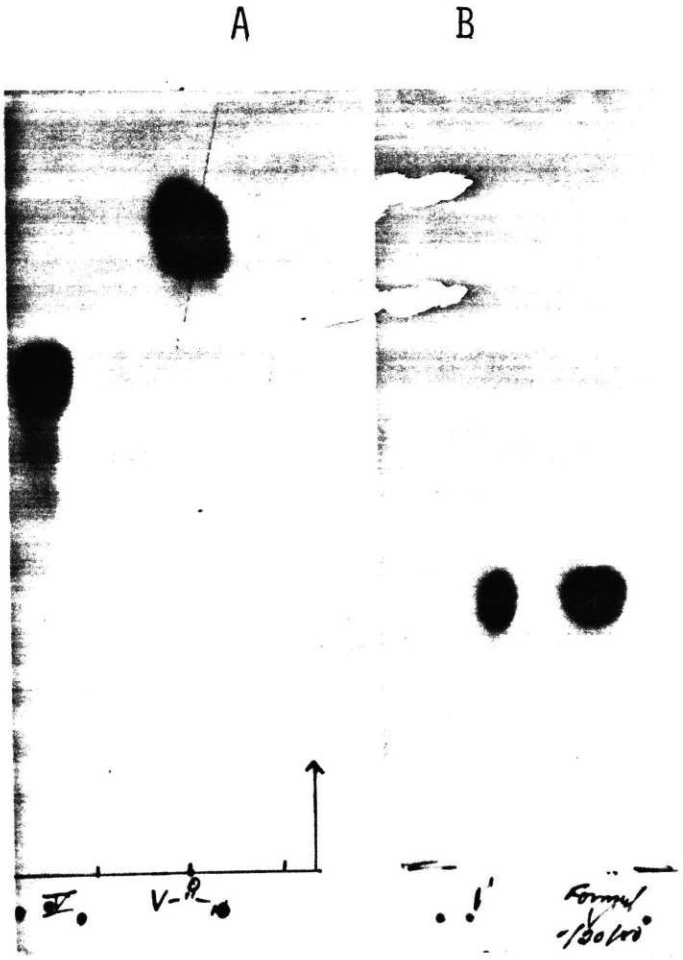
FIGURE 6-9

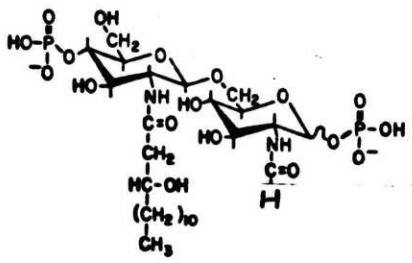
Comparaison of compound V and N-formyl-compound-V by electophoresis.

A) Whatman #1 at 3000 volts, for 30 minutes, at pH 7.1

B) DE-81 paper at 2750 volts, for 45 minutes, at pH 7.1.

FIGURE 6-9





N-FORMYL-V

FIGURE 6-10

Treatment of N-formyl-compound-V with amidase I and amidase II.

A) N-formyl-V with amidase I.

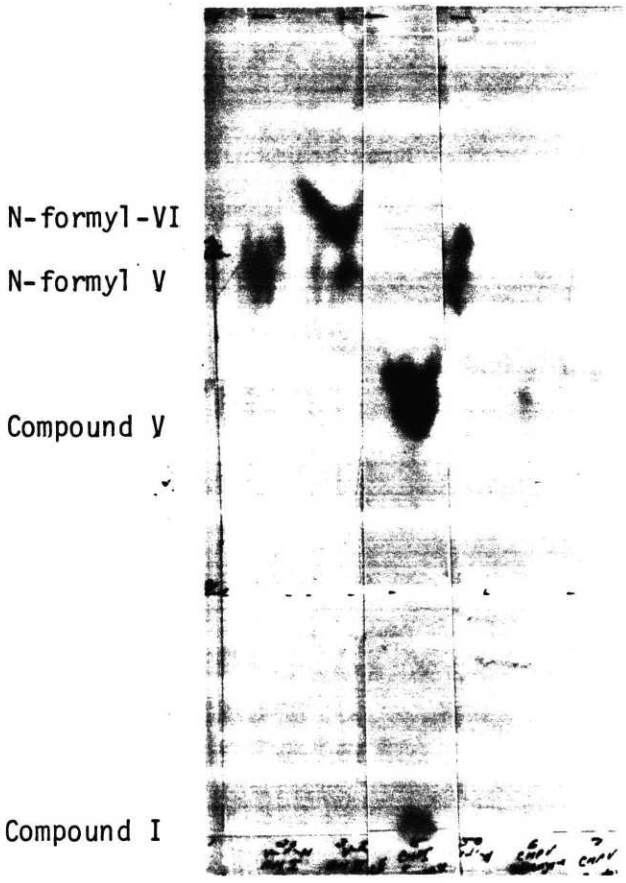
B) N-formyl-V with amidase II.

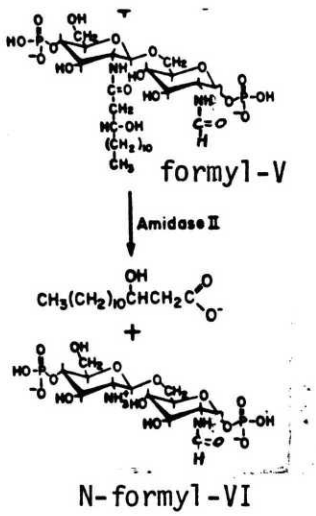
C) Compound I treated with amidase I.

D) N-formyl-V without enzyme

Reaction mixtures contained ∿30 ul N-formyl-V and were prepared like those for assay of the amidase as described in Materials and Methods.

FIGURE 6-10


N-formyl-VI
N-formyl V
Compound V
Compound I
A B C D


formyl-V
Amidase II
N-formyl-VI

deformylate site A demonstrated requirement for a long, hydrophobic acyl substituent for hydrolysis to occur with this enzyme. Also, blocking the amino at site A and neutralizing its positive charge did not relax the specificity of the enzyme; cleavage at site B remained forbidden.

Susceptibility of N-Formyl Compound V to Amidase II.

Amidase II excized the fatty acyl chain at site B of N-formyl compound V. This is shown by the appearance of a novel, faster migrating component either on Whatman-1 or DE-81 paper (fig- 6-11). Amidase II did not release the formyl group since the product N-formyl compound VI was electrophoretically distinct from compound VI on Whatman-1. Thus, positive charge at site A is no requirement for recognition of compound V by amidase II. One must suppose that the presence of a long chain fatty acid at site A is the important factor interfering with proper positioning of compound I on the second enzyme.

MONOSACCHARIDE ANALOGS

N-Myristoyl-Glucosamine Phosphates.

Glucosamine-6-phosphate and α-glucosamine-1-phosphate (Sigma) were acylated with ^{14}C-myristic anhydride (Materials and Methods). Free fatty acid was removed by extraction with petroleum ether and also gel filtration on Biogel P4. N-myristoyl-α-glucosamine-1-phosphate (N-myr-glcN-1-P) migrates as a single spot on silica TLC (R_f∿0.45 solvent I). N-myristoyl-glucosamine-6-phosphate (N-myr-glcN-6-P) contains contaminating N-myr-glcN-1-P, ∿15%; the major component N-myr-glcN-6-P has an R_f of ∿0.34.

Method of Analysis.

Action of the amidases on these substrates was determined by the appearance of free myristic acid (R_f∿0.78) by TLC on silica. Contaminating phos-

FIGURE 6-11

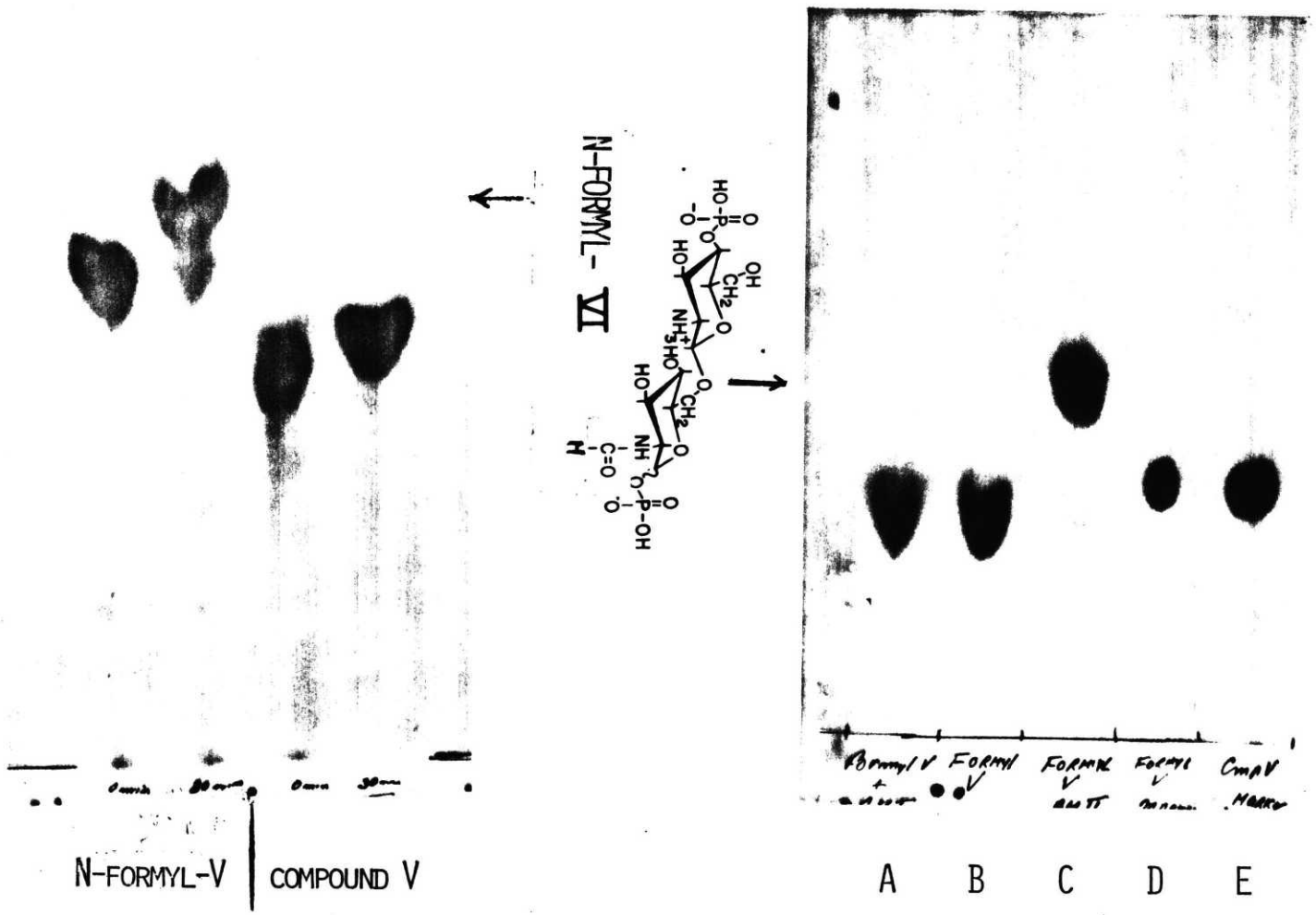
Action of the amidases on N-formyl-compound-V.

A) Comparaison of products of amidase II derived from N-formyl-compound-V and from compound V itself. Reactions were fractionated by electrophoresis on Whatman #1 as described in Materials and Methods. Reaction mixtures contained ∿50 uM of the compound. Two time points are depicted on the electrophoretogram: 0 and 30 minutes.

B Comparaison of the action of amidase I and amidase II on N-formyl-compound-V visualized by electophoresis on DE-81 cellulose (Mat. Met.). A single aliquot of assay mixture was spotted after 30 minute incubation.

a. Formyl-V without enzyme
b. Formyl-V with amidase I.
c. Formyl-V with amidase II.
d. Marker of formyl-V
e. Marker of compound V.

FIGURE 6-11



phatase generates another fast moving product, N-myristoyl-glycosamine (R_f ~0.71). Its structure was confirmed by its identity with the product of pure bovine alkaline phosphatase acting on either N-myr-glcN phosphate (fig. 6-12). Assays with these substrates included 0.37 mM Brij 58, a detergent known not to inactivate the amidases. This prevented the loss of fatty acid on vessel walls, as was observed in preliminary experiments with these substrates.

Action of Amidase I.

Both amidases were purified up to the DEAE cellulose stage. These were tested against the monosaccharide substrates (fig. 6-12). Amidase I did not liberate fatty acid from either N-myr-glcN phosphate. Note, however, that N-myristoyl glucosamine was generated from the N-myr-glcN-6-P preparations but not from N-myr-glcN-1-P. DE-52 purified amidase I still contains one phosphatase isozyme (chapter 3). Since it cannot deacylate the 1-phosphate isomer, a prototype of site A on compound I, amidase I must require the entire disaccharide backbone and possibly both acyl chains in order to correctly bind the substrate for hydrolysis. Also an interesting observation on the remaining phosphatase is possible. This isozyme could not hydrolyze the α-glycosidic phosphate of N-myr-glcN-1-P but does cleave the C_6 substituent of N-myr-glcN-6-P. Thus, when acting on LPS derivatives, it must not affect the glycosidic phosphate but only the C_4 ester.

Action of Amidase II.

Amidase II deacylated N-myr-glcN-1-P although this analog more closely resembles site A of compound I. It however did not affect N-myr-glcN-6-P; very low amounts of fatty acid liberated upon extensive treatment with amidase II actually arise from the N-myr-glcN-1-P contaminant

Neither phosphate on compound I is essential to action of the amidases; thus, the 6 or 1-phosphate on the monosaccharide analogs must serve only to

FIGURE 6-12

Hydrolysis of N-myristoyl-glucosamine-phosphate compounds by the amidases.

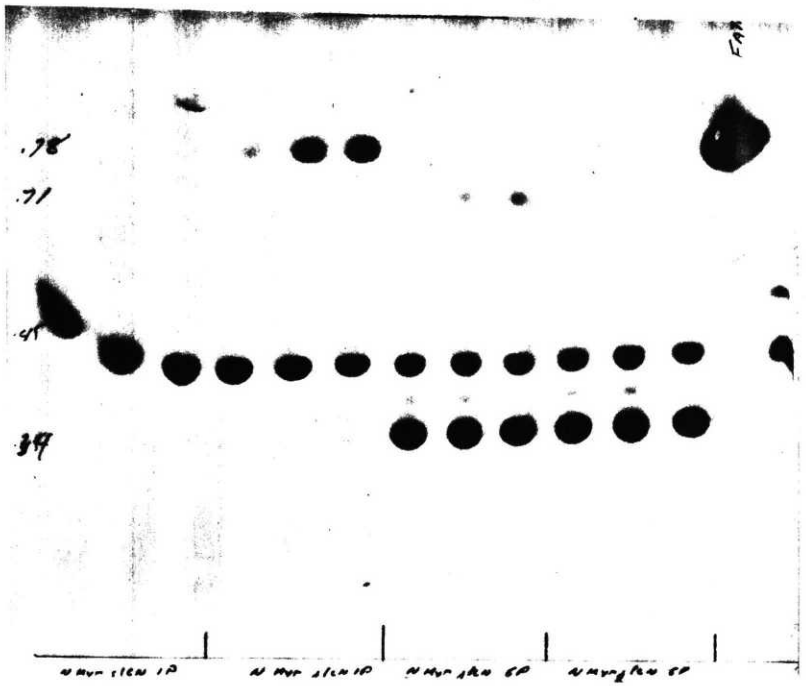
Reaction mixtures were the same as for assay of the amidases (Materials and Methods) with 240 uM substrate.

Substrates: N-myristoyl-α-glucosamine-1-phosphate (N-myr-glcN-1-P)
N-myristoyl-glucosamine-6-phosphate (N-myr-glcN-6-P)

Three time points are depicted: 0, 30 and 60 minutes.

Reaction mixtures were fractionated by TLC on silica gel with ethanol/conc: NH_4OH (6:4).

FIGURE 6-12



FATTY ACID

N-MYRISTOYL-GLUCOSAMINE

N-MYR-GLCN-1-P

N-MYR-GLCN-6-P

solubilize them. Preferential deacylation of α-N-myr-glcN-1-P rather than the 6-phosphate may imply the need for a substituent at C_1, a role fulfilled by the β(1→6)-glycoside of compound I. It is also likely that a C_6-phosphate prevents proper positioning in the active site.

INHIBITORS OF THE AMIDASES

The inhibitory potential of various modified glucosamines and derivatives of LPS were examined so as to elucidate determinants for binding to the active sites. Though not rigorously proven, it was assumed that these compounds inhibit competitively because of their resemblance to authentic substrates

Inhibition of Amidase I.

While maintaining constant concentration of enzyme and substrates (∿30 uM compound I), amidase I was assayed in varying concentrations of inhibitors: di-N-acetyl-chitobiose, N-acetyl-α-glucosamine-1-phosphate, glucosamine-6-phosphate, α-glucosamine-1-phosphate, and compound V. From Dixon plots (1/V vs. [I]) (fig. 6-13) inhibition constants were calculated. Inhibition was not studied at various substrate concentrations; this leaves some doubt as to the nature of the inhibition. To maximize inhibition, the substrate concentration was kept low ($[S_o]/K_m \sim 9.7$).

Neither glucosamine-6-phosphate nor α-glucosamine-1-phosphate, with protonated amino groups, noticeably inhibited amidase I, even up to 800 uM concentrations. The two acetylated compounds, di-N-acetyl-chitobiose and N-acetyl-α-glucosamine-1-phosphate, did inhibit the enzyme; inhibition constants (K_I) were found to be respectively 15 uM and 60 uM. Compound V, a product of amidase I, proved its most potent inhibitor despite a protonated amino at site A; K_I was estimated at 5.6 uM.

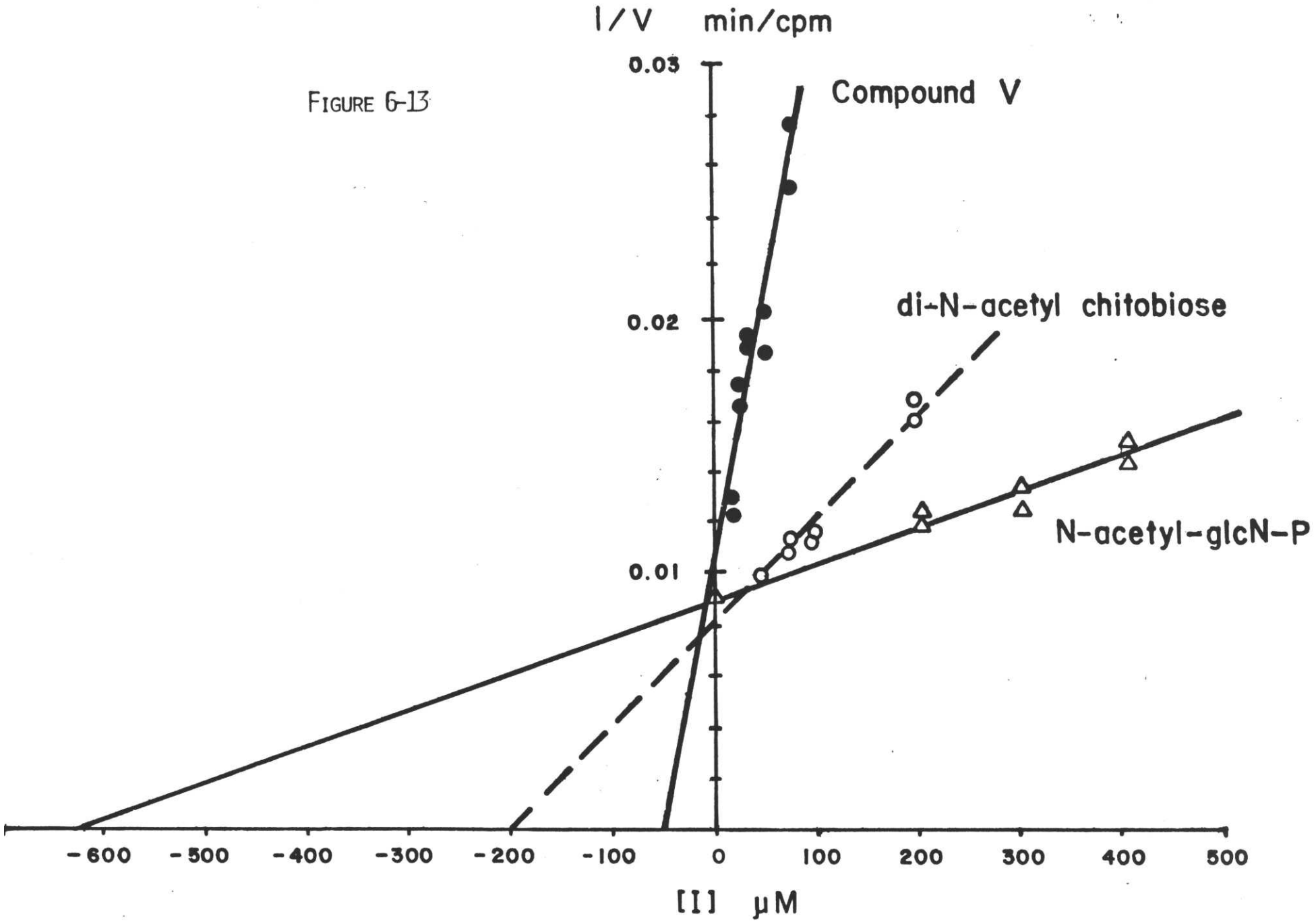
FIGURE 6-13

Inhibition of amidase I by compound V, N-acetyl-α-glucosamine-1-phosphate, and di-N-acetyl-chitobiose (Dixon plots).

Reaction mixtures containing varying concentrations of the inhibitors were composed as described in Materials and Methods. Three time points (7 ul) were fractionated by electophoresis on DE-81 paper: 0.5, 10 and 20 minute. Rates were determined.

$[S_o]$ = 30 uM compound I (18,300 cpm/nmole).

FIGURE 6-13


1/V min/cpm
0.03
0.02
0.01
Compound V
di-N-acetyl chitobiose
N-acetyl-glcN-P
-600
-500
-400
-300
-200
-100
0
100
200
300
400
500
[I] μM

Inhibition of Amidase II.

No significant inhibition of amidase II was discerned with di-N-acetyl-chitobiose, N-acetyl-α-glucosamine-1-phosphate or α-glucosamine-1-phosphate. Concentrations of up to 200 uM with di-N-acetyl-chitobiose and up to 800 uM with the others had no demonstrable effect.

However, compound I was found to be a potent inhibitor of amidase II although that enzyme cannot deacylate it (fig. 6-14). ^{32}P-compound V (51 uM, $[S]/K_m$=2.5) and varying levels of ^{32}P-compound I were incubated with amidase II. Concentrations of compound I were determined from the radioactivity remaining at the origin on electrophoregrams. At 12 uM compound I, amidase II was inhibited by 50%; it was completely inactive above 30 uM. Note that none of the inhibitors of amidase I completely inactivated that enzyme.

Effect of Cations on Amidase I.

Amidase I was assayed in varying concentrations of $CaCl_2$ and $MgCl_2$ with 171 uM compound I (fig. 6-15). Rather than the usual 25 mM citrate buffer, 200 mM formate was used after demonstrating that enzyme was fully active in that buffer. This was to avoid the chelating properties of citrate. Even up to 10 mM concentrations of Ca^{+2} and Mg^{+2} did not inhibit amidase I. At 100 mM divalent cation inhibition was manifested, probably by aggregating the substrate. Both cations behaved similarly. Though it is well established that divalent cations interact with phosphates, they did not inhibit the interaction of compound I with amidase I. This endorses the notion that the phosphate themselves do not interact with the enzymes, as discussed earlier.

Assayed in citrate buffer with varying concentrations of potassium chloride and 100 µM compound I, amidase I was fully active in up to 150 mM KCl (fig. 6-16); its activity declined by 60% at 500 mM KCl and dissappeared above that. It is not clear whether increasing ionic strength influences the physical state of substrate, conformation of the enzyme or both.

FIGURE 6-14

Inhibition of amidase II by compound I.

Reaction mixtures for assay of amidase II contained varying concentrations of ^{32}P-compound I (6190 cpm/nmole).

$[S]_o$ = 51 uM compound V (6190 cpm/nmole)

Mixtures were fractionated by electrophoresis on DE-81. Compound I concentration was determined from radioactivity at the origin. Rates of hydrolysis of compound V were calculated from three time points (7.5 ul): 0.5, 6 and 15 minutes.

FIGURE 6-14

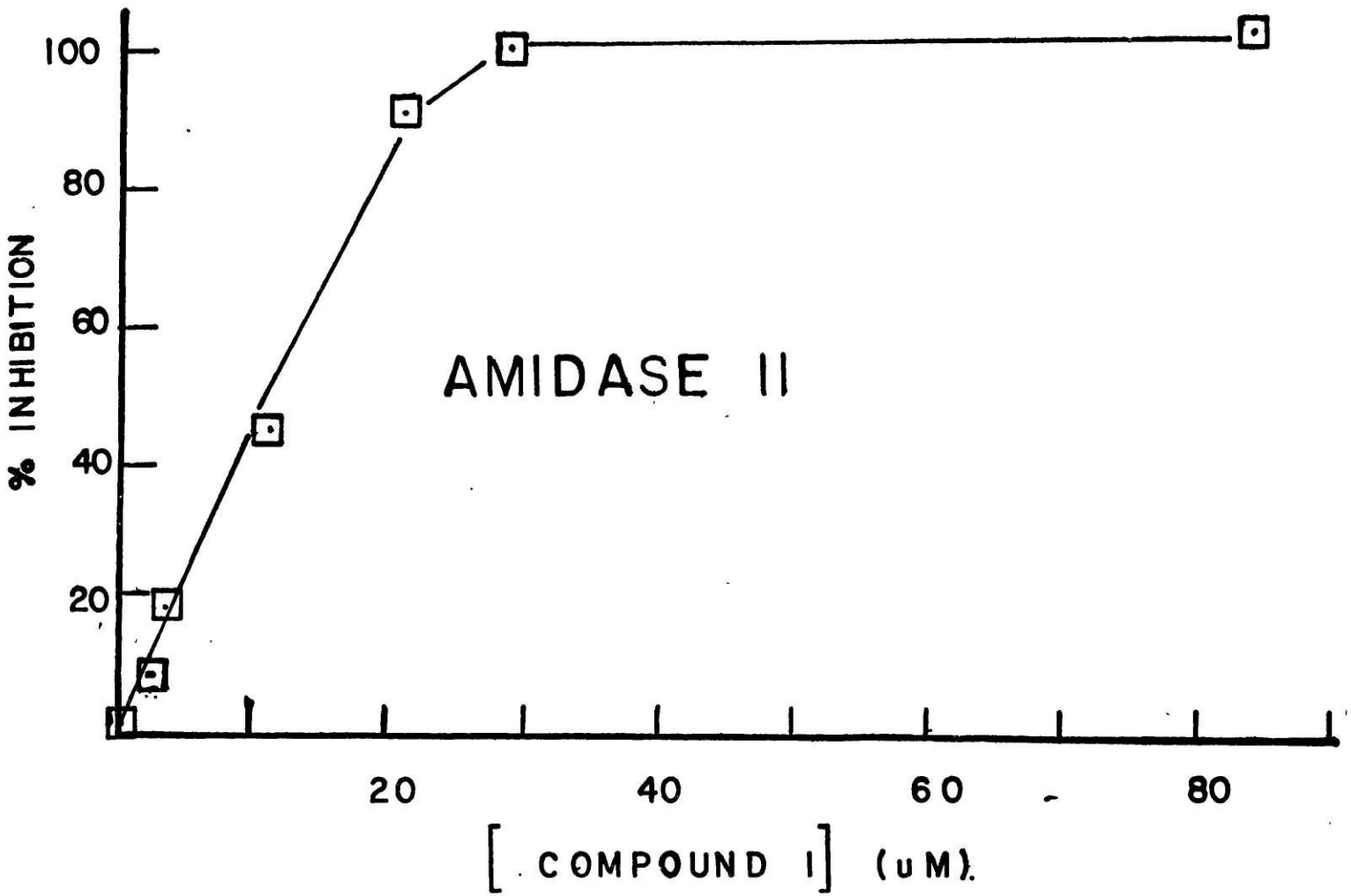


FIGURE 6-15

Effect of divalent cations (Ca^{+2}, Mg^{+2}) on activity of amidase I.

Amidase I was assayed in 70 mM sodium formate, pH 3.0 with varying concentrations of $CaCl_2$ and $MgCl_2$.

Total volume: 15 ul

$[S]_o$= 61 uM compound I

After 16 minutes of incubation at 37 ^{o}C, 5 ul of 0.2M EDTA was added to chelate cations before fractionation by electrophoresis on DE-81 paper.

FIGURE 6-15

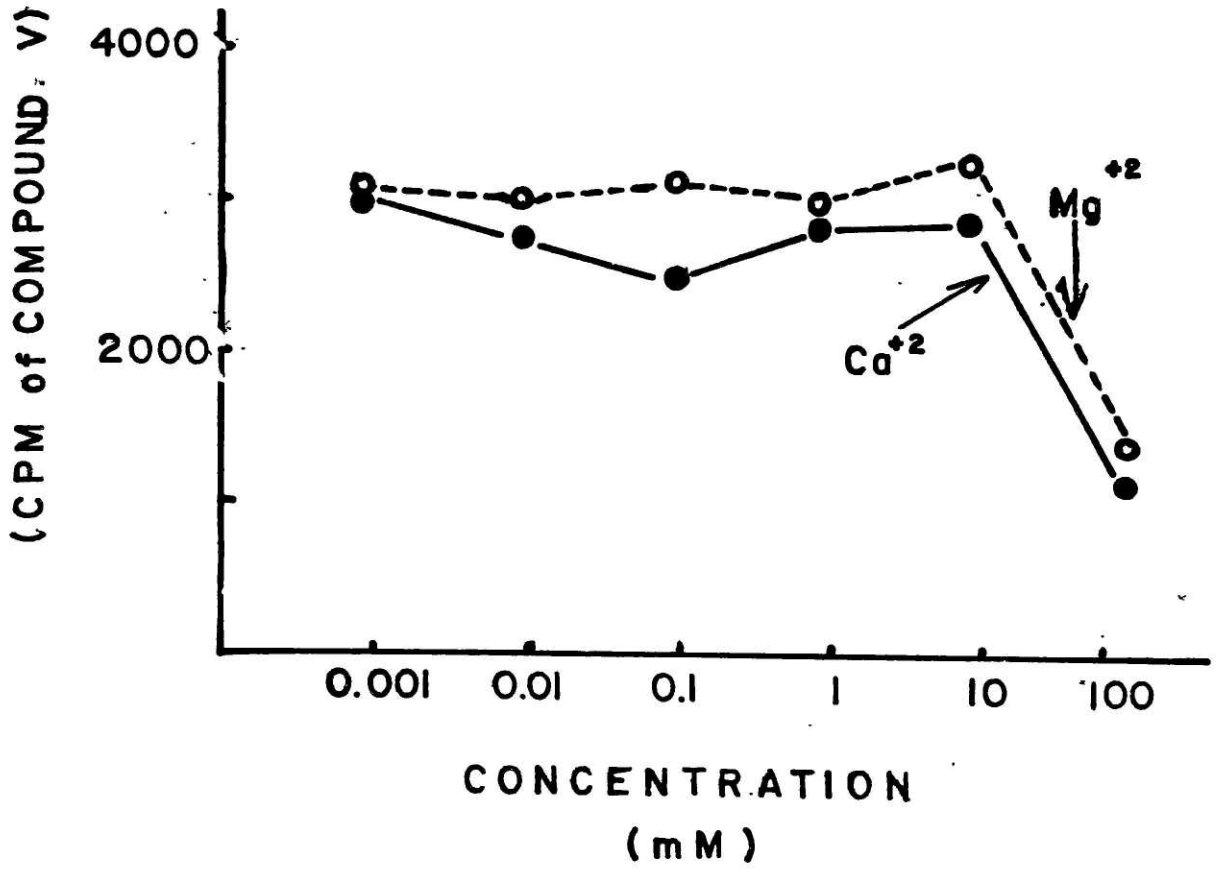

(CPM of COMPOUND V)
4000
2000
Mg+2
Ca+2
0.001
0.01
0.1
1
10
100
CONCENTRATION
(mM)

FIGURE 6-16

Effect of KCl on amidase I activity.

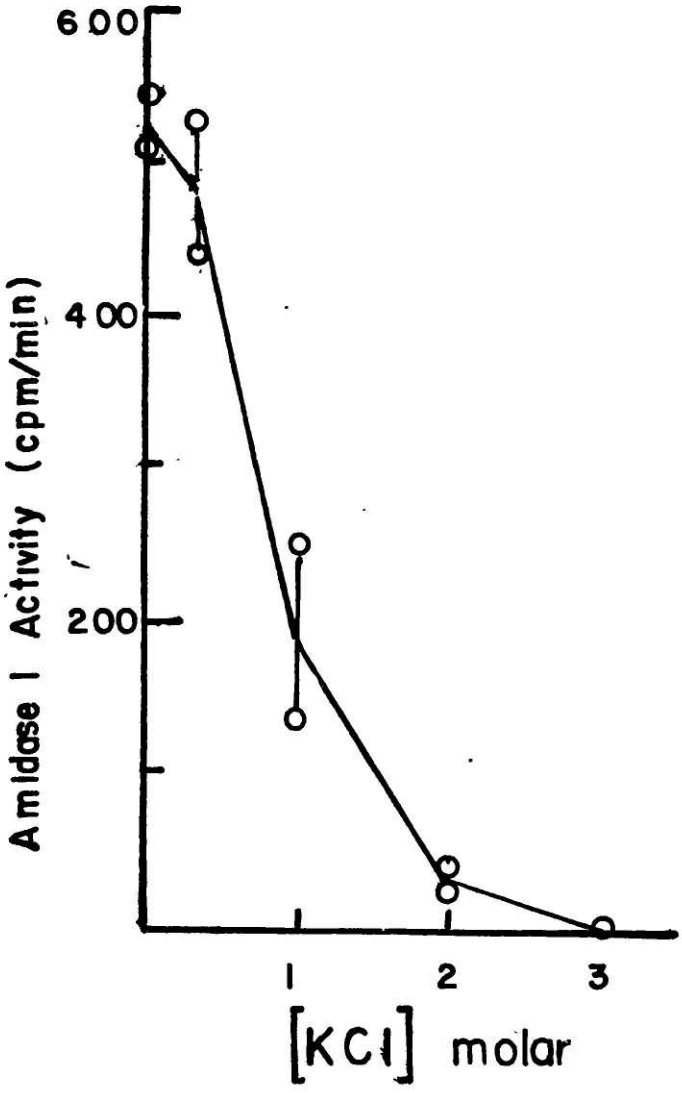
Amidase I was assayed as described in Materials and Methods with varying concentrations of KCl

Total volume: 16.8 ul

$[S]_0$ = 95 uM

Mixtures were fractionated by electrophoresis on DE-81.

FIGURE 6-16



Chapter 7

DISCUSSION

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SPECIFICITY OF THE AMIDASES

In light of the structures of inhibitors and substrates recognized by the amidases (fig. 7-1), certain conclusions and suppositions are proposed concerning the interaction of the enzymes with these molecules.

Amidase I.

Amidase I does not hydrolize N-myristoyl-α-glucosamine-1-phosphate, a monosaccharide analog of site A. This observation alone suggests the necessity for the larger disaccharide structure for proper positioning in the active site. Among the inhibitors evaluated, the strongest are disaccharides, compound V and di-N-acetyl-chitobiose.

In assessing these inhibitors, one recognizes a distinction between acetylated glucosamine derivatives and free amino compounds. Clearly the cationic ammonium of non-inhibitory α-glucosamine-1-phosphate distinguishes it from its inhibitory, acetylated isomer. Di-N-acetyl-chitobiose is also an inhibitor. The active site is optimized to bind a hydrophobic fatty-acyl chain, not a hydrated and charged ammonium. Compound V, with an ammonium at site A, strongly inhibits amidase I, nonetheless.

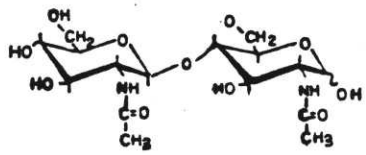
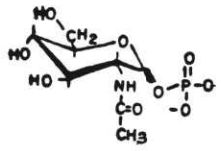
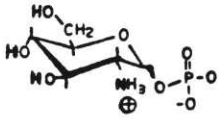
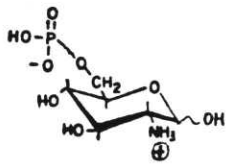
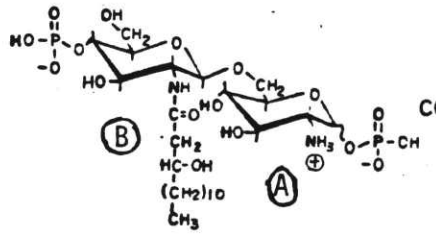
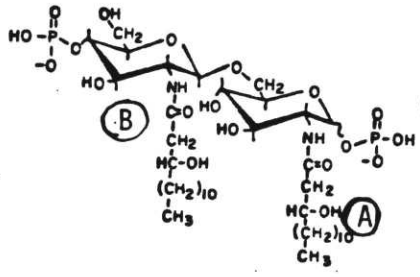
These observations are explained by the model portrayed in figure 7-2. According to this, compound I binds to amidase I via two sites: a catalytic site receiving the A acyl chain and adjoining non-catalytic site receiving the B acyl chain. It is unclear at what site the inhibitor, N-myr-glcN -1-P, binds, but, for purposes of argument, it should bind to the catalytic domain by virtue of its resemblance to the A moiety of compound I. Product inhibition by compound V occurs in an orthodox fashion by its acyl glucosamine occupying the non-catalytic domain; the A ammonium interacts weakly with the catalytic site and can be visualized as hanging loosely. The interaction at the non-catalytic site is essential to proper positioning of the A acyl

FIGURE 7-1

Structures of compounds examined as potential inhibitors or substrates of amidases I and II.

Depicted on two pages.

FIGURE 7-1

STRUCTURES		COMMENTS
	di-N-acetyl chitobiose	Inhibits amidase I, not amidase II
	α-glcNac-1-P	Inhibits amidase I, not amidase II
	α-glcN-1-P	Inhibits neither amidase I nor II
	glcN-6-P	Inhibits neither amidase
	compound V	Hydrolized at site B by amidase II Unchanged by amidase I Inhibits amidase I
	compound I	Hydrolyzed at site A by amidase I Unchanged by amidase II Inhibits amidase II

CONTINUED

FIGURE 7-1

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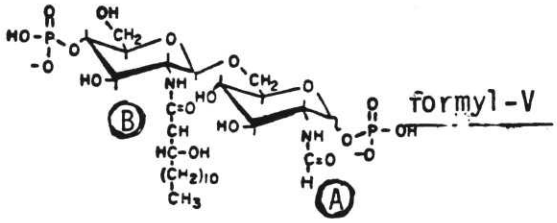
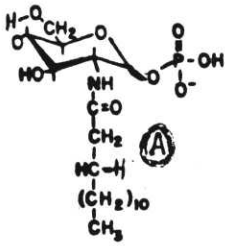
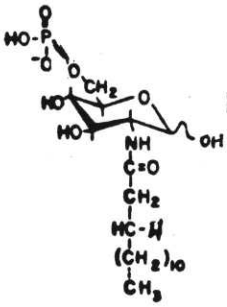
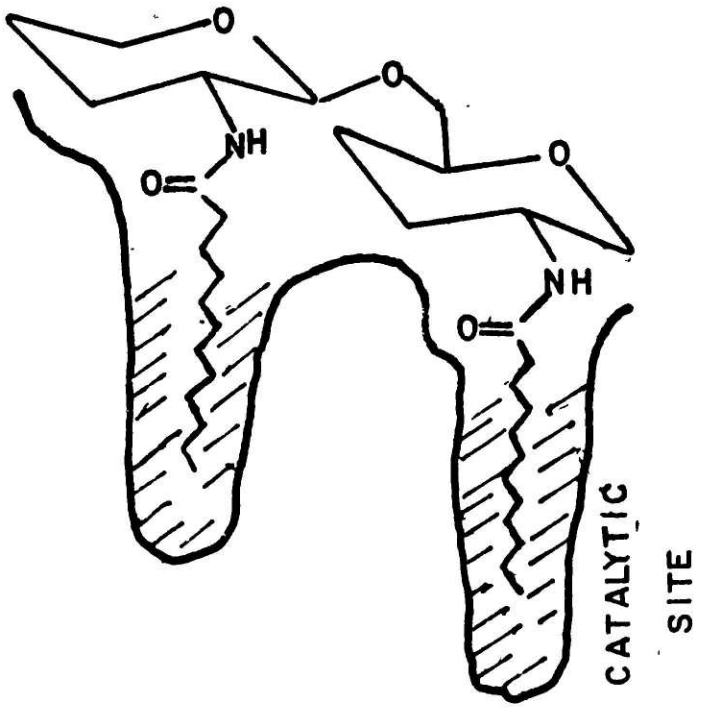
STRUCTURES	COMMENTS
 N-formyl-Compound V	Hydrolyzed at site B by amidase II Not deformylated by either amidase. Site B not affected by amidase I
 N-myristoyl-α-glucosamine 1-phosphate	Deacylated by amidase II Not deacylated by amidase I
 N-myristoyl- glucosamine 6-phosphate	Not deacylated by either amidase.

FIGURE 7-2

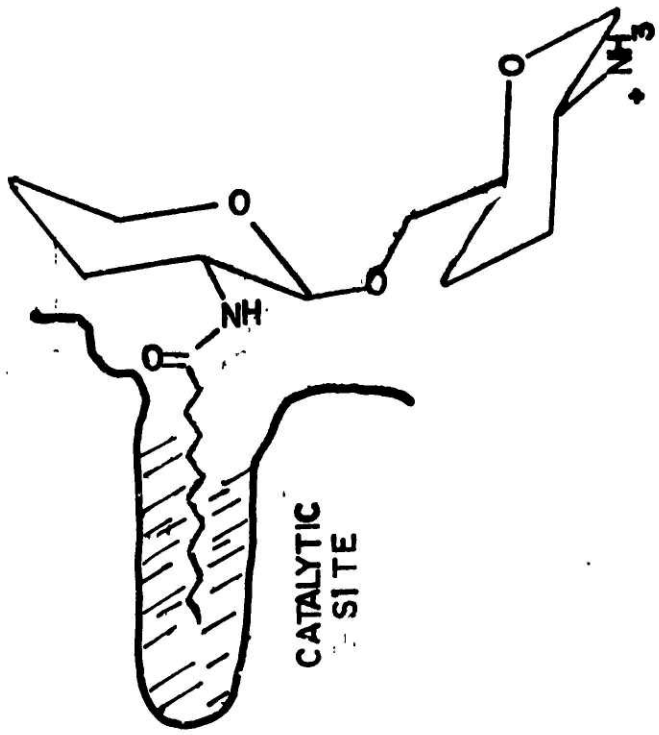
Proposed models for the interactions of amidases I and II with their substrates and inhibitors

Hydroxyls have been omitted from the drawing

FIGURE 7-2



AMIDASE I



AMIDASE II

amide for attack by the active site nucleophile. For this reason the monosaccharide analog, N-myr-glcN-1-P, cannot be hydrolyzed, although it is expected to bind.

Failure of amidase I to deformylate N-formyl-compound V agrees fully with the above model if it also requires that the catalytic domain be hydrophobic and able to distinguish long-chain-acyl glucosamine from the less polar formyl derivative. Although the interaction at the non-catalytic domain is satisfied by this compound, the N-formyl moiety at the catalytic site is too weakly held to be kept in range of the nucleophile.

The role of amidase I in the sequential dyad of amidases is also explained by this model. Amidase I cannot hydrolyze the B-acyl group because that group obligatorily occupies the non-catalytic domain. If the B chain binds to the catalytic domain, it cannot be cleaved for lack of an occupant at the adjoining site, an occupant necessary for activation.

Adjoining non-catalytic domains are important to the specificity of other enzymes. All serine proteases cleave peptide bonds via the same catalytic cascade, but their specificity depends on interaction between amino acid sidechains near the labile bond and complimentary binding sites adjoining the active site (213). Two binding domains were resolved in acetylcholine esterase: an esteratic site recognising the labile ester moiety and an anionic site binding the quaternary ammonium (220). Molecules that bind solely to the anionic site, such as quaternary amines, competitively inhibit hydrolysis of acetylcholine.

Amidase II.

The second amidase interacts differently with its substrates (fig 7-2). Unlike amidase I, this enzyme does hydrolyze the monosaccharide N-myr-glcN-1-P, albeit more slowly than compound V, although that analog mimicks site A. The α-phosphate must satisfy a requirement for a reducing-end substituent,

also met by the β(1-6)-aglycon of compound V. Clearly, an important factor is the interaction of the hydrophobic acyl chain with the active site. More polar analogs such as N-acetylated or non-acylated α-glucosamine-1-phosphate do not inhibit. But, the N-myristoyl derivative is deacylated by the enzyme.

Sequential specificity of amidase II is clarified in the following results. Formylation to alter the charge at site A does not influence cleaage at site B, further endorsing the model that only the site B sugar inteacts specifically with the active site. This dismisses the suggestion that the ammonium at site A ia the determinant distinguishing the inertness of compound I from the susceptibility of compound V to amidase II. Much more revealing is the inhibitory potential of compound I toward amidase II. Although not a substrate, it does bind and inhibit the enzyme. As proposed in chapter 6, amidase II cannot hydrolyse the B-acyl chain of compound I because it is competitively inhibited by site A of the same molecule. The specific binding site of amidase II has greater affinity for the long N-acyl group at site A. In N-formyl-compound V, the more polar formyl substituent cannot compete against the group at sit B. That fatty-acyl site A effectively binds to the catalytic site is demonstrated by hydrolysis of its analog N-myr-glcN-1-P.

If site A of compound I binds to the active site, one must explain why it is not hydrolyzed. An obvious explaination is that the β(1→6) glycoside prevents proper positioning in the active site even though binding occurs. The same rationale explains the inertness of N-myr-glcN-6-P. Lemieux et al. (221) have proposed that binding of oligosaccharide to immunoglobulin is stabilized by hydrophobic interactions and that any drogen bonding is incidental. They have also demonstrated that a galactosyl residue, in binding to antibody, preferentially assumes a conformation such that hydrogen-bonding groups form one hydrophilic surface while the other face is maximally hydro-

phobic. A complimentary hydrophobic domain on the immunoglobulin interacts with the hydrophobic face of the oligosaccharide with a net increase in entropy. If binding to amidase II follows this paradigm, hydrophilic C_6 substituents could conceivably interfere with apposition of the hydrophobic surfaces. In the more stable chair configuration, hydrophilic groups on glucosamine occupy equatorial positions in a pseudo-planar array. Bulky C_6 substituents can freely rotate out of plane and, even without rotating, disrupt hydrophobic interactions below the plane.

Phosphates.

Removal of either C_1 or C_4' phosphate did not interfere with hydrolysis by either amidase. High phosphatase activity in D. discoideum lysosomes is expected to quickly dephosphorylate ingested LPS, leaving partially and fully dephosphorylated substrates for the amidases; deacylation proceeds completely in vivo (172). In the model described above, phosphates are not expected to interact strongly with the active site, especially if the active sites are as hydrophobic as predicted from Lemieux's studies. Failure of divalent cations to inhibit amidase I supports this supposition (Chapter 5).

THe KDO Trisaccharide.

Since OH-LPS is also sequentially hydrolyzed by the amidases, the KDO trisaccharide at C_3' must not interfere. It is indeed surprising that this trisaccharide does not affect hydrolysis of the neighboring C_2' amide by amidase II. With the active site models proposed above, one must imagine that the polysaccharide swings away from the enzyme into the aqueous phase. The natural substrate for these enzymes retain the extended core and O-specific polysaccharide.

UTILITY OF THE AMIDASES

The amidases make possible specific excision of the amide-linked fatty acids from LPS derivatives. Now, through sequential application of alkali and the amidases, all fatty acids on LPS can be released under mild conditions. It is apparent, though not proven, that amidase I hydrolyzes LPS prior to its de-esterification, thus, introducing even greater flexibility in manipulations of the fatty acid composition.

The role of β-hydroxymyristic acid residues in establishing the integrity of the outer membrane can be specifically addressed with these enzymes. LPS forms essential complexes with outer membrane protein and phospholipid (55-57,62). These complexes act as hydrophilic channels (76-78), phage receptors (88,89) and maintain impermeability to hydrophobic agents (79). The incorporation of this unusual fatty acid into LPS suggests a special role in these interactions.

The β-hydroxymyristoyl amides on LPS also appear to play an important role in its endotoxicity. Goodman and Sultzer (119) have shown that alkali-treated LPS, devoid of esterified fatty acid, remains mitogenic to B lymphocytes. Luderitz and coworkers ((120) corroborated that conclusion and found mitogenic activity associated with the lipid A precursor indentified by Osborn *et al*. (22-24) that lacked all non-hydroxylated fatty acids but retained it β-hydroxymyristic chains. They also reported that further removal of β-hydroxymyristic chains by hydroxylaminolysis abolished mitogenic activity. With the *D. discoideum* amidases, it is possible to test the role of β-hydroxylation in interactions with LPS-receptor and other factors pertinent to its endotoxic effect. Characterizing the LPS-receptor and understanding its pharmacology would contribute significantly to management of endotoxemia resulting from most septic infections.

By virtue of their specificity for LPS derivatives, the amidases offer the possibility of novel techniques for quantitating LPS in clinical and research problems. The Limulus amoebocyte lysate (LAL) assay detects extremely low levels of endotoxin (199). Though maximally sensitive to LPS, other components of bacterial membranes can trigger coagulation of the lysate. Contradictory results sometimes arise in tests for septicemia (200). Maitra and coworkers (201) have directly quantitated β-hydroxymyristic acid in hydrolysates of human serum by ga chromatography/mass spectroscopy (GCMS). As little as 22 fmoles of fatty acid were detected. Non-specific hydrolysis of serum released other components indistinguishable from β-hydroxymyristic acid. With the amidases one can specifically release this almost unique fatty acid and directly predict the LPS content.

Amidase I requires recognition of both acyl glucosamine moieties of LPS in order to hydrolyze a potential substrate and is, therefore, more specific than amidase II, capable of hydrolyzing monosaccharide substrates. For this reason the first enzyme is more useful in identifying LPS-like molecules. Fortunately, it is also the first in the sequence of amidases. Such enzymatic analysis can clarify the extent of resemblance to gram-negative LPS of novel endotoxins, such as one isolated from Lysteria monocytogenes (159).

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Sonnet - To Science

Science! true daughter of Old Time thou art!
Who alterest all things with thy peering eyes.
Why preyest thou upon the poet's heart,
Vulture, whose wings are dull realities?
How should he love thee? or how deem thee wise?
Who wouldst not leave him in his wandering
To seek for treasure in the jewelled skies,
Albeit he soared with an undaunted wing?
Hast thou not dragged Diana from her car?
And driven the Hamadryad from the wood
To seek a shelter in some happier star?
Hast thou not torn the Naiad from her flood,
The Elfin from the green grass, and from me
The summer dream beneath the tamarind tree?

Edgar Allan Poe