ENZYMES ASSOCIATED WITH MANNITOL METABOLISM IN THE SPOROPHORE OF AGARICUS BISPORUS (LANGE) IMBACH

By

Nicholas Ian Morton, B.Sc., ARCS.

A thesis presented for the degree of Doctor of Philosphy of the University of London.

Department of Biochemistry,
Imperial College of Science and Technology,
London, SW7 2AZ

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ABSTRACT

Mannitol accumulates in the developing sporophore of Agaricus bisporus to levels around 40% of the dry weight, whereas levels found in the mycelium are considerably lower, 1-5% of the dry weight. The present study investigated the role of some of the enzymes associated with mannitol metabolism in this organism.

Mannitol dehydrogenase is responsible for the NADP mediated interconversion of mannitol and fructose. This enzyme was purified to homogeneity and found to be a tetramer of subunit molecular weight, 29,500.

Mannitol dehydrogenase has a high degree of specificity for NADP(H) as coenzyme and fructose as hexose substrate. However, it has limited activity against sorbitol in addition to mannitol. The enzyme has a high Km for fructose (1.2M at pH7.0). The rates of fructose reduction and mannitol oxidation and substrate Km's show significant variation with pH. The reaction equilibrium is strongly influenced by pH. The mannitol dehydrogenase reaction is sensitive to the $NADP^+$/NADPH ratio. The possible effects of these kinetic properties in vitro on the control of mannitol metabolism in vivo are considered. The level of mannitol dehydrogenase activity varies with development; maximum levels correspond approximately to periods of greatest mannitol synthesis.

Fructokinase and fructose-6-phosphatase activities were detected in the A. bisporus sporophore. The presence of these activities along with mannitol dehydrogenase permits interconversion of mannitol and fructose-6-phosphate generated by the Embden-Meyerhof pathway. The fructokinase enzyme is highly unstable. The phosphatase activity was ascribed to at least three isoenzymes; one is non-specific and present at all stages of development, whereas the other isoenzymes appeared to have a higher degree of specificity and to vary with development. A possible role for the phosphatase enzymes in the control of mannitol metabolism is discussed.

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Some abbreviations used in the text

ADP	Adenosine 5'-diphosphate
AMP	Adenosine 5'-monophosphate
ATP	Adenosine 5'-triphosphate
DEAE	Diethylaminoethyl
EDTA	Ethylenediamintetraacetate
Keq	Equilibrium constant
Km	Michaelis constant
NAD^+	Nicotinamide adenine dinucleotide
NADH	Dihydronicotinamide adenine dinucleotide
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NADPH	Dihydronicotinamide adenine dinucleotide phosphate
PMSF	Phenylmethylsulphonyl fluoride
SDS	Sodium dodecyl sulphate
TEMED	Tetramethyldiaminomethane
tris	Tris (hydroxymethyl) aminomethane

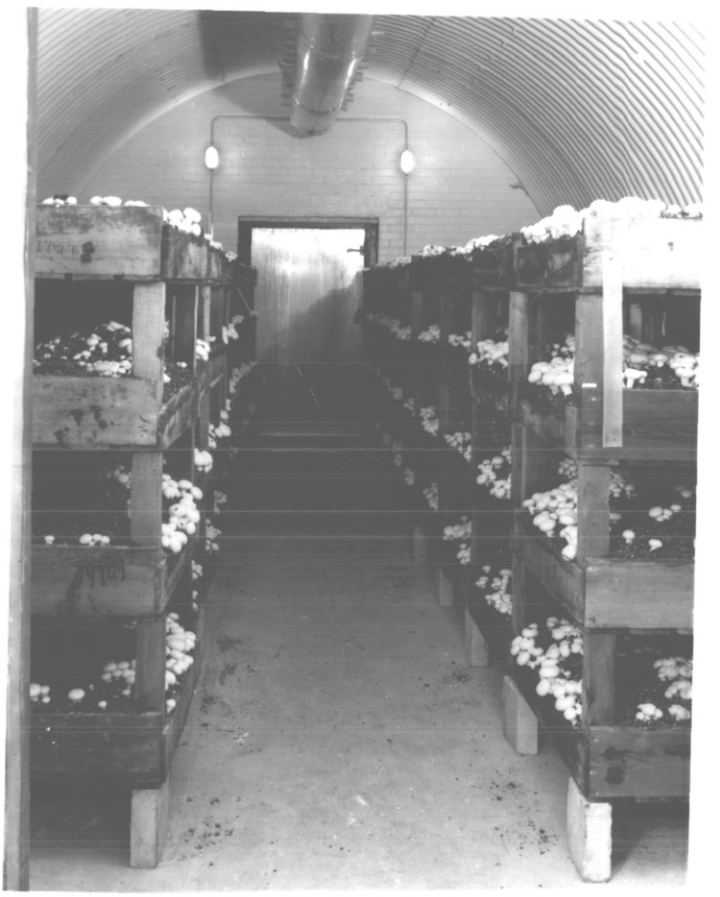
1. INTRODUCTION

1.1. A History of the Cultivation of Agaricus bisporus, the Edible Mushroom

The first known attempts to grow mushrooms of the Agaricus bisporus type took place in France around 1700 (Singer, 1961; Hayes and Nair, 1975). Early crops were grown outdoors or in underground caves in Paris; these caves are still being used to the present day. The crops were derived from an innoculum of soil permeated with mycelium of the wild A. bisporus var. albidus. The art of mushroom growing spread to the rest of Europe and to America. Outdoor cropping became popular and many growers used disused buildings, railway tunnels, etc.; the French developed the use of mushroom house culture, in addition to cave culture, and the Swedes developed the use of glasshouses.

Mushrooms became a delicacy in Europe, particularly in France where the cuisine demanded fresh mushrooms. The French growers used a composting procedure similar to that in use today and during the nineteenth century the French held the lead in culture techniques and learned the benefits of pest control and sterile spawn. Many of the lessons learned about mushroom growing were the result of research at the Pasteur Institute but workers, led by Constantin, often kept progress a secret, and so maintained a monopoly for the Institute in the supply of pure culture

Plate 1: A typical example of a mushroom growing shed showing trays of developing Agaricus bisporus sporophores.



spawn up until the early twentieth century. American mycologists then independently discovered the techniques for growing culture spawn from tissue culture and spores. Until pure culture spawn was available, spawn was produced by digging up wild mycelium ("virgin spawn"), by breaking up and drying spent compost ("flake spawn") or by innoculating old spawn into a dried and shaped mix of horse and cow manure and loam ("brick spawn"). However, the use of pure culture spawn to replace the old methods was widespread by 1918; unlike the French, the Americans did not withold essential information on techniques.

Modern commercial growers grow mushrooms mainly in specially adapted houses to control the moisture content and temperature of the enviroment. The mushrooms are often grown in trays on shelves. Sterile spawning is used and closer monitoring and control of composting process have taken place. Mushroom production can now be highly mechanised and large canning plants are used for packaging some crops.

1.2. Basidiomycetes.

The Basidiomycotina or Basidiomycetes are a subdivision of the division Eumycota in the kingdom of Fungi. The Basidiomycetes are characterised by the formation of basidiospores on a basidium (Ainsworth, 1971). The basidia are formed on fruit-bodies, also known as "sporophores" or "basidiocarps",

and are initiated as a binucleate cell (Hawker, 1966). The two nuclei fuse, the process of karyogamy, and a meiotic division follows immediately. Karyogamy followed by meiosis also occurs in the formation of ascospores in the Ascomycetes. However, similarities in reproduction between the two groups end here and the typical Basidiomycete then continues fruiting by the development of four sterigmata, each with an apical sac-like swelling on the basidia. The swellings are the basidiospore initials and a single haploid nucleus migrates into each from the basidium, though two or more nuclei may enter in some species. A cell wall forms around the nucleus to form the basidiospore as an integral, exogenous spore, separated from the basidium.

The Basidiomycetes are varied in form and habitat but taxonomically can be divided into three classes (Ainsworth, 1971, 1973), namely;

1. Teliomycetes. The basidiocarp is replaced by teliospores (encysted probasidia) which are grouped in sori or scattered within the host tissue. The members of this class are parasitic on vascular plants.
2. Hymenomycetes. The basidiocarp is usually well developed and the basidia are typically organised as a spore bearing layer or "hymenium". The hymenium is exposed at the primordium stage (in species said to be "gymnocarpous") or before the spores have quite matured ("semiangiocarpous"). The basidiospores are ballistospores,

i.e. forcibly ejected as the term suggests. Hymenomycetes are microrhizal fungi or, occasionally, saprophytic or parasitic.

3. Gasteromycetes. The basidiocarp is again well developed and basidia are typically organised as a hymenium. The basidiocarp is closed at least until the spores are mature ("angiocarpous"). The basiospores are not ballistospores. The Gasteromycetes are also normally microrhizal fungi or, occasionally, saprophytic or parasitic.

The Hymenomycetes are divided into two subclasses; the Phragmobasidiomycetidae and the Holobasidiomycetidae (Ainsworth, 1973). The former are characterised by phragmobasidia in which the metabasidium (the stage of the basidium in which meiosis occurs) is divided by primary septa. The Holobasidiomycetidae and all the Gasteromycetes have holobasidia in which the metabasidium is not divided by primary septa but may become adventitiously septate.

Within the Holobasidiomycetidae occurs the order Agaricales; this order is characterised by the hymenium covering lamellae on the lower surface of the pileus or lining tubes which are easily separable from the pileus (McNabb and Talbot, 1973). Within the Agaricales occurs the family Agaricaceae, characterised by lamellae free or practically so; an annulus typically present on the stipe; spore deposit blackish, fuscous, olive-fuscous, or some shade of chocolate brown; spores lacking an apical germ pore; stipe and pileus clearly separable (Smith, 1973). In

Plate 2: Developing sporophores of Agaricus bisporus .

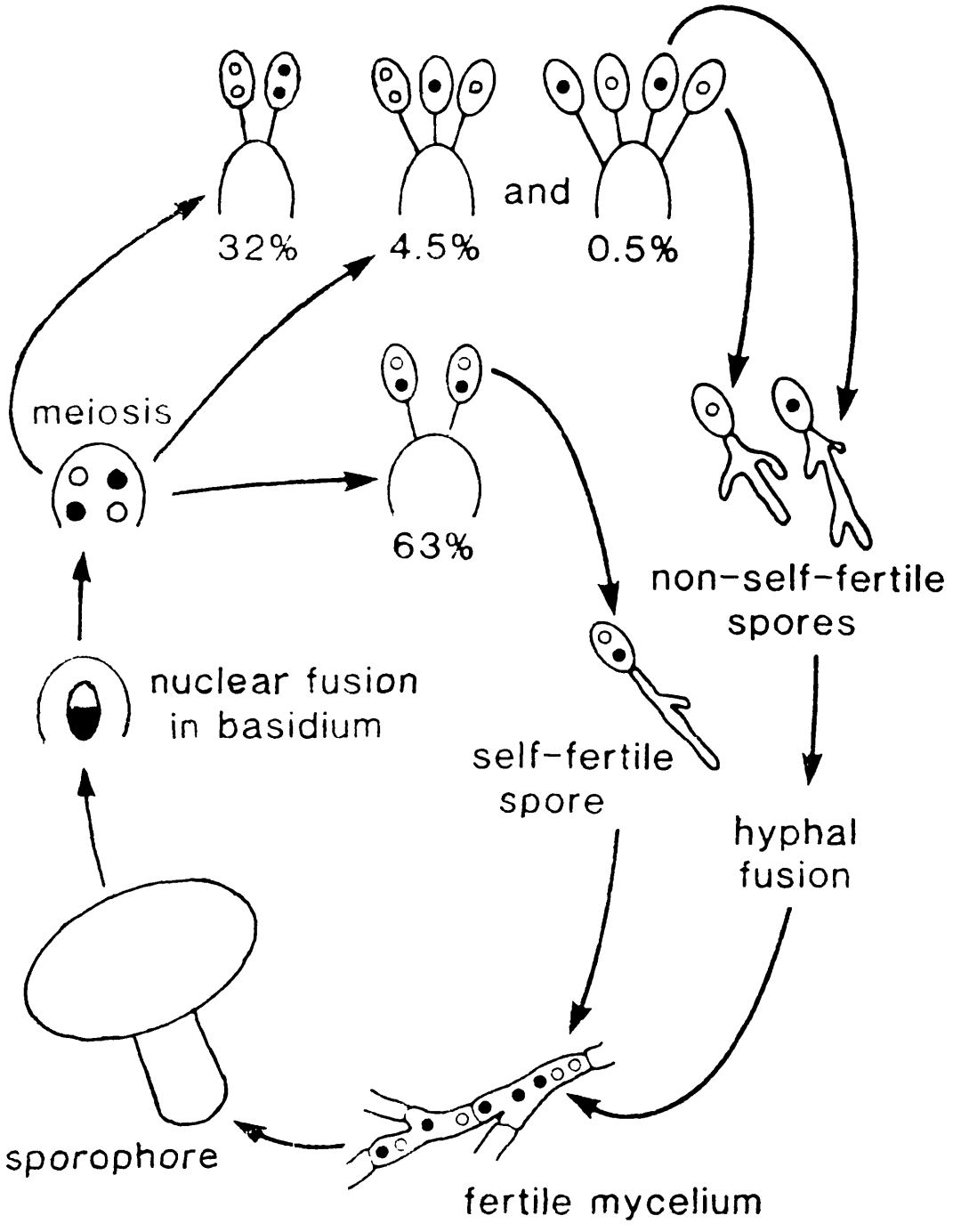


turn, the genus Agaricus occurs within the Agaricaceae typified by ovoid to ellipsoid and smooth spores (Smith, 1973). The genus contains many species with some variety. Many of the species are edible and can be cultivated, including Agaricus bisporus which is commonly cultivated in the U.K..

1.3. The Growth and Propogation of Agaricus bisporus.

The life-cycle of Agaricus bisporus (Lange) Imbach is shown in Figure 1 (Elliott, personal communication). As the specific name "bisporus" indicates, the basidia generally bear only two spores as against the typical four spores described above for Basidiomycetes. However, not all of the basidia are of the two-spored type. Most of the spores produced are dikaryotic and heterokaryotic (Elliott and Langton, 1981). A. bisporus is a secondarily homothallic species, i.e. there are two nuclear types produced by separation of a single mating allele and in the normal dikaryotic heterokaryotic spores a nucleus of each type is present. Such spores are capable of germinating and producing fertile mycelium. Infertile mycelium can be produced from irregular spores; these spores may receive homoallelic nuclei or they may be haploid, formed by the receipt of a single nucleus. Fertile mycelium can be produced by the fusion of homoallelic mycelium with compatible, homoallelic mycelium carrying the opposite mating factor (Elliott, 1978).

Figure 1: Life-cycle of Agaricus bisporus (after Elliott).



Open and closed circles indicate nuclei of different mating types.

The heterokaryotic mycelium produces simple mycelial strands in the vegetative phase. Modern commercial practice is to propogate the mycelium on sterile grain which acts as spawn and is used to seed a compost medium; the compost itself is produced in such a way that it acts as a selective substrate for A. bisporus. The compost is prepared from horse manure and wheat straw. This is the traditional growth medium and, whilst other mixtures employing such ingredients as corn-cobs, legume hay, gypsum, ammonium nitrate, muriate of potash and dried brewers grains or wheat straw and dried blood have been developed, the horse manure and wheat straw mix is the most popular today (Hayes and Nair, 1975). The straw and manure are arranged in stacks and water and nitrogenous supplements are added to initiate the fermentation. The stacks are turned to mix and maintain aerobic conditions. High microbial activity results in high temperatures within the stacks which, in turn, select for specific groups of thermophilic micro-organisms. The early part of composting is performed outside but a second stage is performed inside buildings where temperatures and aeration are controlled, the "peak-heat stage", to complete the process. The compost is pasteurised and, in most modern growing units, placed in trays, prior to spawning (Hayes and Nair, 1975).

The mycelium of the A. bisporus spawn grows and colonises the compost. Left in this condition a few sporophores (fruit-bodies) might develop in time (Eger, 1961).

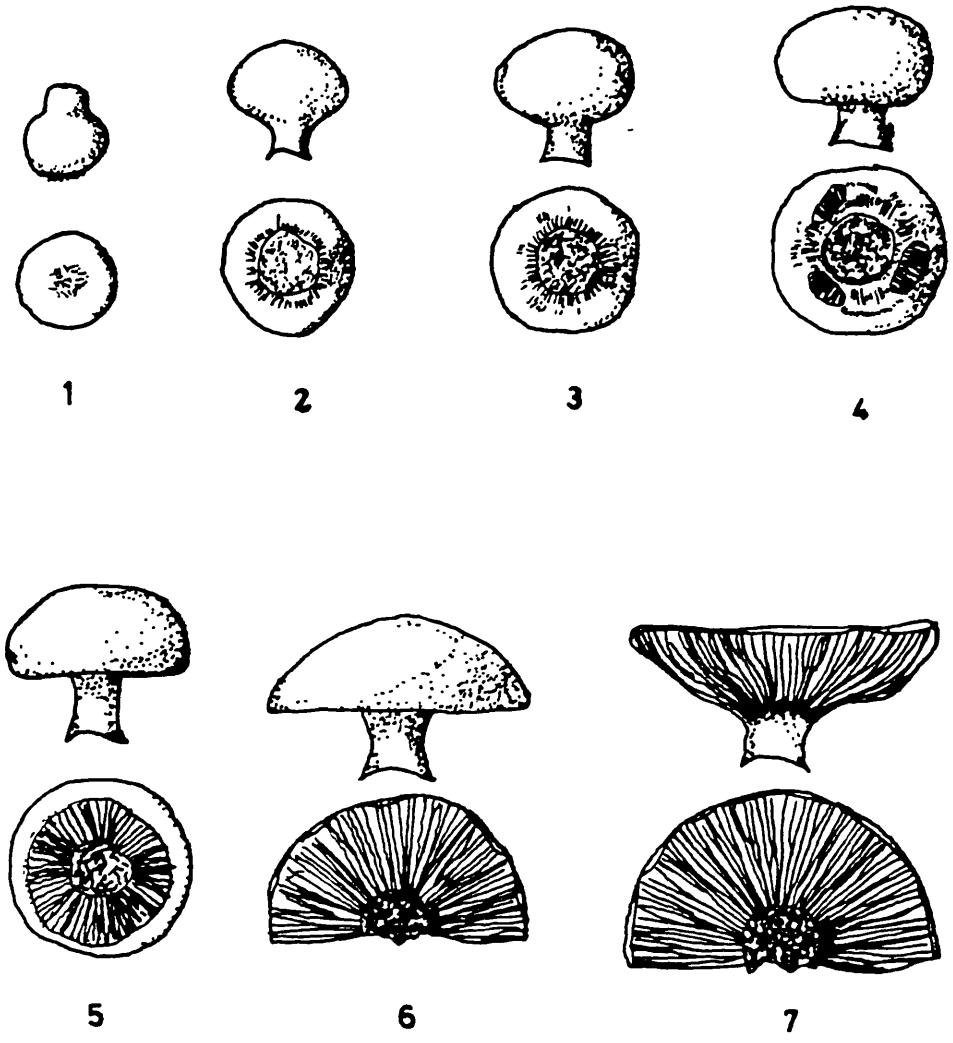
However, to promote fruiting, growers cover the surface with a 3 - 5 cm layer of "casing". This consists of buffered peat or soil, normally a 1:1 (v/v) mixture of peat and chalk. It is unknown how this process stimulates production but microorganisms, carbon dioxide and volatile metabolites have been considered as important by various workers (Long and Jacobs, 1974). Long and Jacobs (1974) were able to demonstrate that microorganisms are not essential for sporophore production by fruiting A. bisporus in a sterile enviroment. However, under such sterile conditions, fruiting did not take place unless activated charcoal was included as a component of the casing mixture.

Fruiting commences about two weeks after casing and the yield capacity of the culture diminishes with time so that it is normal commercial practice to terminate cropping after five or six weeks. Sporophore production is not even; there are four or five peaks of production or "flushes" occurring at intervals of about ten days. At sporophore initiation the mycelium aggregates forming fruit-body primordia, some of which abort without further development. The primordia are roughly spherical with a diameter of about 1 mm. These then form "pinheads" which were classified as stage 1 sporophores by Hammond and Nichols (1975); Table 1 and Figure 2 summarise their classification into seven arbitrary stages from the pinheads, through "buttons" and "cups", to "flats". Sporophore development takes about ten days. Even at stage 1 the sporophore is differentiated

Table 1: Description of stages of development of the *A. bisporus* sporophore (after Hammond and Nichols, 1975).

Stage	Approximate diameter of the pileus (mm)	Description
1	5	"Pinhead" - velum not differentiated
2	20-30	"Button" - velum visible and intact but not stretched
3	30-40	"Closed Cup" - velum stretched but still intact
4	30-40	"Cup" - velum starting to tear
5	30-50	"Cup" - velum torn, pileus still cup shaped, gills clearly visible
6	40-60	"Flat" - upper surface of the pileus convex, gill surface flat or slightly concave
7	50-70	"Flat" - gill surface curving upwards

Figure 2: Appearance of sporophore at stages 1-7 (after Hammond and Nichols, 1976a).



into three tissue types; the "stipe" or "stalk" and the "pileus" and the "gill" tissues of the cap. The gills bear the basidia and each basidium bears two, three or four spores which complete the life-cycle.

Studies on the developmental stages of fruit-body initiation and growth are difficult, as are studies on the biochemical differences between fruit bearing vegetative mycelium and sporophore mycelium. This is because most strains of A. bisporus produce very few fruit-body primordia when grown axenically and these are normally produced asynchronously and will not develop further than the aggregate primordium stage. Such difficulties are not experienced with many other Hymenomycetes, including Coprinus species which are members of the Agaricaceae closely related to A. bisporus and can be fruited on artificial media and therefore have been used extensively for research. Two strains of A. bisporus have been isolated which produce many aggregates during growth in axenic culture. These aggregates have been shown to be similar in some biochemical aspects to normal sporophore initials but they too will progress no further than the aggregate stage (Elliott and Wood, 1978).

The above problems inhibit progress on understanding the biochemistry of fruiting in A. bisporus despite its commercial importance. Vincent-Davis (1972) found there was a difference in cell wall composition between sporophore and vegetative mycelium; there is a higher level of chitin and

α1-3 glucan and less β glucan in the sporophore. There is also a difference in lipid composition; sporophores contain free sterol which is absent in mycelium (Holtz and Schisler, 1971). A further study by Lindberg (1950) demonstrated differences between mycelium and fruit-bodies in the production of phenol oxidase type enzymes; axenically grown mycelium produces laccase, the fruit-bodies produce a carbon monoxide sensitive polyphenol oxidase and mycelium grown in compost and bearing fruit-bodies contains a mixture of both enzyme types.

Soluble carbohydrates have been more intensively studied in recent years. Trehalose occurs in both mycelium and sporophore at roughly 4% of the dry weight early in the fruiting cycle (stages 1 and 2, see 1.3.) and this level then drops in both tissues during sporophore growth (Hammond and Nichols, 1976a). The similarity in levels between the tissues led to the suggestion that in A. bisporus trehalose acts as a translocatable energy source that is freely mobile between mycelium and sporophore (Hammond, 1979; Hammond and Nichols, 1976a). Cochrane (1958) had previously proposed a role for trehalose as a translocatable substrate in fungi generally. Sucrose occurs in the mycelium at levels up to approximately 1.2% of the dry weight with only traces in the sporophore. Glucose levels are around 0.7% of the dry weight of the mycelium at "casing" and then fall as the sporophore develops and levels in the sporophore itself never exceed 0.15% of the dry weight (Hammond and Nichols, 1976a).

A non-structural polysaccharide, probably glycogen, is also present in both mycelium and sporophore of A. bisporus (Hammond, 1979; Hammond and Nichols, 1976b). As levels are approximately similar in sporophore (12% of the dry weight) and sporophore bearing mycelium (18.3% of the dry weight), it has been suggested that, like trehalose, this polysaccharide is mobile between tissues and that both trehalose and non-structural polysaccharide act as reserve materials which are utilised during sporophore growth (Hammond, 1979).

However, the most abundant carbohydrate in A. bisporus sporophores is the hexose polyol, mannitol.

1.4. Mannitol in Agaricus bisporus

Mannitol was discovered in fungi in the nineteenth century (see Cochrane, 1958); it was shown to occur in the sporophores of many Basidiomycetes and Ascomycetes. The almost universal occurrence of mannitol in Basidiomycetes is illustrated by a study of ninety-one species carried out by Bonnet (1959). The polyol was present in all but five species and nearly always in combination with trehalose. Mannitol is also present in Phycomycete fungi, algae and some flowering plants (Lewis and Smith, 1967).

In A. bisporus the level of mannitol accumulating in the growing sporophore reaches values of up to 50% of the dry

weight (McConnell and Esselen, 1947; Rast, 1965; Hammond and Nichols, 1976a). Mannitol is also found in the mycelium but at lower levels, approximately 1-5% of the dry weight (Hammond and Nichols, 1976a).

Despite the high levels recorded, the role of mannitol in the developing mushroom has yet to be determined conclusively. Lewis and Smith (1967) considered possible roles for mannitol in a variety of organisms; as a translocatable substrate, as a reserve store of both carbohydrate and reducing power, as a regulator of coenzyme levels and as an osmoregulator. Since Hammond and Nichols (1976a) found mannitol levels to be considerably lower in the mycelium than the sporophore in A. bisporus, they concluded it is unlikely that it acts as a translocatable substrate in this organism, with the mycelium "feeding" the sporophore, because this would have to occur against a steep concentration gradient. Dütsch and Rast (1972) considered mannitol was unlikely to have a role as a storage of carbohydrate or reduced NADP as mannitol oxidation would not be favoured if the low $NADP^+$/NADPH ratio, found in other organisms (Glock and McLean, 1955; Horecker, 1965), was also true for A. bisporus. However, this argument may not be valid since Hammond (unpublished data) has detected a ratio of $NADP^+$:NADPH of 1:1 in A. bisporus sporophores, considerably higher than the ratio of between 1:6 and 1:30 found in most of the mammalian tissues studied by Glock and McLean (1955). Nevertheless, the view that mannitol is not used

as a storage substance is supported by the low rate of mannitol oxidation in the sporophore. Mannitol only disappears late in the development of the fruit-body after sporulation and during senescence (Hammond and Nichols, 1976a) or under conditions of stress, e.g. after harvest (Hammond and Nichols, 1975). Dütsch and Rast (1972) suggested that mannitol is a growth regulator which acts by controlling coenzyme levels - specifically, the availability of $NADP^+$ to the pentose phosphate pathway. They showed that NADPH produced by the pentose phosphate pathway was reoxidised by the reduction of fructose to mannitol. The ratio of the oxidised to reduced form of the coenzyme has been implicated in the control of this pathway in Escherichia coli (Model and Rittenburg, 1967), the blue-green alga Anabaena cylindrica (Batt and Brown, 1974), other micro-organisms (Eagon, 1963) and in rat liver (Eggleston and Krebs, 1974). Furthermore, a link between mannitol synthesis and the pentose phosphate pathway has been reported in the grape-rotting fungus, Diplodia viticola (Strobel and Kosuge, 1965).

Hammond (1979) believes the most likely role for mannitol in A. bisporus is that of an osmotic agent; mannitol is proposed to maintain the water content of the sporophore thereby supporting the organ by turgor pressure and counteracting the effects of "transpiration". This view is supported by the comparison of water and mannitol contents of sporophores produced during a flush (1.3.) with the

relatively small number of sporophores produced between flushes, the "interflush" sporophores; there is an increase in water content coinciding with a similar increase in mannitol content in the flush sporophores relative to interflush sporophores (Hammond and Nichols, 1979). It is suggested that the higher mannitol levels produced during a flush are to overcome the greater water stress which would result from many fruit-bodies "transpiring" above the soil. A scheme for osmotic support in the sporophore of the agaric fungus Coprinus cinereus has been proposed and is supported by strong evidence (Moore, Elhiti and Butler, 1979). Expansion of the cap (pileus) cells is accompanied by a rapid increase in water content and degree of vacuolisation. Simultaneously there is an amplification of the urea cycle; it is proposed that this amplification increases the amounts of osmotically active solutes in the cap cells which, in turn, drive the process of water uptake (Moore, Elhiti and Butler, 1979). The stipe also accumulates osmotically active substances but here it is believed carbohydrates rather than amino compounds are important (Moore, Elhiti and Butler, 1979); no single specific carbohydrate is identified as responsible, although trehalose is present in large amounts. Mannitol is ruled out as the responsible carbohydrate as only low levels are found in C. cinereus. Mannitol does, however, appear to have an osmotic role in some marine fungi where it maintains the correct differential of water potential between the

mycelium and the external marine medium; e.g. in the Hyphomycete Dendryphiella salina (Jennings and Austin, 1973).

The precise function of mannitol in the developing sporophore of A. bisporus therefore remains disputed and uncertain. Nevertheless, there is little doubt that the synthesis of mannitol is intimately connected with the fruiting process and its synthesis and subsequent turnover are of great significance in the life-cycle of A. bisporus.

1.5. The Enzymology of Mannitol Metabolism

1.5.1. Mannitol Dehydrogenase

The production of polyols is assumed to be by direct reduction of sugars or sugar-phosphates, and the metabolism of a polyol is assumed to start with direct oxidation or with phosphorylation to a polyol-phosphate followed by oxidation to sugar-phosphate (Lewis and Smith, 1967). Enzymes capable of carrying out such reactions, namely polyol and polyol-phosphate dehydrogenases, are listed by Touster and Shaw (1962). The list is extended for fungal enzymes known to oxidise mannitol or mannitol-phosphate by Lewis and Smith (1967). Both NAD^+ and $NADP^+$ dependent enzymes have been reported.

A mannitol-phosphate dehydrogenase activity has not been found in A. bisporus. However, Edmundowicz and Wriston

(1963) did report the presence of an $NADP^+$ dependent mannitol dehydrogenase in A. campestris. The enzyme was shown to reduce fructose to mannitol and to oxidise mannitol to fructose but was inactive against mannitol-1-phosphate or sorbitol. An improved purification procedure was used by Ruffner et al (1978) in preparing mannitol dehydrogenase from A. bisporus. They compared the enzyme with the data of Edmundowicz and Wriston (1963) and some of the important kinetic parameters of the two preparations are summarised in Table 2. As this table shows, there were some very large differences in the Km values obtained by the two groups and, in addition to these, there were differences in specificity for some of the substrates. These differences are summarised in Table 3. Ruffner et al showed the purified enzyme was highly specific for fructose but had some activity against polyols other than mannitol, notably sorbitol. Edmundowicz and Wriston found the same specificity for fructose apart from a trace of activity against fructose-6-phosphate but, in the polyol oxidising direction of the reaction, they found no activity against sorbitol. Edmundowicz and Wriston detected a significant reaction when NADH and NAD^+ were used as coenzyme with fructose and mannitol respectively. In contrast, Ruffner et al found the purified enzyme preparation did not catalyse a detectable reaction between NADH and any sugar, and the preparation was active against NAD^+ only at low levels and only in the presence of sorbitol. The reasons for the differences in kinetic and specificity data between

Table 2: Some kinetic properties of fungal mannitol dehydrogenases (MDH)

Preparation	Km				pH optimum	
	Fructose (mM)	Mannitol (mM)	NADPH (μM)	$NADP^+$ (μM)	Fructose reduction	Mannitol oxidation
MDH from *A. campestris* (Edmundowicz and Wriston, 1963)	1.4	0.46	16	22	-	-
MDH from *A. bisporus* (Ruffner *et al*, 1978)	190	34	5.25	-	8.0	8.75
MDH from *Diplodia viticola* (Strobel and Kosuge, 1965)	-	128	-	82	7.0	8.5
MDH from *Cephalosporium chrysogenum* (Birken and Pisano, 1976)	1000	71.5	-	-	-	9.5

Table 3: Selected data on the specificity of the mannitol dehydrogenase enzymes of Edmundowicz and Wriston (1963) and Ruffner *et al* (1978).

Reaction	Reaction rate	
	Purified preparation of Edmundowicz and Wriston	Purified preparation of Ruffner *et al*
D-Fructose-NADH	5-10	0
D-Mannitol-NAD^+	5-10	0
D-Sorbitol-NAD^+	0	1
D-Fructose-6-phosphate-NADPH	Trace	0
D-Sorbitol-$NADP^+$	0	21
D-Mannitol-1-phosphate-$NADP^+$	0	0
Perseitol-$NADP^+$	-	6
D-Arabitol-$NADP^+$	-	3
L-Arabitol-$NADP^+$	-	2

Results are expressed as percent of the activity with mannitol and $NADP^+$ in the polyol oxidising direction and with fructose and NADPH in the hexose reducing direction.

the two reports are unclear. However, it should be noted that the mannitol dehydrogenase preparation of Edmundowicz and Wriston was purified 13 fold whereas that of Ruffner et al was purified 84 fold. The purer preparation may have been less subject to anomolies created by competition between mannitol dehydrogenase and other enzymes for added substrates. Furthermore, both sets of workers followed the mannitol dehydrogenase reaction spectrophotometrically at 340 nm; other reactions which produce or consume compounds optically active at 340 nm are less likely to occur in the purer preparation and, therefore, less likely to generate misleading results.

Ruffner et al (1978) also compared the enzyme with other fungal mannitol dehydrogenases; those isolated from Diploida viticola (Strobel and Kosuge, 1965), Dendryphiella salina (Holligan and Jennings, 1972), Aspergillus candidus (Strandberg, 1969) and Cephalosporium chrysogenum (Birken and Pisano, 1976). The Diplodia enzyme was found to be relatively unspecific for both ketose and polyol substrates but had an absolute requirement for $NADP^+$ and NADPH. It was also found that mannitol accumulation in D. viticola paralleled closely the level of glucose-6-phosphate dehydrogenase activity; thus, Strobel and Kosuge (1965) concluded, mannitol synthesis is dependent on the supply of NADPH generated by glucose-6-phosphate dehydrogenase activity as part of the pentose phosphate pathway. This should be compared and contrasted with the role suggested

for mannitol in A. bisporus by Dütsch and Rast (1972; described above), in which NADPH synthesis by the pentose phosphate pathway is dependent on the supply of $NADP^+$ generated by the reduction of fructose to mannitol.

An NAD^+-linked mannitol-1-phosphate dehydrogenase activity and a mannitol-1-phosphate activity have been reported in Aspergillus species (Yamada et al, 1961; Lee, 1967). Strandberg (1969) detected, in addition to these, an $NADP^+$-linked mannitol dehydrogenase activity with high specificity for both the coenzyme and the polyol in a strain of A. candidus. Neither a fructose-6-phosphatase activity nor a glucose isomerase activity could be found by Strandberg (1969) and, without either of these, it is difficult to envisage how fructose is supplied to the dehydrogenase for reduction to mannitol, and so it is unlikely that mannitol dehydrogenase acts anabolically to produce mannitol in A. candidus. Strandberg (1969) was also unable to detect a mannitol kinase activity but did detect a fructokinase activity. The activities present and absent in A. candidus are summarised in Figure 3 (after Strandberg, 1969). According to this scheme mannitol is produced from fructose-6-phosphate via mannitol-1-phosphate but this pathway cannot be reversed since no mannitol kinase activity is present; the mannitol is therefore converted to fructose by mannitol dehydrogenase which acts catabolically, and the fructose, in turn, is further metabolised by the detected fructokinase activity.

Figure 3: Activities present and absent in the Aspergillus candidus system (according to Strandberg (1969)).

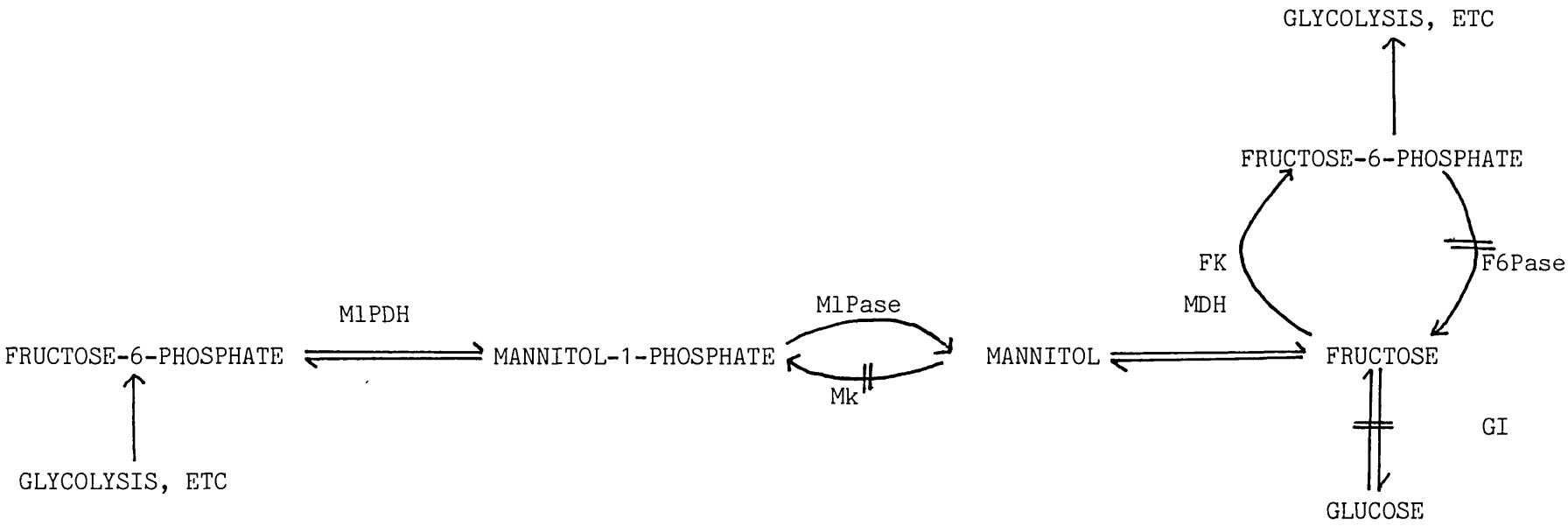
→ or ⇌ activity present

⫲→ or ⇌ activity absent

MlPDH - mannitol-1-phosphate dehydrogenase MlPase - mannitol-1-phosphatase Mk - mannitol kinase

MDH - mannitol dehydrogenase Fk - fructokinase F6Pase - Fructose-6-phosphatase

GI - glucose isomerase



The Dendryphiella mannitol dehydrogenase activity was found to have low specificity for both the polyol and the $NADP^+$ coenzyme but the activity was not extensively studied and the assays were performed on only one crude extract (Holligan and Jennings, 1972).

The polyol dehydrogenase isolated from Cephalosporium (Birken and Pisano, 1972) was different from the above as it was a series of isoenzymes which, as a mixture, showed broad substrate specificity with respect to polyols, was able to utilise NAD(H) or NADP(H) as coenzyme and had two main activities; namely the oxidation of sorbitol (or xylitol) with NAD^+ and the oxidation of mannitol with $NADP^+$. A summary of the kinetic parameters found with the Diplodia and Cephalosporium enzymes is included in Table 2. On the basis of these kinetic parameters and similarities in polyol specificities, Ruffner et al (1978) propose a possible functional relationship between the Diplodia viticola mannitol dehydrogenase and the Agaricus bisporus mannitol dehydrogenase.

Ruffner et al (1978) showed that the mannitol dehydrogenase activity from A. bisporus was sensitive to the ratio of $NADP^+$:NADPH, particularly in the polyol oxidising direction of the reaction. From their work, and the observation that under normal circumstances vegetative hyphae and fruit-bodies accumulate mannitol throughout their life (Rast, 1965), Ruffner et al (1978) conclude that mannitol dehydrogenase functions as a polyol synthesising enzyme

under normal physiological conditions, (cf. the deduced role for the enzyme in Aspergillus candidus, above). This role in A. bisporus is consistent with the pattern of mannitol accumulation during sporophore growth (Hammond and Nicols, 1976a) and with the equilibrium constant for the reaction

Mannitol + $NADP^+ \rightleftharpoons$ Fructose + NADPH + H^+ of 5×10^{-10}

observed by Edmundowicz and Wriston (1963) with mannitol dehydrogenase prepared from A. campestris.

1.5.2. Fructose-6-phosphatase

For the mannitol dehydrogenase in A. bisporus to be active in mannitol synthesis, there must be a supply of fructose. In assessing the role of mannitol dehydrogenase in Aspergillus above, Strandberg (1969) considered two possible sources of fructose; the result of fructose-6-phosphatase activity against fructose-6-phosphate and the result of glucose isomerase activity against glucose. Strandberg found neither activity but Edmundowicz and Wriston (1963) found a trace of apparent mannitol dehydrogenase activity in their extract with fructose-6-phosphate as substrate. One possible explanation they offered for this was the presence of a phosphatase in their preparation of mannitol dehydrogenase; it was suggested that the unidentified phosphatase is active against fructose-6-phosphate and able to catalyse the production of the true substrate for

the dehydrogenase reation, fructose. Hammond and Nichols (1977) studied the metabolism of ^{14}C-labelled fructose-6-phosphate. They found significant conversion into mannitol; after 30 min conversion ranged from 7.9% in the stipe to 33.0% in the pileus and after 2h conversion ranged from 27.0% in the stipe to 48.3% in the gills. Therefore, it seems likely a fructose-6-phosphatase activity exists in the cell.

Fructose-6-phosphatase activity could be attributable to non-specific phosphatase enzymes. Such enzymes have been isolated from a large range of organisms. Many fungal phosphatases were identified some years ago and broadly classified into acid and alkaline types depending on the pH optimum for activity (Cochrane, 1958). Non-specific fungal phosphatases can have a role in metabolic control; normally this is related to phosphate levels. Aspergillus nidulans produces a constitutive alkaline phosphatase, two repressible acid phosphatases and a repressible alkaline phosphatase; expression of the repressible enzymes is dependent upon the composition of the growth medium, particularly the phosphate content (Dorn and Riviera, 1966). A similar response to phosphate is shown by Neurospora crassa which produces an alkaline phosphatase (Nyc, Kadner and Crocken, 1966) and an acid phosphatase (Nyc, 1967), both of which are repressed by inorganic phosphate. The repression of non-specific phosphatases has been linked to sporophore production in the agaric fungus, Coprinus

lagopus (North and Lewis, 1971). In this organism, constitutive acid phosphatase is produced together with an alkaline phosphatase which is repressible by phosphate in the mycelium but not the sporophore; it was suggested by North and Lewis that the repressible enzyme has a role in controlling phosphate metabolism during fruiting.

The fructose-6-phosphatase activity could be attributable to enzymes with a specificity for fructose-6-phosphate. Sugar phosphatase enzymes with high specificity have been reported from many sources and are often crucial in the regulation of carbohydrate metabolism; for example, the fructose-1,6-bisphosphatase activity isolated from many organisms (Pontremoli and Horecker, 1971). The enzyme is involved in the regulation of the relative amounts of carbohydrate directed towards breakdown via the Embden-Meyerhof pathway (glycolysis) and towards glucose synthesis (gluconeogenesis) (Atkinson and Walton, 1967). Fructose-bisphosphatase from all organisms studied, except the slime mold Polysphondylium pallidum, can be inhibited by AMP, and the enzyme from many sources has been reported to show allostery with respect to the binding of fructose bisphosphate (Pontremoli and Horecker, 1971).

An additional level of regulation is shown by fructose-bisphosphatases from mammalian liver and kidney; enzyme active at neutral pH can be converted by proteolysis to a form with an alkaline optimum pH (Horecker et al, 1975).

This conversion can be induced by added proteases *in vitro* and may occur through the mediation of lysosomal proteases *in vivo*. Both the neutral and alkaline form of the rabbit liver and kidney enzyme are activated by EDTA. The EDTA activation of the neutral form, but not of the alkaline form, is accompanied by a shift in pH optimum. Both forms of the rabbit liver and kidney enzyme are also activated by divalent metal ions; the alkaline enzyme shows greater activity when assayed in the presence of Mn^{2+} than in the presence of Mg^{2+} whereas the reverse is found with the neutral form (Horecker *et al*, 1975). Kinetic evidence indicates that the enzyme has separate binding sites for the divalent cation and the metal chelator in these activation processes (Rosenberg *et al*, 1973). EDTA can be replaced as an activator by naturally occurring chelators such as oleate and by physiological levels of histidine in studies on the rabbit liver fructose bisphosphatase. Some sulphydryl compounds have been found to affect the activity of both enzyme forms in a variety of ways but the role of these substances in regulation is unclear (Horecker *et al*, 1975). A form of the enzyme was discovered by Benkovic *et al* (1974) which has only a small tryptophan containing peptide removed from its N-terminus; this form of the enzyme may be important in regulation as it has greatly reduced sensitivity to AMP inhibition.

A fructose-1,6-bisphosphatase has also been isolated from the muscle of both vertebrates and invertebrates and this

was initially considered unusual since muscle is not capable of gluconeogenesis (Horecker et al, 1975). The enzyme has been purified from rabbit skeletal muscle and differs from the liver and kidney enzyme in amino acid composition and immunological properties. It is inhibited by low concentrations of AMP, Ca^{2+} and Zn^{2+}. The muscle fructosebisphosphatase is thought to have a role as a heat generating ATPase by acting in conjunction with phosphofructokinase to catalyse a cyclic synthesis and hydrolysis of fructose-1,6-bisphosphate. The heat produced is thought to raise the temperature of bumblebee flight muscle, where the bisphosphatase is active, in preparation for flight. And vertebrates may use the heat produced in muscle by this cycle to regulate body temperature (Horecker et al, 1975).

The skeletal muscle enzyme may, therefore, be subject to hormonal or neuronal regulation. The relative levels of the neutral and alkaline forms of the liver enzyme are affected by external stimuli such as fasting or exposure to cold temperatures (Horecker et al, 1975). The levels of the alkaline fructose bisphosphatase in mammalian liver can be increased by the administration of adrenal glucocorticoid hormones (Kvam and Parks, 1960; Weber et al, 1961).

Fructose-1,6-bisphosphatase has an important role in plant photosynthesis and here too there are examples of regulation;

the enzyme from spinach leaves is active in light but almost inactive in darkness except at high concentrations of Mg^{2+} ions (Chehabar and Wolosiuk, 1980). The light regulatory mechanism involves the photochemical reduction of thioredoxin which then converts the enzyme to the active form at low (physiological) concentrations of Mg^{2+} ions. The thioredoxin activation of the enzyme can be mimicked by preincubation with fructose-1,6-bisphosphate. High concentrations of Mg^{2+} ions give a fast and reversible activation of the enzyme but activation by the thioredoxin system or by preincubation with the substrate is a slow "hysteretic" type effect.

Fructose-1,6-bisphosphatase has been isolated from bacteria. The enzyme from Bacillus licheniformis has been purified and shown to be inactivated by incubation in the presence of AMP or the absence of Mn^{2+} ions, though the AMP inactivation is prevented by addition of phosphoenolpyruvate to the mixture (Opheim and Bernlohr, 1975).

Regulation of levels of fructosebisphosphatase has been observed in fungi; synthesis of the enzyme in the yeasts Saccharomyces cerevisiae (Grancedo et al, 1965; Foy and Bhatacharjee, 1978) and Candida utilis (Rosen et al, 1965) can be derepressed under conditions of limited hexose. Thus a large range of regulatory mechanisms from a diverse range of organisms has been observed with the phosphatase enzymes specific for fructose-1,6-bisphosphate .

Specific phosphatases active against monophosphate esters are also known to occur and are often important in the regulation of metabolism; glucose-6-phosphatase is perhaps the most extensively studied enzyme of this type (see, for example, Nordlie, 1971) and is involved in the control of mammalian carbohydrate metabolism, particularly the control of blood glucose levels (Ashmore and Weber, 1959; Nordlie, 1969). The mammalian enzyme has an additional activity; it can catalyse the synthesis of glucose-6-phosphate from glucose and a variety of phosphoryl donors (Nordlie, 1971). The phosphohydrolase and phosphotransferase activities of the enzyme are selectively altered by many metabolites and the actions of regulatory molecules can be modified by altering the pH of the system or by the addition of detergents (Nordlie, 1971). Detergents also affect enzyme activities in the absence of other compounds (Nordlie, 1971); optimal concentrations of some detergents activate the enzyme but supraoptimal concentrations inhibit, and any level of other detergents, including bile acids, inhibit. The effects of detergents on both enzyme activites may be related to conformational changes in the enzyme molecule which, in turn, may be related to the protein-lipid nature of the enzyme. The list of regulatory substances is long and involves some complex interactions but is well reviewed by Nordlie,(1971). Important inhibitors include intermediates of the tricarboxylic acid cycle, most notably citrate, some amino acids and long-chain fatty acyl-coenzyme A esters and fatty acids which affect both enzyme

activities in a pH and concentration dependent manner. Various nucleoside di- and triphosphate compounds inhibit the phosphatase reaction and act as phosphoryl donors in the phosphotransferase reaction. Orthophosphate inhibits both activities. Inhibition is also shown by molybdate, certain divalent cations, including Mg^{2+}, Ca^{2+}, Zn^{2+}, and Cu^{2+}, and some sulphydryl reagents. Pyruvate activates the enzyme, and phosphoenolpyruvate inhibits the phosphohydrolase activity competitively and acts as a phosphoryl donor in the transferase reaction. Lysolecithin activates both activities and other phospholipids are necessary for activity and appear to maintain the enzyme in a stable, active conformational state. Inhibition of the glucose-6-phosphatase activity alone has been demonstrated with a number of substances including iodide, arsenate, borate and silicic acid (Nordlie, 1971). Both activities of the enzyme are regulated at the physiological level by variation in the dietary and hormonal status of the mammal (Ashmore and Weber, 1959; Nordlie, 1971).

The enzyme has high Km values for its substrates relative to physiological levels of these substrates; the Km for glucose-6-phosphate is 1-2mM compared with hepatic glucose-6-phosphate levels of 0.05mM; the Km for glucose is 80-90mM compared with normal physiological values of 5mM (Clark and Lardy, 1975). Such a variance between Km and physiological substrate level indicates the enzyme may be particularly susceptible to regulation <u>in vivo</u>, especially

through competitive inhibition by ATP and other nucleotides (Clark and Lardy, 1975).

In addition to its detection in a range of mammalian tissues (above), glucose-6-phosphatase has been reported in plants, bacteria and fungi (Nordlie, 1971).

1.5.3. Fructokinase.

Under normal physiological conditions, mannitol is not metabolised by the A. bisporus sporophore (Dütsch and Rast, 1972). However, mannitol has been shown to be used as a respiratory substrate in harvested and stored sporophores (Hammond and Nichols, 1975) and has been proposed as a possible respiratory substrate for the senescing sporophore (Hammond and Nichols, 1976a). Hammond and Nichols, 1976a) suggested that the utilisation of mannitol in respiration is by a reversal of the normal substrate flow; such a scheme requires the presence of a fructokinase activity capable of reversing the fructose-6-phosphatase reaction described above. In common with the phosphatase activity, a fructokinase activity could be specific or general; the former case requires an enzyme specific for fructose, while the latter requires an enzyme active against a range of hexoses.

Hexokinases, particularly from yeast and mammalian sources, have been extensively studied (Colowick, 1973; Purich et al,

1973). They are widespread in occurrence and a glucko-kinase reaction is an important step in the Embden-Meyerhof pathway. The phosphorylation of many other hexoses is also essential in preparation of these sugars for further methabolism.

The yeast hexokinase is actually a mixture of three isoenzymes, designated A, B and C, though B and C are conformational rather than structural isozymes (Purich et al, 1973). Unlike some of the mammalian enzymes described below, product inhibition by glucose-6-phosphate does not seem to be important in regulating yeast hexo-kinases (Purich et al 1973). Kosow and Rose (1971) have suggested that orthophosphate, citrate, malate, 3-phospho-glycerate and ribonucleoside 5-triphosphates can function as activators of hexokinase B below pH7.0. Kosow and Rose ascribe the activation to the existence of two conformational states; an inactive form preferred in the acid range, and an active form favoured by the binding of various polyanions to an allosteric site or by alkaline pH. Colowick (1973) reports, in addition, that the B isozyme (designated PII by Colowick) is inhibited by ADP and GDP which effectively means the enzyme can be regulated by the ADP:ATP concentration ratio. The regulatory properties are much less marked with the A (or PI) isozyme (Colowick, 1973). Less is known about the regulation of the C isozyme as this hexokinase is

often converted to the B isozyme during purification. However, this conformational change in itself may be considered regulatory as it leads to an increase in specific activity (Ramel et al, 1971).

The mammalian enzyme is also a mixture of isozymes, types I - IV, which occur in different ratios in different tissues; brain and kidney contain predominantly type I, skeletal muscle type II, adipose tissue, heart and intestine types I and II and liver type IV (Clark and Lardy, 1975). A dimension to regulation of carbohydrate metabolism is added by the observations that relative levels of these isozymes can be affected by dietary and hormonal status of the animal (Clark and Lardy, 1975) or, in the case of rat epididymal fat pad tissue, by the animal's stage of development (Purich et al, 1973). The four isozymes can be distinguished by differences in electrophoretic mobility and catalytic properties (Clark and Lardy, 1975) and also in their susceptibilities to thermal and proteolytic inactivation (Purich et al, 1973). The four isozymes show a large variation in Km for glucose (Katzen et al, 1965) and for fructose (Colowick, 1973); types I, II and III have similar values but type IV has a very high (>800mM) Km for fructose. Because of this, type IV is generally referred to as a glucokinase. However, it is a true

hexokinase; mannose and 2-deoxyglucose are reasonably good phosphoryl acceptors, as is fructose, albeit at concentrations unlikely to exist under physiological conditions. Furthermore, when the Michaelis constants for mannose, 2-deoxyglucose and fructose are normalised with respect to the Km for glucose, the specificities of all four isozymes are very similar (Purich et al, 1973). There are also differences in the regulation of the activity of these enzymes; type IV is again the exception as the other three isozymes all show extreme sensitivity to glucose-6-phosphate inhibition and a weaker sensitivity to ADP inhibition (Clark and Lardy, 1975).

The inhibition of the type I enzyme by glucose-6-phosphate can be attenuated by orthophosphate though orthophosphate alone does not activate the enzyme (Purich et al, 1973). The orthophosphate deinhibition of the type I enzyme found in brain and erythrocytes maintains the glucose-6-phosphate level which, in turn, maintains the fructose-6-phosphate level and therefore the rate of the phosphofructokinase reaction. This allows glycolysis to proceed at high rate in the presence of otherwise inhibitory concentrations of glucose-6-phosphate (Purich et al, 1973). A mechanism for alleviation of glucose-6-phosphate inhibition by phosphate has been proposed by Ellison et al (1975) based on kinetic data;

these authors suggest that an enzyme-phosphate complex is formed which has similar properties to the free form apart from a relatively high dissociation constant for glucose-6-phosphate. Phosphate appears to bind at an allosteric site whereas glucose-6-phosphate is associated either at the active site or at an allosteric site which overlaps the catalytic site (Ellison et al, 1975).

The type III enzyme does not show the phosphate attenuation of glucose-6-phosphate inhibition. However, this isozyme tends to occur in glycogen-rich tissues such as heart or skeletal muscle where the glycolytic pathway can continue to operate at a high level, even if the phosphorylation of glucose by hexokinase is inhibited. In these tissues, glucose-6-phosphate will be supplied by phosphorylase activity against glycogen (Purich et al, 1973). The type II isozyme shows biphasic inhibition by glucose-6-phosphate; inhibition appears promptly on addition of glucose-6-phosphate and a further hysteretic inhibition appears at least 12 sec later (Kosow and Rose, 1972).

The mammalian hexokinase may be regulated by Mg^{2+} ions but it seems likely that these ions take effect by complexing the inhibitory ATP^{4-} ion to form the active substrate, $MgATP^{2-}$, rather than directly acting on

the enzyme (Purich et al, 1973). Thus the balance of Mg^{2+} and ATP^{4-} ions is important. There have been reports that the type I enzyme is stimulated by citrate (Kosow et al, 1973) and by catecholamines (Harrison and Gray, 1971). Mammalian hexokinases are often partitioned between a soluble pool and a mitochondrial particulate pool, with up to 80% appearing in the latter (Purich et al, 1973). The balance of the equilibrium between soluble and particulate forms could be an important form of regulation and glucose-6-phosphate, adenine nucleotides and other metabolites have been found to solubilise in vitro preparations of the particulate form. However, these effects were only observed at concentrations of these metabolites considerably higher than physiological levels; the mode of in vivo regulation of this equilibrium, if any, remains to be established (Purich et al, 1973).

The hexokinases described above are generally most active against glucose but have some activity against fructose and other hexoses. However, fructose can also be phosphorylated by enzymes with a specificity for this substrate. Many bacterial hexokinases exist; some of these have low substrate specificity and others are specific for a group of structurally related sugars (Colowick, 1973). Yet other enzymes are specific for just one hexose, e.g. a highly specific fructokinase

activity occurs in Streptomyces violaceoruber (Sabater et al, 1972); the activity is not constitutive but can be induced by growth on fructose. This enzyme shows unusual kinetics associated with changes in molecular weight (Sabater and Delafuente, 1975); the saturation curve of the enzyme for ATP in the presence of equimolar amounts of Mg^{2+} or Co^{2+} ions (to form the $MgATP^{2-}$ or $CoATP^{2-}$ complex) is sigmoidal but becomes hyperbolic on addition of a 10mM excess of Mg^{2+} or Co^{2+} ions. In the presence of excess Mg^{2+}, the molecular weight of the enzyme is approximately 20,000. This is also the case in the presence of fructose. However, in the presence of high concentration of $MgATP^{2-}$ and in the absence of both excess Mg^{2+} and fructose, the molecular weight appears to be approximately 80,000. Furthermore, the saturation curves for fructose give intermediate inflections indicating negative cooperativity. Sabater and Delafuente conclude that sigmoidal saturation curves for ATP are found under conditions promoting the association of enzyme subunits into a tetramer of 80,000 daltons; single subunits show hyperbolic kinetics. As well as bacterial enzymes, a fructokinase has also been reported in green pea seeds (Medina and Sols, 1956).

All the above hexokinase and fructokinase activities transfer the phosphate group to position 6 on the glycosidic ring. However, there is an enzyme specific for fructose

which transfers the phosphate group to position 1 in mammalian liver and muscle (Slein et al, 1950). This enzyme has been purified to homogeneity from bovine liver by Raushel and Cleland (1977a) and shown to have limited activity against a number of sugars apart from D-fructose which contain the minimum molecule of (2R)-2-hydroxymethyl-3,4-dihydroxytetrahydrofuran; for example, L-sorbose, D-tagatose, D-psicose, D-xylulose, L-ribulose, D-sedoheptulose, L-galactoheptulose, D-mannoheptulose and D-ribose. The enzyme is inhibited by $MgADP^-$ competitively with $MgATP^{2-}$ as the varied substrate and non-competitively with fructose as the varied substrate. In contrast, D-fructose-1-phosphate inhibits the enzyme competitively with fructose as the varied substrate and non-competitively with $MgATP^{2-}$ as the varied substrate (Raushel and Cleland, 1977a). The kinetic data and other data from isotope exchange and isotope partitioning experiments and pH studies (Raushel and Cleland, 1977b) indicate a random enzyme mechanism, with fructose release slower than the catalytic step but release of fructose-1-phosphate in the forward direction of the reaction faster than the catalytic step.

The preceeding introduction has included a survey of fungal mannitol dehydrogenases and of sugar phosphatases and hexokinases from various sources. Emphasis has been placed on their role in metabolic regulation. The present work was undertaken to investigate the possible role of mannitol dehydrogenase, phosphatases (specifically a fructose-6-

phosphatase) and kinases (specifically a fructokinase) in the regulation of mannitol metabolism in the edible mushroom, Agaricus bisporus. Information gained from such a study would increase understanding of carbohydrate, particularly polyol, metabolism in Basidiomycetes and could lead to commercial applications in the growth of this important edible fungus.

Mannitol dehydrogenase is known to exist in A. bisporus and has been investigated by Ruffner et al (1978); the present study was carried out with a view to extending the above work using rigorously purified enzyme and giving special consideration to the observation that it is sensitive to the $NADP^+$/NADPH ratio (1.5.1.). Other possible forms of regulation of mannitol dehydrogenase were also to be considered.

The fructose-6-phosphatase and fructokinase activities have not been identified in A. bisporus but have been postulated in schemes for the production and turnover of mannitol in this organism (above). Sections 1.5.2. and 1.5.3. have shown the various ways in which phosphatases and kinases, often in conjunction with each other, can act to regulate carbohydrate metabolism. Confirmation of the presence of these enzymes in A. bisporus was required together with investigations to discover whether the activities were attributable to specific or general enzymes and whether the enzymes were involved in regulation.

One form of regulation considered for many of the enzyme catalysed reactions discussed in Section 1.5. is variation in the levels of the enzymes themselves. Since mannitol production is associated with early stages of sporophore development and mannitol catabolism only with the senescing sporophore, experiments were carried out to examine the possibility that levels of enzymes involved in mannitol metabolism vary with sporophore age.

2. Materials and Methods

2.1. Production, Harvest and Storage of *Agaricus bisporus* Sporophores

A commercial compost was prepared and spawned with *Agaricus bisporus* (Lange) Imbach strain "Darlington's 649" by the Mushroom Unit at the Glasshouse Crops Research Institute, Littlehampton. The compost was packed into 51 plastic buckets which were watered regularly and mycelium was allowed to colonise the compost. After two to three weeks at 18°C compost contained dense white mycelium and was "cased" to a depth of approximately 2cm with a peat/chalk (1:1) mixture. The buckets were maintained at 18°C in a humid atmosphere and watered regularly.

At harvest, sporophores were removed from the bed by hand and approximately 5mm of the base of the stipe was trimmed off to remove attached compost and casing materials.

For sporophore storage, the stipe was separated from the rest of the fruit-body and sliced transversely to a thickness of approximately 2mm. The pileus plus gill tissue was sliced radially to a thickness of about 2mm. All tissue slices were immediately frozen in liquid nitrogen and stored at -20°C.

2.2. Enzyme Assays

2.2.1. Assay of Mannitol Dehydrogenase Activity

Mannitol dehydrogenase was assayed by following the production or consumption of NADPH at 340nm in the spectrophotometer. All assays were carried out at 25°C in a total volume of 1.0ml. The reduction of fructose was assayed by incubating an aliquot of the extract to be tested in 50mM tris/HCl buffer, pH 7.5, containing 800mM D-fructose and 0.25mM NADPH. The oxidation of mannitol was assayed by incubating an aliquot of the extract to be tested in 50mM sodium carbonate buffer, pH10.4, containing 200mM D-mannitol and 1mM $NADP^+$. One unit of mannitol dehydrogenase activity is defined as that which catalyses the oxidation of 1nmol of NADPH min^{-1} in the above system.

The products of fructose reduction or mannitol oxidation were identified by chromatography (2.5.5a.).

2.2.2. Test for Irreversible pH Inactivation of Mannitol Dehydrogenase.

Aliquots (10µl) of purified enzyme (2.3.1.) were incubated at 25°C for 30 min with 0.99ml of a selection of 1mM buffers over a range of values between pH4.0 and pH11.0. The buffers used were acetate, pH4.0 - 5.5; tris-maleate/NaOH, pH5.0 - 7.5; tris/HCl, pH7.0 -9.5; sodium carbonate,

pH9.0 - 11.0. Each solution was then dialysed at 4°C for 5h against 1l of 50mM tris/HCl buffer, pH7.5, containing 25mM mercaptoethanol. Each solution was then made up to a total volume of 2.0ml with 50mM tris/HCl buffer, pH7.5, containing 25mM mercaptoethanol. A sample of each (100µl) was assayed for mannitol dehydrogenase activity by following fructose reduction (2.2.1.), to assess the effects of holding the enzyme preparations at a variety of pH's prior to assaying under the standardised conditions above. All results quoted (3.1.3.) are an average of at least two experiments showing less than 10% variation.

2.2.3. Determination of Mannitol Dehydrogenase Substrate and Coenzyme Specificity.

Aliquots (10µl) of purified enzyme were assayed for mannitol oxidation and fructose reduction under standardised conditions (2.2.1.) except that NAD^+ (1mM) and NADH (0.25mM) were used in place of $NADP^+$ and NADPH respectively.

Further assays were carried out for oxidation of 200mM D-sorbitol, D-galactitol, ribitol, L-arabitol or erythritol in place of D-mannitol under the standardised conditions (2.2.1.). Assays were carried out for reduction of 800mM D-mannose, D-glucose, L-sorbose or D-fructose-6-phosphate in place of D-fructose under the standardised conditions All results quoted (3.1.4.) are an average of at least two experiments showing less than 10% variation.

2.2.4. The Determination of the Effect of pH, Substrate Concentration and Inhibitors on Purified Mannitol Dehydrogenase.

Aliquots (10µl) of purified enzyme preparation were assayed for the rate of fructose reduction at 25°C in 1.0ml of a selection of 50mM buffers over a range of values between pH6.5 and pH10.5 with varying substrate and co-enzyme concentrations. The buffers used were tris-maleate/NaOH, pH6.5-7.5; tris/HCl, pH7.0-9.5; sodium carbonate, pH9.0-10.5. The substrate and co-enzyme concentrations used were i) D-fructose varied in the range 10 to 2000 mM with NADPH fixed at 0.25mM; ii) NADPH varied in the range 2 to 250µM with D-fructose fixed at 800mM. The choice of these concentrations for the fixed reactants is further discussed in sections 3.1.5. and 3.2.

Aliquots (10µl) of purified enzyme preparation (2.3.1.) were assayed for the rate of mannitol oxidation at 25°C in 1.0ml of a selection of 50mM buffers over a range of values between pH6.5 and pH10.8 with varying substrate and coenzyme concentrations. The buffers used were tris-maleate/NaOH, pH6.5-7.5; tris/HCl, pH7.0-9.5; sodium carbonate, pH9.0-11.5. The substrate and coenzyme concentrations used were i) D-mannitol varied in the range 2.5 to 200mM with a fixed excess $NADP^+$ (1mM); ii) $NADP^+$ varied in the range 10 to 2000µM with a fixed excess of D-mannitol (200mM).

For each pH value, the rate of reaction was plotted against the variable reactant concentration on a Lineweaver-Burke plot. These were used to determine the apparent maximum rate of reaction (Vmax) and Km for the variable reactant at each pH.

Aliquots (10μl) of purified enzyme preparation (2.3.1.) were assayed for rate of fructose reduction at 25^{o}C in 1.0ml of 50mM tris/HCl buffer, pH7.5, containing 800mM D-fructose, NADPH at a non-saturating concentration of 6.9μM and variable concentration of $NADP^{+}$ in the range 0 to 345μM to assess the effects of the antagonistic form of the coenzyme ($NADP^{+}$) on the rate of fructose reduction.

Aliquots (10μl) of purified enzyme preparation (2.3.1.) were assayed for rate of mannitol oxidation at 25^{o}C in 1.0ml of 50mM sodium carbonate buffer, pH10.4, containing excess D-mannitol (200mM), $NADP^{+}$ at a non-saturating concentration of 20μM and variable concentration of NADPH in the range 0 to 400μM to assess the effects of the antagonistic form of the coenzyme (NADPH) on the rate of mannitol oxidation where the enzyme is not saturated with the coenzyme. The series of incubations was repeated except $NADP^{+}$ was set at a saturating concentration of 1mM and NADPH concentration was varied in the range 0 to 1mM to examine the possibility of different behaviour when the $NADP^{+}$ coenzyme was present in excess. The whole series of incubations (i.e. with $NADP^{+}$ concentrations of 20μM and 1mM) was repeated but

with the sodium carbonate buffer replaced by 50mM tris/HCl buffer, pH7.5, to examine any differences in behaviour at a pH nearer physiological pH but further from the optimum pH for mannitol oxidising activity.

Aliquots (100µl) of purified enzyme preparation (2.3.1.) were incubated at room temperature for 30 min in a total volume of 1.0ml of iodoacetate (100mM) or mercuric chloride (100mM) in 50mM tris/HCl buffer, pH7.5, or in buffer alone as a control. Samples were assayed for mannitol oxidising activity and fructose reducing activity under the standardised conditions (2.2.1.). The activity of the mannitol dehydrogenase incubated in iodoacetate or mercuric chloride was calculated relative to the control incubation. The experiment was repeated using 10mM iodoacetate in place of the stronger solution above; the effect of this reduced iodoacetate concentration was monitored over an extended period of 300 min. The experiment was then repeated for both iodoacetate concentrations (10mM and 100mM) but with NADPH (0.25mM) or D-fructose (800mM) included in the incubations.

All results quoted for the effects of pH, substrate concentration and inhibitors on the activity of purified mannitol dehydrogenase (sections 3.1.5.-3.1.7.) are an average of at least two incubations showing less than 10% variation.

2.2.5. Determination of Keq for Mannitol Dehydrogenase

Purified mannitol dehydrogenase preparation (10µl) was incubated with 36.91µM $NADP^+$ and 500µM D-mannitol in 1.0ml of 50mM sodium carbonate buffer, pH10.18. The reaction was allowed to reach equilibrium as determined by a constant absorbance at 340nm. From the increase in absorbance at 340nm and the molar extinction coeffecient of NADPH at this wavelength (6.22×10^3), the increase in concentration (M) of NADPH during the course of the reaction was calculated. An equimolar decrease in concentration of $NADP^+$ was assumed. The molar stoichiomety of 1:1 for NADPH produced and fructose produced, determined by Edmundowicz and Wriston (1963), was assumed to calculate the increase in concentration (M) of fructose during the course of the reaction. An equimolar decrease in concentration of mannitol was assumed. From the calculated changes in concentration and known initial concentrations of fructose, mannitol, $NADP^+$ and NADPH, the final concentration of each was calculated. The concentration of H^+ ions (M) at equilibrium was known from the measured pH. These data allowed Keq for the reaction

$$\text{Mannitol} + NADP^+ \rightleftharpoons \text{Fructose} + NADPH + H^+$$

to be calculated.

The result quoted (3.1.8.) is an average of two experiments showing less than 10% variation.

2.2.6. Assay of Phosphatase Activity.

2.2.6.1. *p*-Nitrophenylphosphate Assay.

An aliquot of the enzyme extract to be tested was incubated at 25°C with 10mM *p*-nitrophenylphosphate in either 50mM acetate buffer, pH4.5, or 50mM tris-maleate/NaOH buffer, pH6.5, in a total volume of 1.0ml. Samples (100µl) of the incubation were removed 0, ½, 1, 2 and 3 min after addition of the extract and added to 0.9ml of 1.0M sodium hydroxide. This treatment of the samples terminates the enzyme activity and generates the yellow nitrophenate ion. The absorbance at 410nm was used to determine the concentration of nitrophenate ion by comparison with a standard curve of nitrophenol treated with sodium hydroxide as above. One unit of phosphatase activity is defined as that which catalyses the production of 1nmol of *p*-nitrophenol min^{-1} at 25°C and pH4.5.

2.2.6.2. Assay by Determination of Liberated Inorganic Phosphate.

An aliquot of the enzyme to be tested was incubated at 25°C with either 100mM D-glucose-6-phosphate or 200mM D-fructose-6-phosphate in a total volume of 2.0ml. Incubations were carried out in solutions buffered with either 50mM acetate buffer, pH4.5, or 50mM tris-maleate/NaOH buffer, pH6.5. Samples (0.25ml) were removed at 0, ½, 1, 2, 3 and 5 min

after addition of the extract and added to 0.7ml of water. Immediately, 0.2ml of 2.5% (w/v) ammonium molybdate, 5N sulphuric acid was added. The solutions were mixed, then 0.1ml of Fiske-Subbarow reducing agent (Sigma Chemicals, Poole) was added and the solutions mixed again. The blue colour was allowed to develop for 10 min at which time the absorbance at 620nm was measured. The concentration of inorganic phosphate at each time interval was determined by comparison with a standard curve using known concentrations of inorganic orthophosphate treated as above.

2.2.7. The pH-Activity Profile of Partially Purified Acid Phosphatase.

Aliquots of partially purified acid phosphatase preparation were assayed for activity with ρ-nitrophenylphosphate as substrate over a range of pH vlaues. Incubations (1.0ml) were carried out at 25°C of enzyme preparation containing 16.7μg of protein with 10mM ρ-nitrophenyl-phosphate in a variety of 50mM buffers. The buffers used were citrate, pH3.0-4.5; acetate, pH3.75-5.5; tris-maleate/NaOH, pH5.0-7.5; tris/HCl, pH7.0-9.5. The incubations were sampled and the concentrations of ρ-nitrophenol produced were determined as above (2.2.6.1.).

2.2.8. The Determination of the Effect of Substrate Concentration on a Partially Purified Preparation of Acid Phosphatase.

Samples of the acid phosphatase preparation containing 16.7µg of protein were assayed for activity against ρ-nitrophenylphosphate at pH4.5 as in section 2.2.6.1. except that the concentration of ρ-nitrophenylphosphate was varied; the range used was 0.025 to 10mM. Further samples of the preparation containing 83.5µg of protein were assayed for activity against fructose-6-phosphate as in section 2.2.6.2. except that the concentration of fructose-6-phosphate was varied; the range of concentrations used was 2.0 to 200mM. The incubations were repeated with the D-fructose-6-phosphate replaced succesively by each of the following; D-glucose-6-phosphate, D-fructose-1-phosphate and D-fructose-1,6- bisphosphate.

For each series of incubations the rate of reaction was plotted against substrate concentration on a Lineweaver-Burke plot to determine the maximum rate of reaction (Vmax) and the Km for the substrate. Each result quoted (4.1.) is an average of at least two experiments showing less than 10% variation.

2.2.9. Assay of Fructokinase Activity

2.2.9.1. Fructokinase Assay Coupled to Phosphoglucose Isomerase and Glucose-6-phosphate Dehydrogenase.

The production of fructose-6-phosphate was coupled to phosphoglucose isomerase and glucose-6-phosphate dehydrogenase. Aliquots of the enzyme extract to be tested were incubated at 25°C in 50mM tris/HCl buffer, pH8.5, containing 80mM D-fructose, 2.5mM ATP, 7.5mM magnesium sulphate, 3 units of phosphoglucose isomerase (Sigma Chemicals, Poole), 3 units of glucose-6-phosphate dehydrogenase (Sigma Chemicals, Poole) and 1mM $NADP^+$ in a total volume of 1.0ml. The production of NADPH was followed spectrophotometrically at 340nm. One unit of activity is defined as that which catalyses the production of 1.0nmol of fructose-6-phosphate min^{-1} from fructose under the above conditions.

2.2.9.2. Radioisotope Assay for Fructokinase.

The following incubates were mixed; 50mM tris/HCl buffer, pH8.5, (13.75µl), 200mM D-fructose in 50mM tris/HCL buffer, pH8.5, (25µl), 0.2µCi $µl^{-1}$ U-^{14}C-D-fructose (Radiochemical Centre, Amersham; 5µl) and 25mM ATP, 75mM magnesium sulphate in 50mM tris/HCl buffer, pH8.5 (6.25µl). Enzyme extract (12.5µl) containing protein at a concentration of 10-20mg ml^{-1} was added and the mixture incubated at 25°C. Samples (2.5µl) were taken over a 40 min period and applied to Whatman No. 1 chromatography paper over 10µl of 50mM mercuric chloride to kill the reaction. The chromatogram was

developed and autoradiographed and the radioactive products of the reaction were counted as detailed in sections 2.5.5b. and 2.5.6.

2.2.9.3. Fructokinase Assay Linked to Cresol Red.

The method of Darrow and Colowick (1962), in which the amount of ATP hydrolysed is determined as its acid equivalent, was used. A stock assay solution was prepared by mixing 7ml of 0.006% (w/v) cresol red, 1.6% (w/v) $MgCl_2.6H_2O$ with 1.5ml of 0.1M ATP, neutralising with 0.1M NaOH until the indicator became reddish purple, adding 3ml of 0.1M glycylglycine/NaOH buffer, pH9.0, and diluting with distilled water to 30ml. Aliquots (100µl) of the enzyme extract to be tested were added to 2.5ml of stock assay solution and 0.4ml of 0.6M D-fructose and the change in absorbance at 560nm and 25°C was followed in a spectrophotometer. The method was calibrated by measuring the absorbance at 560nm of the usual reaction mixture before and after the addition of 0.1ml of standard HCl in place of the enzyme extract.

2.2.10. Assay of Glucokinase Activity.

2.2.10.1. Glucokinase Assay Coupled to Glucose-6-phosphate Dehydrogenase.

Assays for glucokinase activity were carried out as those for fructokinase activity in section 2.2.9.1. but with

D-fructose replaced by D-glucose and with phosphoglucose isomerase omitted from the incubation.

2.2.10.2. Radioisotope Assay for Glucokinase.

Assays for glucokinase activity were carried out as those for fructokinase activity in section 2.2.9.2. but with D-fructose and U-^{14}C-D-fructose replaced by D-glucose and U-^{14}C-D-glucose respectively.

2.3. Enzyme Purification Procedures.

2.3.1. Purification of Mannitol Dehydrogenase.

After preliminary experiments the following procedure was adopted in a typical purification. All stages of the purification were carried out at 4°C. Frozen slices (2.1.) of sporophores (340g) harvested at stage 2 of development (1.3.) were allowed to thaw and homogenised in a "Vortex" mixer with 25ml of 10mM tris/HCl buffer, pH7.5, containing 50mM mercaptoethanol and 0.25mM PMSF. The homogenate was centrifuged at 15,000g for 30 min and the supernatant retained. Nucleic acids were precipitated by the addition of excess protamine sulphate to give a final concentration of approximately 0.25% (w/v). The suspension was centrifuged as above. The supernatant was retained and precipitated by the gradual addition of solid ammonium sulphate (heavy metal free). The fraction precipitating between 55 and

80% saturation with ammonium sulphate was isolated by centrifugation and dissolved in a minimum volume (approximately 5ml) of 10mM buffer (above). This preparation was applied to a Sephadex G100 column (33 x 2.5cm), equilibrated with 10mM buffer (above), and eluted with the same buffer; eighty 5ml fractions were collected. The active fractions, which eluted as a single peak in the void volume of the column, were bulked and concentrated by ultrafiltration in an Amicon pressure cell with a PM10 membrane (exclusion limit 10,000 daltons) to a volume of approximately 8ml. The concentrate was applied to a column of DEAE-Sephadex A50 (30 x 1.4cm), equilibrated with 10mM buffer (above), and eluted with the same buffer; sixty 4ml fractions were collected. The active fractions, which eluted as a single peak in the void volume of the column, were bulked and applied to a column of Matrēx Gel Red A (Amicon; 5 x 1.0cm), equilibrated with 10mM buffer (above). The column was then washed with the same 10mM buffer and 4ml fractions were collected until no further protein emerged. A linear 100ml gradient (Bock and Ling, 1954) of 0 to 4.0M sodium chloride in 10mM buffer (above) was applied to elute bound protein and 2ml fractions were collected. The active fractions, which eluted as a single peak corresponding to a salt concentration of 2.5-3.0M, were bulked and dialysed for 16h against 10l of 10mM mercaptoethanol and 0.05mM PMSF. The preparation was concentrated in an Amicon pressure cell as above. Glycerol was added to give a final concentration of 50% (v/v) and

the preparation was stored in this form at -20°C. There was no significant loss of activity after storage for 1 month in this condition.

2.3.2. Purification of Acid Phosphatase.

After preliminary experiments the following procedure was adopted in a typical purification. All stages of the purification were carried out at 4°C. Frozen slices (2.1.) of sporophores (240g) harvested at stage 2 of development (1.3.) were allowed to thaw and homogenised in a "Vortex" mixer with 15ml of 10mM tris/HCl buffer, pH7.8, containing 50mM mercaptoethanol and 0.25mM PMSF. The homogenate was centrifuged at 15,000g for 30 min and the supernatant retained. Nucleic acids were precipitated by the addition of excess protamine sulphate to give a final concentration of approximately 0.25% (w/v). The suspension was centrifuged as above. The supernatant was retained and precipitated by the gradual addition of solid ammonium sulphate (heavy metal free); the acid phosphatase activity, as measured by activity against *p*-nitrophenylphosphate at pH4.5 (2.2.6.1.) precipitated between 50 and 80% saturation. This fraction was isolated by centrifugation (as above) and dissolved in a minimum volume (approximately 5ml) of 10mM buffer (above). This preparation was then applied to a Sephadex G100 column (33 x 2.5cm), equilibrated with 10mM buffer (above), and eluted with the same buffer; eighty 5ml fractions were collected. The activity (assayed as above) eluted as a

single peak in the void volume of the column. The active fractions were bulked and concentrated by ultrafiltration in an Amicon pressure cell using a PM10 membrane (exclusion limit 10,000 daltons) to a volume of approximately 6ml. This concentrate was applied to a column of DEAE-Sephadex A50 (30 x 1.4cm), equilibrated with 10mM Buffer (above), and the column was washed with the same buffer. Fractions (4ml) were collected until no further protein emerged. A 100ml linear gradient of increasing ionic strength from 0 to 1.0M sodium chloride in 10mM buffer (above) was used to elute bound protein from the column (Bock and Ling, 1954); 2ml fractions were collected. The fractions were assayed for activity against *p*-nitrophenylphosphate (above) and the active fractions, eluting at a salt concentration of approximately 0.6M, were bulked and dialysed for 16h against 2l of 50mM acetate buffer, pH4.5, containing 0.5% (w/v) sodium azide. The dialysed preparation was stored at 4°C and no significant loss of activity was shown after a period of 3 weeks.

2.3.3. Purification of Fructokinase.

After preliminary experiments, the following procedure was adopted in a typical purification. All stages of the purification were carried out at 4°C. The activity was monitored by assaying for fructokinase activity at each stage as described in section 2.2.9.1. Freshly harvested stage 2 sporophores (1.3.) were sliced, in the same manner

as sporophores for storage (2.1.), to a thickness of 2mm. The slices (200g) were homogenised in a "Vortex" mixer with 20ml of 10mM tris/HCl buffer, pH9.0, containing 50mM mercaptoethanol and 0.25mM PMSF. The homogenate was centrifuged at 15,000g for 30 min and the supernatant retained. Nucleic acids were precipitated by the addition of excess protamine sulphate to give a final concentration of approximately 0.25% (w/v). The suspension was centrifuged as above. The supernatant was retained and solid ammonium sulphate (heavy metal free) was added gradually to a saturation of 55%. The suspension was centrifuged as above. The precipitate was retained and redissolved in a minimum volume (approximately 5ml) of 10mM buffer (above). The resuspended precipitate was applied to a column of Sephadex G200 (36 x 2.5cm), equilibrated with 10mM buffer (above), and eluted with 10mM buffer (above); eighty 4ml fractions were collected. The ten active fractions, which eluted as a single peak in the void volume of the column were bulked and applied to 1g of DEAE-Sephadex A50 matrix. Prior to application, the gel matrix was equilibrated in three changes of 100ml of 10mM buffer (above) and the final buffer rinse supernatant discarded to leave gel slurry. The matrix and the enzyme preparation were mixed and the matrix allowed to settle. The supernatant was discarded and the matrix was rinsed by mixing with 100ml of 10mM buffer (above), allowing the matrix to settle and discarding the supernatant. The rinse procedure was repeated twice more successively. The matrix was then mixed with 100ml

of 0.5M sodium chloride in 10mM buffer (above), allowed to settle and the supernatant discarded. This procedure was repeated with 1.0M sodium chloride in 10mM buffer (above). The matrix was then mixed with 100ml of 2.0M sodium chloride in 10mM buffer (above) and allowed to settle. The supernatant was removed and retained and concentrated to a final volume of approximately 20ml by ultrafiltration in an Amicon pressure cell with a PM10 membrane (exclusion limit 10,000). Glycerol was added to give a concentration of 50% (v/v) and the preparation was stored at -20°C.

2.4. Determination of Variations in Enzyme Activities with Age of Developing Sporophore.

Sporophores were harvested over the 13 day period of a "flush" (1.3.); a minimum of 2 specimen fruit-bodies was harvested for each stage sampled. The first appearance of stage 1 "pinheads" (1.3.) was designated as day 0 and the age in days for all samples recorded.

Sporophores up to day 4 were extracted complete. Older fruit-bodies were divided into stipe and pileus plus gill tissue by breaking off the stipe at the junction with the pileus.

At early stages of development, up to day 6, when the sporophores were still small, all the harvested tissue was extracted. At later stages, the sample size was

restricted to a fresh weight of about 5g by taking radial slices of the pileus plus gill tissue and longitudinal sections of the stipe.

The fresh weight of each sample was recorded and some samples were dried in an oven (at 80°C) to constant weight to determine dry weight. The tissues of the remaining samples were sliced to a thickness of approximately 2mm and a volume equivalent to 25% (weight of buffer/weight of tissue) of 10mM tris/HCl buffer, pH7.5, containing 50mM mercaptoethanol and 0.25mM PMSF, was added. The sample was homogenised in a "Vortex" mixer and the homogenate was centrifuged in a microfuge for 10 min and the supernatant retained. Nucleic acids were precipitated by the addition of 3% (w/v) protamine sulphate to a final concentration of 0.25% (w/v). The suspensions were centrifuged as above. Some samples from the late stages of development (days 9-11) required a third centrifugation (as above) to obtain a clear extract. Soluble protein concentrations were determined in each extract (2.5.1.).

An aliquot (50µl) of each extract was diluted to 5.0ml with 10mM tris/HCl buffer, pH7.5, containing 25mM mercaptoethanol and dialysed against 1l of the same buffer for 6h. At the end of this time, the volume of the dialysed solution was measured and the concentration of protein in the extract was estimated (2.5.1.) allowing for all dilution. Selected

dialysed extracts were also assayed for mannitol dehydrogenase activity (2.2.1.).

An aliquot (1-25µl) of each undialysed extract was assayed for mannitol dehydrogenase activity (2.2.1.). Further aliquots (100µl) of undialysed extracts were assayed for phosphatase activity against *p*-nitrophenylphosphate (2.2.6.1.) and against fructose-6-phosphate and glucose-6-phosphate (2.2.6.2.). These assays were carried out at pH4.5 and pH6.5 to examine any difference in pattern of phosphatase activity with pH. Aliquots (12.5µl) of undialysed extracts were assayed for fructokinase activity by the radioisotope method (2.2.9.2.).

Aliquots (5 or 10µl) of undialysed extracts were subjected to polyacrylamide-gel electrophoresis at pH8.0 (2.5.3.1a) and stained for protein (2.5.4.1.) or for acid phosphatase activity (2.5.4.4.). Further aliquots (10µl) of undialysed extracts were subjected to polyacrylamide-gel electrophoresis at pH5.0 (2.5.3.1b). Gels were stained for protein (as above), or for mannitol dehydrogenase activity (2.5.4.2.), or stained for mannitol dehydrogenase activity followed by staining for protein on the same gel (2.5.4.3.); the gels stained for both enzyme activity and protein were used to identify the protein band corresponding to mannitol dehydrogenase on gels stained for protein alone. The gels stained for protein were scanned for absorbance at 550nm to determine the relative intensity of the mannitol dehydrogenase band.

The study was carried out on three separate flushes. Results quoted (6.1.) for soluble protein levels are means of two or three determinations of levels in sporophores of the same age but from separate flushes; these results showed less than 10% variation. Similarly, results for mannitol dehydrogenase activity levels are means of two or three determinations showing less than 15% variation and results for phosphatase activity levels are means of two determinations showing less than 10% variation. Results for fructokinase activity levels are from a single determination only. Estimates of relative mannitol dehydrogenase concentrations in extracts from the intensity of the enzyme band stained for protein on polyacrylamide-gels are also single determinations.

2.5. General Analytical Techniques.

2.5.1. Determination of Protein in Solution.

The concentration of proteins in solution was estimated by a spectrophotometric method based on that of Warburg and Christian (1942). The optical absorbance at 260 and 280nm was measured and the protein concentration calculated from the equation of Layne (1957);

$$1.55A_{280} - 0.76A_{260} = \text{Protein concentration (mg ml}^{-1}\text{)}.$$

The protein concentration of the final purified preparation

of mannitol dehydrogenase was confirmed by the method of Lowry et al (1951) as modified by Miller (1959). A solution containing 10% (w/v) Na_2CO_3, 0.05% (w/v) $CuSO_4$ and 0.1% potassium tartrate in 0.5M NaOH was freshly prepared; 1ml of this mixture was added to 1ml of the protein solution to be tested. Prior to mixing, the protein solution was diluted to give a concentration in the acceptable range of 40-200μg ml^{-1}. The samples were mixed well and allowed to stand for 10 min at room temperature before adding 3ml of a 1:11 dilution of Folin-Ciocalteau phenol reagent. Incubation was continued for a further 30 min to allow the development of a blue colour which was measured at 560nm. The method was calibrated by testing standard solutions of bovine serum albumin of known concentration.

2.5.2. Spectrophotometry

Unless otherwise stated, optical absorbances of solutions were measured in 1ml or 3ml silica cuvettes, with a 1cm light path, using a Gilford 2000 recording spectrophotometer.

2.5.3. Polyacrylamide-gel Electrophoresis.

2.5.3.1. Gels for Native Proteins.

a) The method of gel preparation was based on that described by Weber, Pringle and Osborne (1972). Gels containing 8% (w/v) polyacrylamide (1% cross-linked) were prepared by

mixing 11.2ml water, 0.8ml 1.0M tris/acetate buffer, pH8.0, supplemented to 1.5% (w/v) with disodium EDTA, 2ml 1.5% (w/v) ammonium persulphate and 2ml 1% (w/v) TEMED. The solution was degassed and 4ml of a stock solution of 40% (w/v) acrylamide, 0.4% (w/v) NN'-methylenebisacrylamide was then added. The gel mixture was carefully pipetted into gel tubes (8.4cm in length and 0.5cm internal diameter) and allowed to polymerise overnight at room temperature.

The gels were inserted into a Shandon Disc Electrophoresis Unit and the reservoirs filled with 40mM tris/acetate buffer, pH8.0, supplemented to 0.0375% (w/v) disodium EDTA (TAE buffer). The whole unit was placed in a cold room at $4^{o}C$ and a tracking dye of saturated bromophenol blue in 50% (w/v) sucrose was applied to the gels. A current of 7mA gel^{-1} was applied for 2h to remove potentially harmful ions prior to the application of protein samples; the top reservoir acted as the cathode.

At the end of this time, the TAE buffer in the anode and cathode compartments was renewed and protein samples applied. The samples were prepared by mixing protein suspensions (15-20µg in a maximum volume of 50µl) with 50% (w/v) sucrose solution in a ratio of 2:1 (v/v). The unit was maintained at $4^{o}C$ and a current of 7mA gel^{-1} was applied for either 2½ or 3h; the top reservoir acted as the cathode.

The gels were then removed and stained for protein or acid phosphatase activity (2.5.4.).

b) Gels were prepared as above except that 1.0M acetate buffer, pH5.0, supplemented to 1.5% (w/v) with disodium EDTA, replaced the 1.0M tris/acetate buffer, pH8.0, similarly supplemented with disodium EDTA, in the gel mixture.

Harmful ions were removed from the gels as above except that 40mM acetate buffer, pH5.0, supplemented to 0.0375% (w/v) with disodium EDTA replaced the TAE buffer. At the end of this time, the buffer in each compartment was renewed and samples (prepared as above) were applied. The unit was maintained at 4°C and a current of 7mA gel^{-1} was applied for 2½h; the bottom reservoir acted as the cathode.

The gels were then removed and stained for protein or mannitol dehydrogenase activity (2.5.4.).

2.5.3.2. Gels for Denatured Protein.

Gels containing 8% (w/v) acrylamide (1% cross-linked) and 0.1% (w/v) SDS were prepared by mixing 10ml 0.2M potassium phosphate buffer, pH7.2, supplemented with 0.02M disodium EDTA, 2ml 1.5% (w/v) ammonium persulphate and 2ml 1% (w/v) TEMED. The mixture was degassed. Then 4ml acrylamide stock solution (2.5.3.1.) and 2ml 1% (w/v) SDS were added. The mixture was carefully pipetted into gel tubes (dimensions as in section 2.5.3.1.) and allowed to polymerise at room temperature overnight.

The gels were inserted into the electrophoresis unit (2.5.3.1.) and the reservoirs filled with 0.1M potassium phosphate buffer, pH7.2, supplemented to 0.01M with disodium EDTA and 0.1% (w/v) with SDS. Bromophenol blue/sucrose mixture (2.5.3.1.) was applied as tracking dye to each gel. A current of 6mA gel^{-1} was applied until the tracking dye reached the lower reservoir to remove harmful ions; the upper reservoir acted as the cathode and the unit was maintained at room temperature.

The buffer in the anode and cathode compartments was then renewed and the protein samples were applied. The protein samples (5-50µg) were denatured by suspending them in 60µl 200mM potassium phosphate buffer, pH7.2, containing 1% (w/v) SDS, 1% (w/v) mercaptoethanol and 10mM disodium EDTA, and boiling for 3 min. The denatured samples were mixed 2:1 (v/v) with bromophenol blue/sucrose solution (as above) before application to the gels. Electrophoresis was carried out at room temperature by the application of a current of 6mA gel^{-1} for 3h; the upper reservoir acted as the cathode. Samples prepared and subjected to electrophoresis as described here included extracts from A. bisporus at various stages of purification and "marker" proteins with subunits of known molecular weight.

The gels were removed after electrophoresis and stained for protein (2.5.4.1.). Where gels were used to estimate subunit molecular weight, the mobility of each protein

sample was calculated relative to the bromophenol blue dye front. From the marker proteins, a standard curve of Log (molecular weight of subunit) against mobility was plotted (3.1.2.) and the molecular weight of the mannitol dehydrogenase subunit determined by its mobility.

2.5.4. Gel Staining Procedures.

2.5.4.1. Detection of Protein in Gels.

Proteins were stained and fixed by soaking in 0.25% (w/v) Coomassie Brilliant Blue R in methanol: glacial acetic acid: water, 5:1:4 by volume, for 3h at room temperature. Gels were destained over several days in several changes of methanol: glacial acetic acid: water, 10:3:27 by volume, at room temperature.

2.5.4.2. Detection of Mannitol Dehydrogenase Activity in Gels.

Gels were incubated at 25°C in the dark for a period of 5 to 20 min in 50mM sodium carbonate buffer, pH10.4, containing 100mM D-mannitol, 1mM $NADP^+$, 0.002% (w/v) phenazine ethosulphate and 0.032% (w/v) nitro blue tetrazolium. Gels were destained in several change of water over a period of 2 days at room temperature in the dark.

2.5.4.3. Detection of Mannitol Dehydrogenase Activity and Protein in the Same Gel.

Gels were incubated in the mannitol dehydrogenase activity staining mixture (2.5.4.2.) but, instead of destaining in water at the end of the incubation, were placed immediately in Coomassie Brilliant Blue R stain for proteins (2.5.4.1.) for 3h. Gels were then destained over several days at room temperature in the destain solution described in section 2.5.4.1.

2.5.4.4. Detection of Phosphatase Activity in Gels.

The method of staining for acid phosphatase activity was based on the "post-incubation capture" technique of Allen and Hyncik (1963). Gels were incubated in 50mM acetate buffer, pH4.5, containing 50mM D-fructose-6-phosphate or other sugar phosphate (6.1.3.) and 15mM calcium chloride, for 2 to 20 min at room temperature. The gels were washed thoroughly with water and placed in a solution of 3mM lead nitrate for a minimum of 30 min to precipitate lead phosphate in the area of enzyme activity in the gel. The gels were then rinsed in several changes of distilled water over a period of 1 to 2h. They were then treated with a solution of 5% (v/v) ammonium sulphide for 2 min to convert the lead phosphate to a coloured lead sulphide precipitate and finally rinsed again in several changes of distilled water.

For detection of alkaline phosphatase activity, the procedure above was used except that 50mM tris/HCl buffer, pH8.5, replaced the 50mM acetate buffer, pH4.5.

2.5.5. Determination of Native Molecular Weight of Purified Mannitol Dehydroenase by Gel Filtration.

Aliquots (0.25ml) of purified mannitol dehydrogenase preparation (0.33mg ml^{-1} ; see section 2.3.1. for purification details) and "marker" proteins of known native molecular weight (1mg ml^{-1}), with solid sucrose added to give a final sucrose concentration of 30% (w/v), were applied to a column (12 x 1.5cm) of Sephadex G200. The column was previously equilibrated with 50mM tris/HCl buffer, pH7.5, and samples were eluted with the same buffer at a flow rate of 0.445ml min^{-1}. The eluant was passed through a Cecil CE 212 spectrophotometer flow cell and protein estimated at 280nm. The elution volume of each substance was estimated from the peak absorbance at 280nm and that for mannitol dehydrogenase was confirmed by collecting 0.1ml fractions which were assayed for enzyme activity (2.2.1.). From the marker proteins, a standard curve of Log (molecular weight) against elution volume was plotted (3.1.2.) according to the method of Andrews (1964). The molecular weight of the mannitol dehydrogenase enzyme was determined from its elution volume and the standard curve. The result quoted (3.1.2.) is a mean of two experiments showing less than 5% variation.

2.5.6. Paper Chromatography of Sugars.

a) The incubation products of the mannitol dehydrogenase reaction (2.2.1.) were applied to Whatman No.1 paper and separated by descending chromatography for 22h at room temperature in a mixture ethyl methyl ketone: acetic acid: borate saturated water, 9:1:1 by volume. "Markers" of D-fructose, D-glucose, D-mannitol and D-sorbitol were simultaneously chromatographed. Sugars and sugar alcohols were detected by dipping the chromatogram in a saturated solution of lead tetraacetate in glacial acetic acid and allowing the chromatogram to dry. Sugars and sugar alcohols appeared as white spots against a brown background. The products of the mannitol dehydrogenase incubation were identified by comparing the mobilities of the chromatographed compounds with those of the known markers.

b) The incubation products of the fructokinase reaction (2.2.9.2.) were applied to Whatman No.1 paper and separated by descending chromatography for 24h at room temperature in a mixture propan-1-ol: ethyl acetate: water, 7:1:2 by volume. "Markers" of D-fructose, D-glucose, D-fructose-6-phosphate and D-glucose-6-phosphate were simultaneously chromatographed and cut from the chromatogram after it had been developed and dried. The markers were detected by dipping the chromatogram in a reagent based on the aniline phthalate reagent of Partridge (1949) and heating to 110°C for 10 min. The reagent contained 1.7g o-phthalic acid, 1.0ml aniline, 5ml acetic acid, 5ml 40% (v/v) trichloroacetic

acid and 90ml ethanol. Sugars and sugar phosphates were detected as brown spots against a yellow background. The remainder of the chromatogram was autoradiographed for 3 days using Fuji RX-3 film to locate radioactive products of the fructokinase reaction. These were identified by comparing their mobilities with those of the known markers. The radioactive areas of the chromatogram were removed and the relative amount of each radioactive product in each sample of a fructokinase incubation was determined by the method of Corbett et al (1975); counting was carried out in a "Beckman LS-230" liquid scintillation counter.

2.5.7. Determination of Cellular pH.

An estimate of the gross cellular pH of A. bisporus sporophores was obtained by homogenising sporophores, harvested at various stages of development, with no additives in a "Vortex" mixer. The cellular debris was removed by centrifugation at 15,000g for 30 min and the pH of the resulting supernatant was measured on a meter with a micro-probe.

3. Mannitol Dehydrogenase.

3.1. Results.

3.1.1. Enzyme Purification.

Table 4 summarises the purification procedure for mannitol dehydrogenase enzyme from 340g of frozen sporophore slices harvested at stage 2 of development (see Section 2.3.1. for details). There was an increase in yield at the stage of the Matrēx Gel Red A column; this may have been due to the removal of an enzyme inhibitor. The level of purification achieved was illustrated by SDS polyacrylamide-gel electrophoresis of denatured samples; the final preparation showed a single band (Plate 3). A single protein band and a corresponding single mannitol dehydrogenase were shown by polyacrylamide-gel electrophoresis of a native sample of the enzyme preparation (Plate 4).

3.1.2. Molecular Weight.

SDS polyacrylamide-gel electrophoresis of the purified enzyme with protein markers of known subunit molecular weight (2.5.3.2.) gave a value of 29,500 for the molecular weight of the denatured enzyme subunit (Fig. 4).

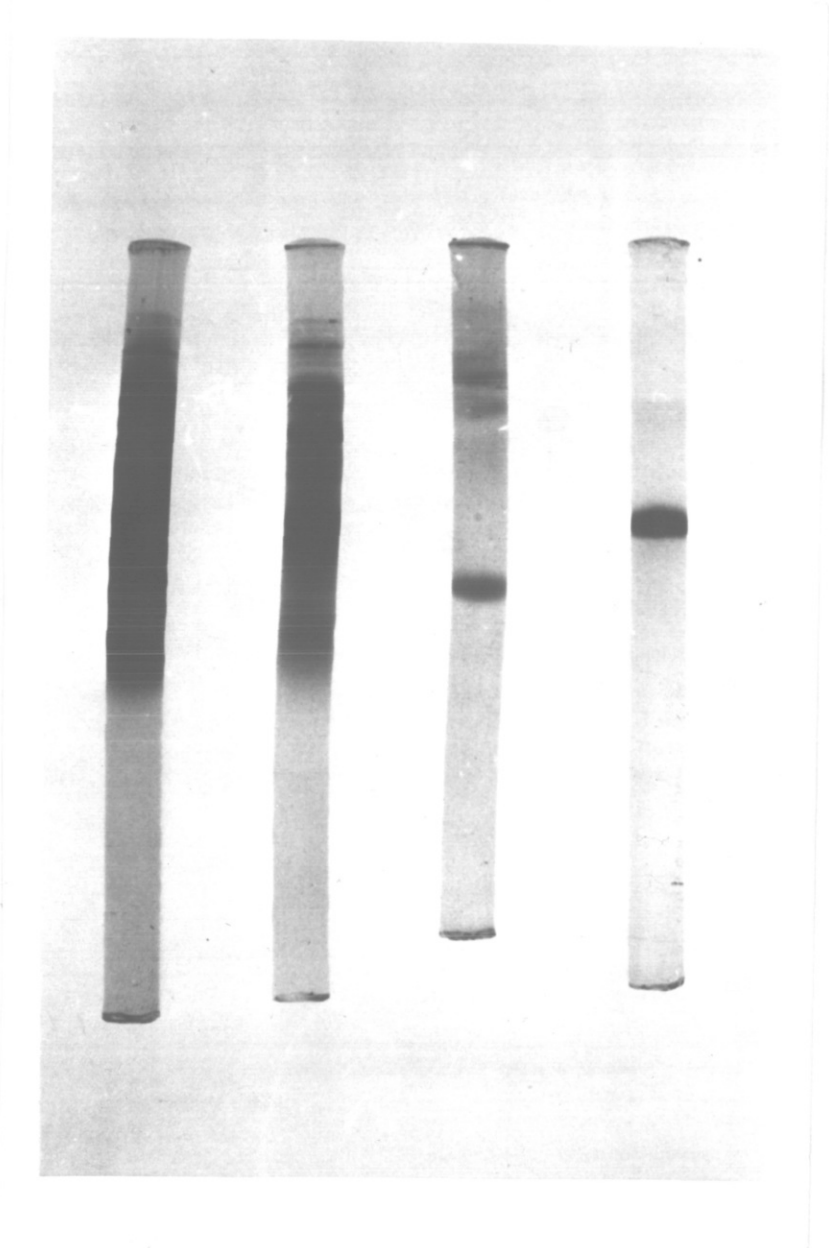
The molecular weight of the native enzyme was estimated to be 115,000 (Fig. 5) by gel filtration on a Sephadex

Table 4: Purification of mannitol dehydrogenase from 340g of frozen A. bisporus sporophore slices.

Fraction	Volume (ml)	Protein (mg)	Total Activity (Units x 10^{-3})	Specific Activity (Units x 10^{-3} mg^{-1})	Purification (Fold)	Yield (%)
Crude Extract.	225	5310	5478	1.032	1.00	100
Protamine sulphate precipitated extract.	213	4622	5214	1.128	1.09	95.2
55-80% saturated ammonium sulphate fraction.	8.7	569	1356	2.388	2.31	24.8
Concentrated eluate from Sephadex G100 column.	7.7	128	508	3.954	3.83	9.27
Eluate from DEAE-Sephadex A50 column	27.5	2.70	115	42.42	41.1	2.10
Concentrated eluate from Matrex Gel Red A column	2.10	0.924	179.4	194.4	188.4	3.27

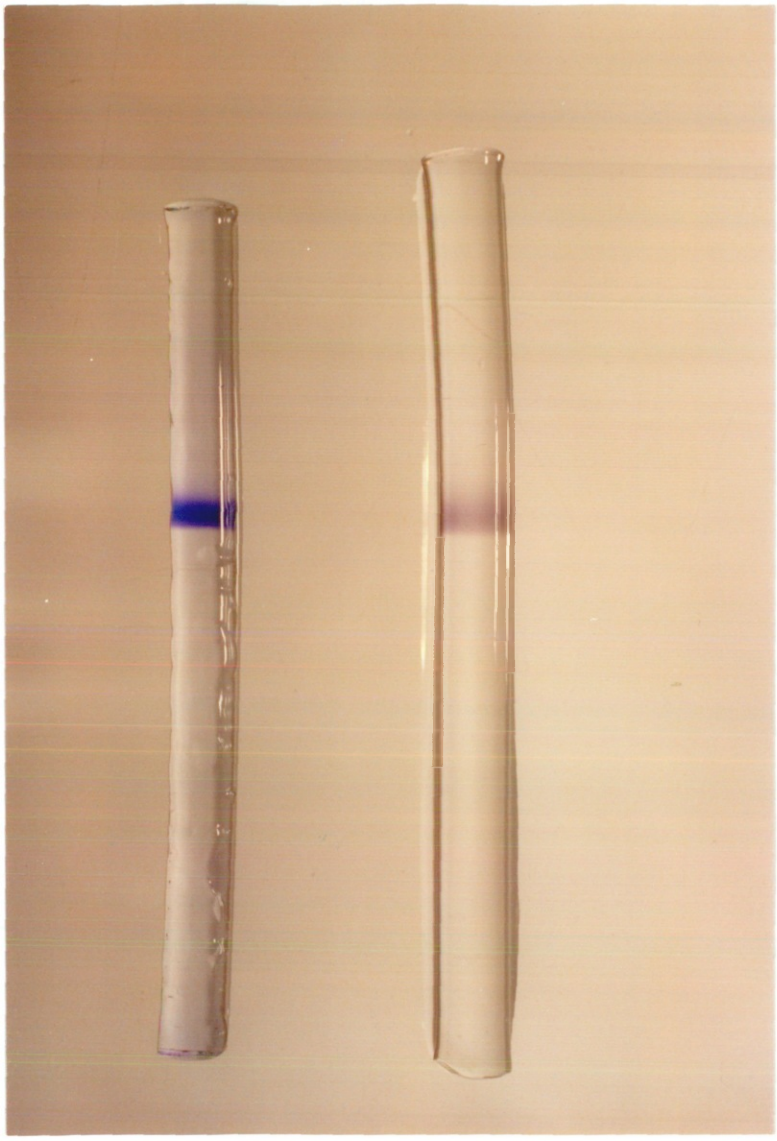
See text for details. Units are as defined in Section 2.2.1.

Plate 3: SDS polyacrylamide-gel electrophoresis of denatured samples of fractions isolated during the purification of mannitol dehydrogenase.



Samples of the following fractions (from left to right) were denatured and examined on SDS polyacrylamide-gels (2.5.3.2.); crude extract, eluate from Sephadex G100, eluate from DEAE-Sephadex A50 and eluate from Matrex Gel Red A.

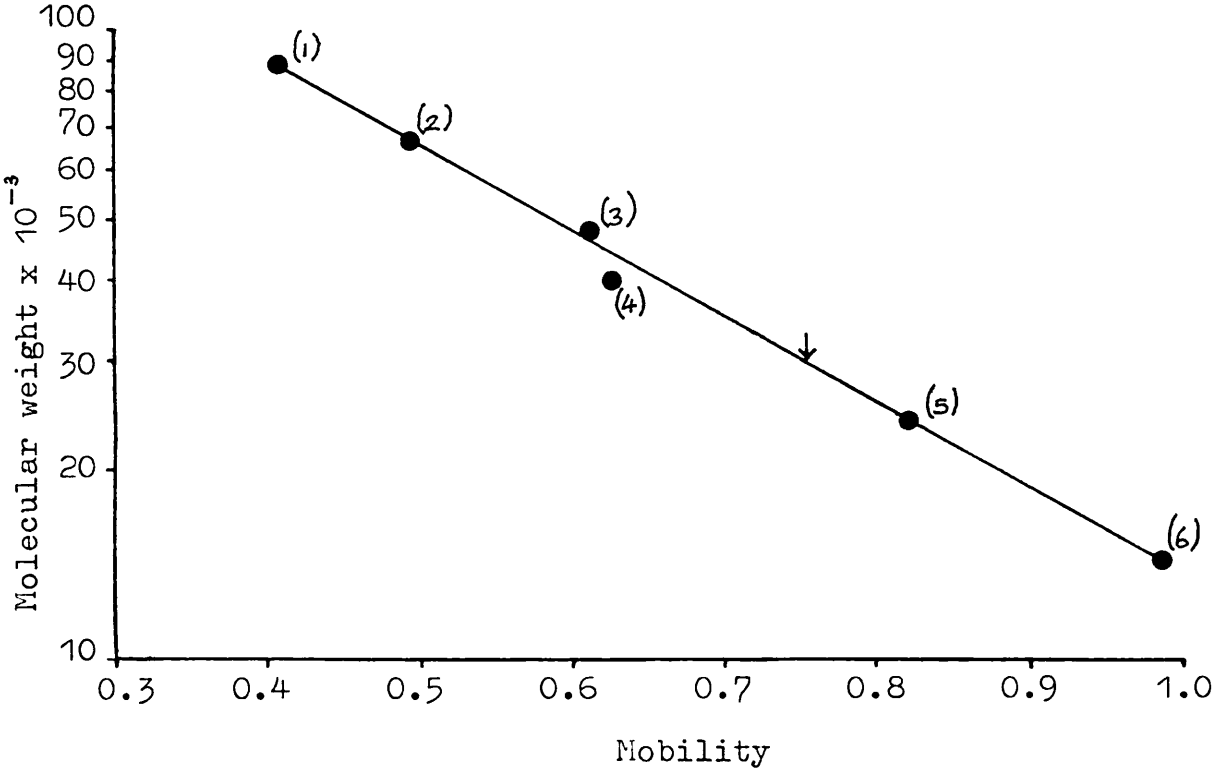
Plate 4: Gel electrophoresis of native samples of purified mannitol dehydrogenase preparation.



Native samples of the preparation were subjected to electrophoresis at pH5.0 (2.5.3.1b.) and stained for protein (left) and mannitol dehydrogenase activity (right) as described in Section 2.5.4.

Figure 4: Molecular weight of denatured mannitol dehydrogenase.

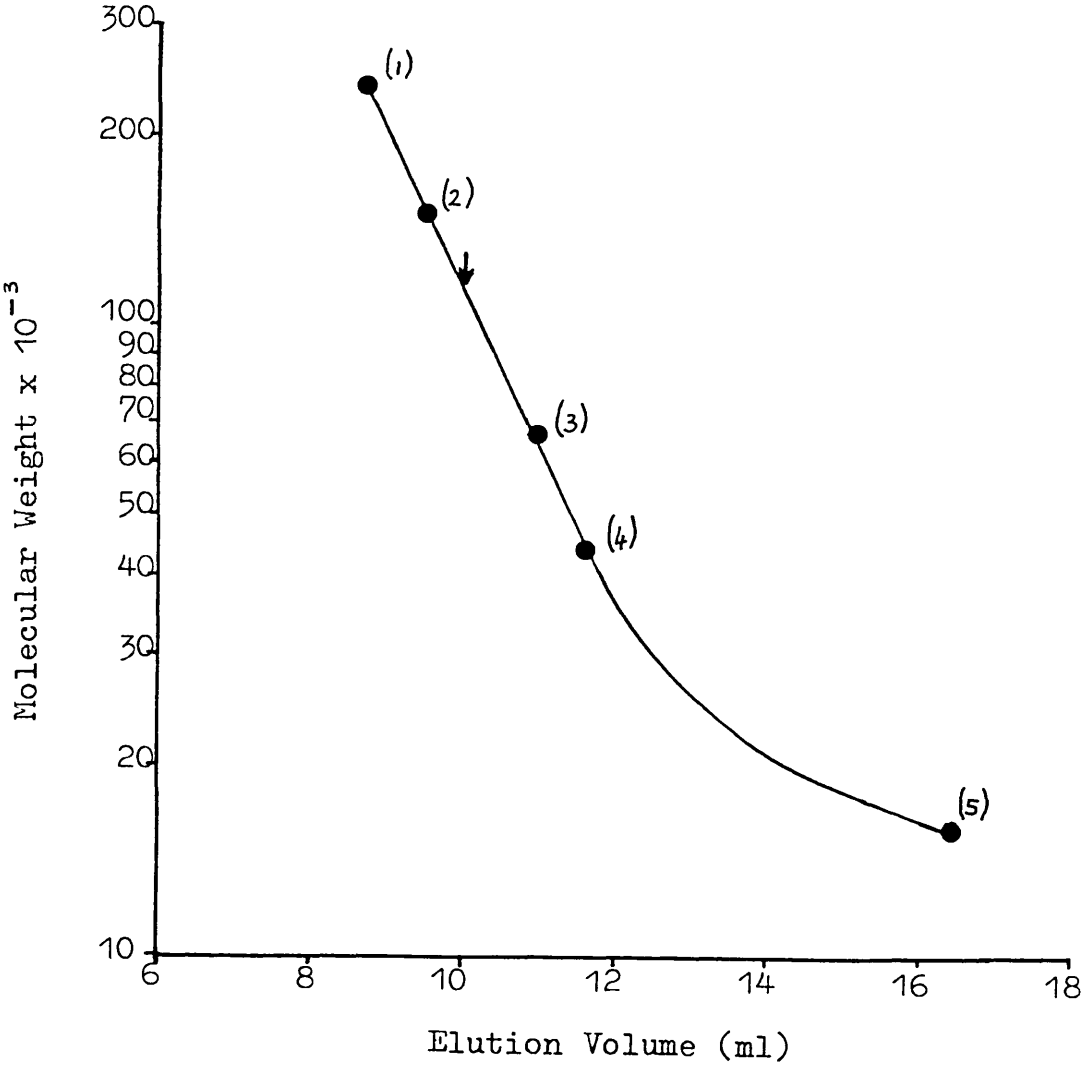
Electrophoresis was carried out in 8% (w/v) polyacrylamide-gels containing 0.1% (w/v) SDS (2.5.3.2.). Protein markers were (1) Bacillus stereothermophilus methionyl-tRNA synthetase (kindly donated by Dr. C. J. Bruton, Dept. of Biochemistry, Imperial College), (2) bovine serum albumin (Boehringer),



(3) B. subtilis tryptophanyl-tRNA synthetase (kindly donated by C. J. B.), (4) rabbit muscle aldolase (Boehringer), (5) bovine chymotrypsinogen (Boehringer) and (6) lysozyme (Boehringer). The arrow indicates the mobility of mannitol dehydrogenase.

Figure 5: Molecular weight of native mannitol dehydrogenase by gel filtration.

Protein markers and mannitol dehydrogenase preparation were individually applied to a Sephadex G200 column, eluted at a constant flow rate and elution volumes recorded (2.5.5.). Markers used were (1) catalase, (2) horse liver alcohol dehydrogenase, (3) bovine serum albumin, (4) ovalbumin and



(5) horse myoglobin (all Boehringer). The arrow indicates the elution volume of mannitol dehydrogenase.

G200 column with protein markers of known molecular weight (2.5.5.). This figure is approximately four times that of the subunit molecular weight (above) suggesting the native enzyme is a tetramer.

3.1.3. Irreversible pH Effects.

Aliquots of the purified enzyme were preincubated in a variety of 50mM buffers prior to assay under standard conditions (2.2.2.). Resultant activities, relative to an index of 100 for the enzyme preincubated at pH7.5, are given in Table 5. There was a loss of activity when the enzyme was preincubated below pH6.5; this loss was 20% at pH6.2 and rose to 90% of the activity as the pH was decreased to pH4.6. This was presumably due to irreversible denaturation of the enzyme and this effect made it impossible to determine values for kinetic parameters at pH's below 6.5.

3.1.4. Test for Substrate and Coenzyme Specificity.

Incubations of the purified enzyme with a variety of substrates were carried out as detailed in Section 2.2.3. Relative rates of reaction are summarised in Tables 6a and b. A high degree of specificity towards NADP(H) was shown. Although specificity towards D-mannitol was not complete, since a relative rate of 7.7% occured with D-sorbitol, the enzyme was specific for D-fructose; no

Table 5: Effects of preincubation of mannitol dehydrogenase in buffers of varying pH on activity.

pH of preincubation buffer.	Relative rate of fructose reduction.
10.9	105
9.5	97
8.8	102
8.0	96
7.5	100
7.0	97
6.5	98
6.2	80
5.6	67
5.0	41
4.6	10

Table 6a: Substrate and coenzyme specificity of mannitol dehydrogenase for the reaction in the ketose reducing direction (expressed relative to activity with fructose and NADPH = 100).

Substrate	Relative Activity	
	NADPH as coenzyme	NADH as coenzyme
D-Fructose	100	0
D-Glucose	0	0
D-Galactose	0	0
L-Sorbose	0	0
D-Fructose-6-phosphate	0	0

Table 6b: Substrate and coenzyme specificity of mannitol dehydrogenase for the reaction in the polyol oxidising direction (expressed relative to activity with mannitol and $NADP^+$ = 100).

Substrate	Relative Activity	
	$NADP^+$ as coenzyme	NAD^+ as coenzyme
D-Mannitol	100	0
D-Sorbitol	7.7	0
D-Galactitol	0	0
Ribitol	0	0
L-Arabitol	0	0
Erythritol	0	0

See Methods Section 2.2.3. for details.

reaction could be detected with any other sugar.

With D-mannitol as substrate, D-fructose was identified as the sole reaction product by paper chromatography (2.5.6a.), and with D-fructose as substrate, D-mannitol was similarly identified as the sole product of the reaction.

3.1.5. Kinetic Properties.

Values of the maximum rate (Vmax) of fructose reduction and mannitol oxidation and apparent Km values for all substrates were determined at a number of different pH values (2.2.4.) above pH6.5 (3.1.3.). Table 7 summarises the results for the reaction in both directions at the respective pH optima as observed under the experimental conditions employed.

Figure 6 shows the variation of apparent Km for fructose with varying pH. The Km obtained was approximately 1.23M at pH7.3 and this value decreased with an increase in pH. High concentrations of fructose showed substrate inhibition of the enzyme; this inhibition was apparent for fructose concentrations greater than approximately 1.2M below pH9.9 and for fructose concentrations greater than approximately 0.8M above pH9.9. Substrate inhibition by fructose was therefore shown for fructose concentrations of the same order of magnitude as the Km for fructose; hence "saturation" of the enzyme with fructose was

Table 7: Kinetic constants of mannitol dehydrogenase.

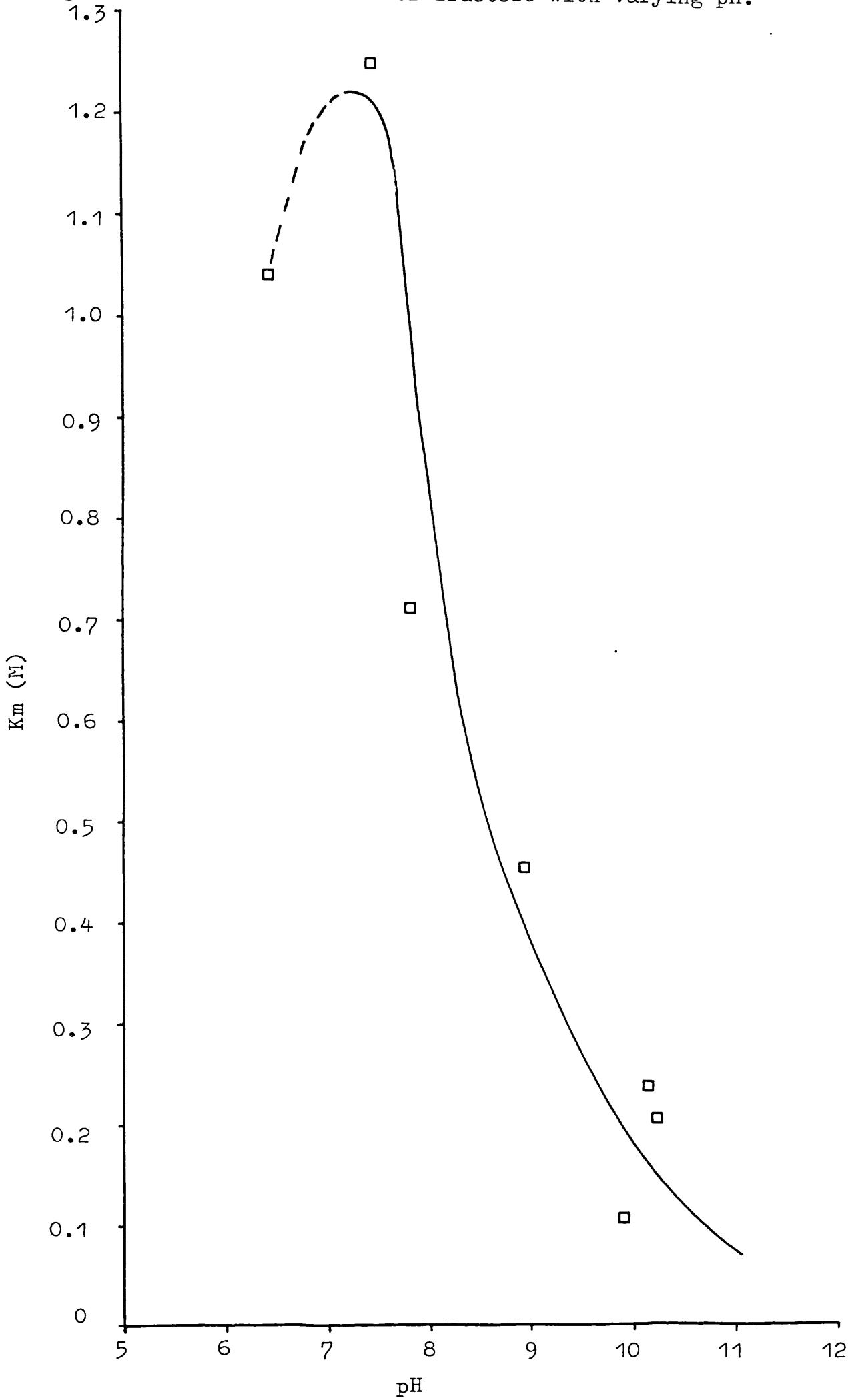
Reaction	Km (mM)	Vmax (μmoles NADPH consumed or produced min^{-1} mg^{-1} protein)
Fructose reduction	Fructose = 1.20×10^{3} NADPH = 1.70×10^{-2}	591
Mannitol oxidation	Mannitol = 16.2 $NADP^{+}$ = 8.2×10^{-2}	70.2

The results summarised are those for fructose reduction at pH7.0 and mannitol oxidation at pH10.25, the respective pH optima.

See Methods Section 2.2.4. for details.

Values of Km for fructose were determined under conditions of NADPH saturation in various 50mM buffers at various pH's. See Methods Section 2.2.4. for details.

Figure 6: Variation of Km for fructose with varying pH.

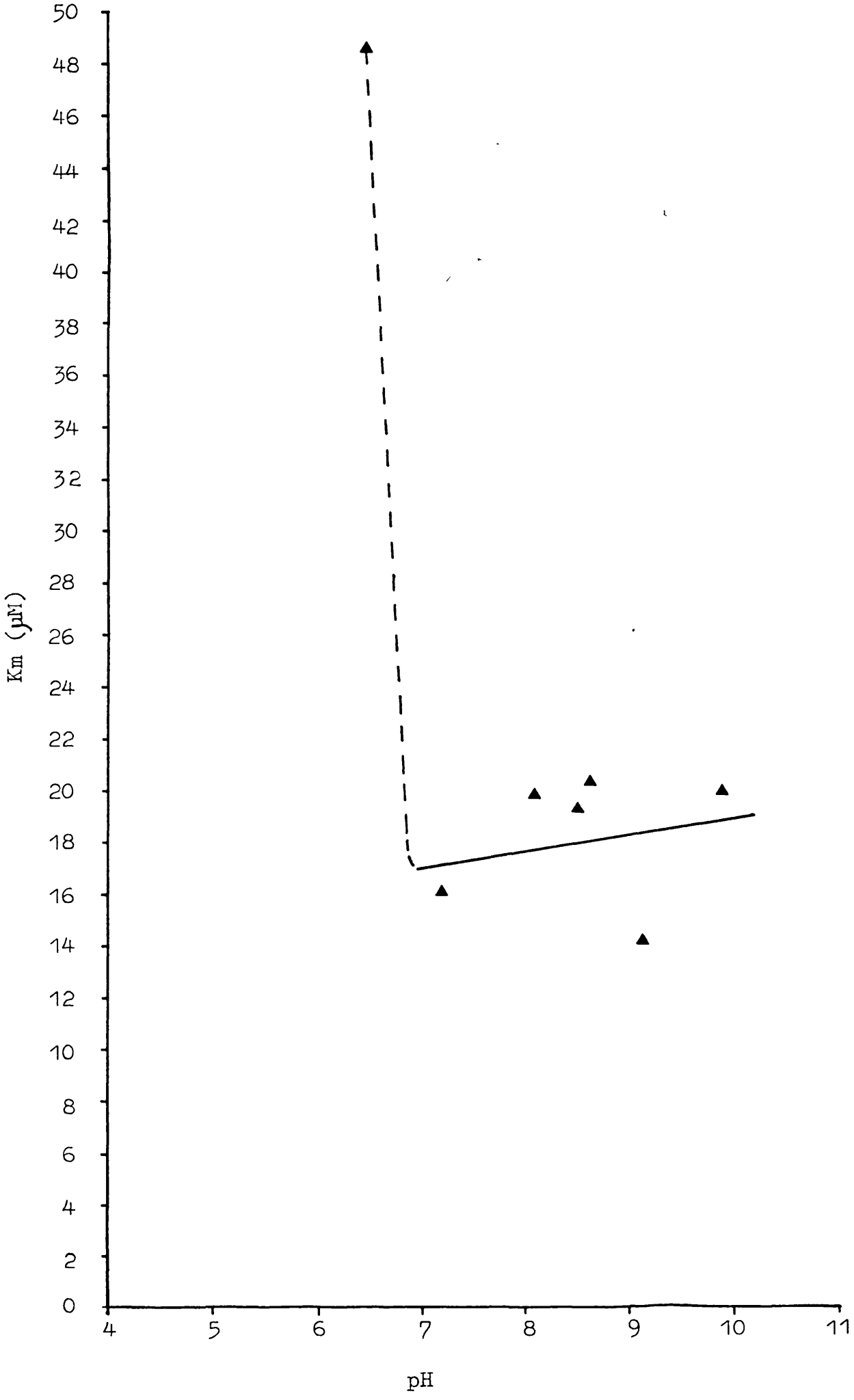


impossible under experimental conditions. In evaluating the properties of the enzyme with respect to NADPH, fructose concentrations were fixed at 800mM. At this sugar concentration the variation of the rate of fructose reduction was apparently first order with respect to NADPH, i.e. points fell on a straight line on a Lineweaver-Burk plot. The variation of apparent Km for NADPH with varying pH under these conditions is shown in Figure 7; the gradient of the straight line in the region above pH7 was calculated by regression analysis and this gradient is not significantly different from zero when analysed by the "t-test". There was a large rise in Km below pH7 which may have been due to denaturation of the enzyme; possibly there was a limited amount of irreversible denaturation as has been shown to occur below pH6.5 (3.1.3.).

The variation of Vmax for fructose reduction with varying pH is illustrated by Figure 8. The values were determined under conditions of a) fixed fructose concentration (800mM) with NADPH concentrations variable and b) fixed saturating concentration of NADPH (0.25mM) with fructose concentrations variable (2.2.4.). The two methods gave different graphs ("a" and "b", Figure 8) but with both methods the rate of fructose reduction rose as the pH dropped towards pH7. The variation of Vmax for mannitol oxidation with varying pH is shown by graph "c" on Figure 8. These results were derived from incubations with a) variable $NADP^+$ and fixed, saturating mannitol concentrations and b) variable mannitol

Values of Km for NADPH were determined under conditions of fixed fructose concentrations in various 50mM buffers at various pH's. See Methods Section 2.2.4. for details.

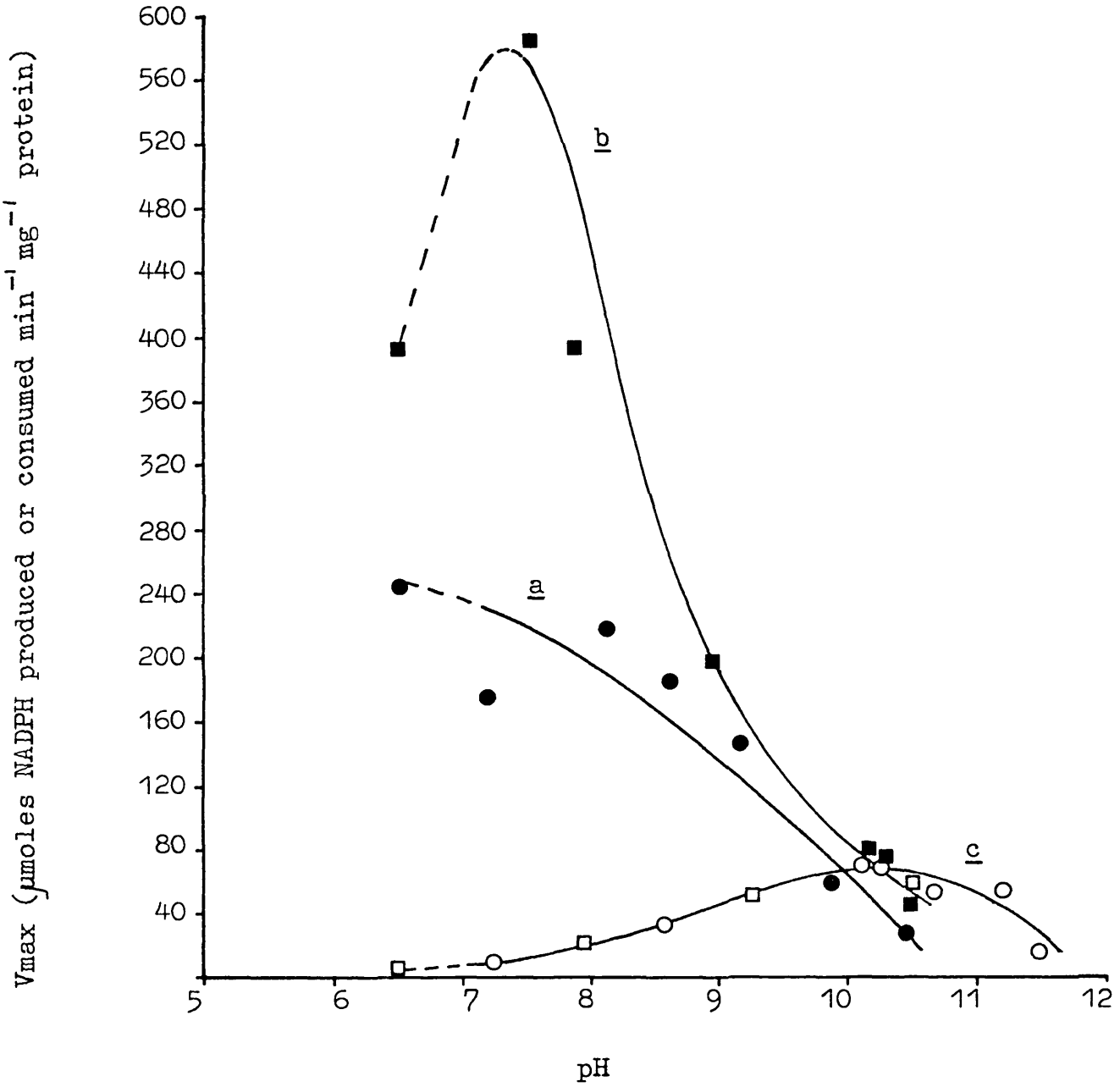
Figure 7: Variation of Km for NADPH with varying pH.



Values of Vmax were determined for a) fructose reduction under conditions of fixed fructose concentration with variable NADPH concentration (●), b) fructose reduction under conditions of NADPH saturation with variable fructose concentration (■), c) mannitol oxidation under conditions of mannitol saturation and variable $NADP^+$ concentration (○) and under conditions of $NADP^+$ saturation and variable mannitol concentration (□).

See Methods Section 2.2.4. for details.

Figure 8: Variation of Vmax for fructose reduction and mannitol oxidation with varying pH.



and fixed, saturating $NADP^+$ concentrations (2.2.4.); the results from the two methods fell on the same curve. A Vmax of 70.2 μmoles mannitol oxidised min^{-1} mg^{-1} protein, at an optimum pH of 10.25, was obtained.

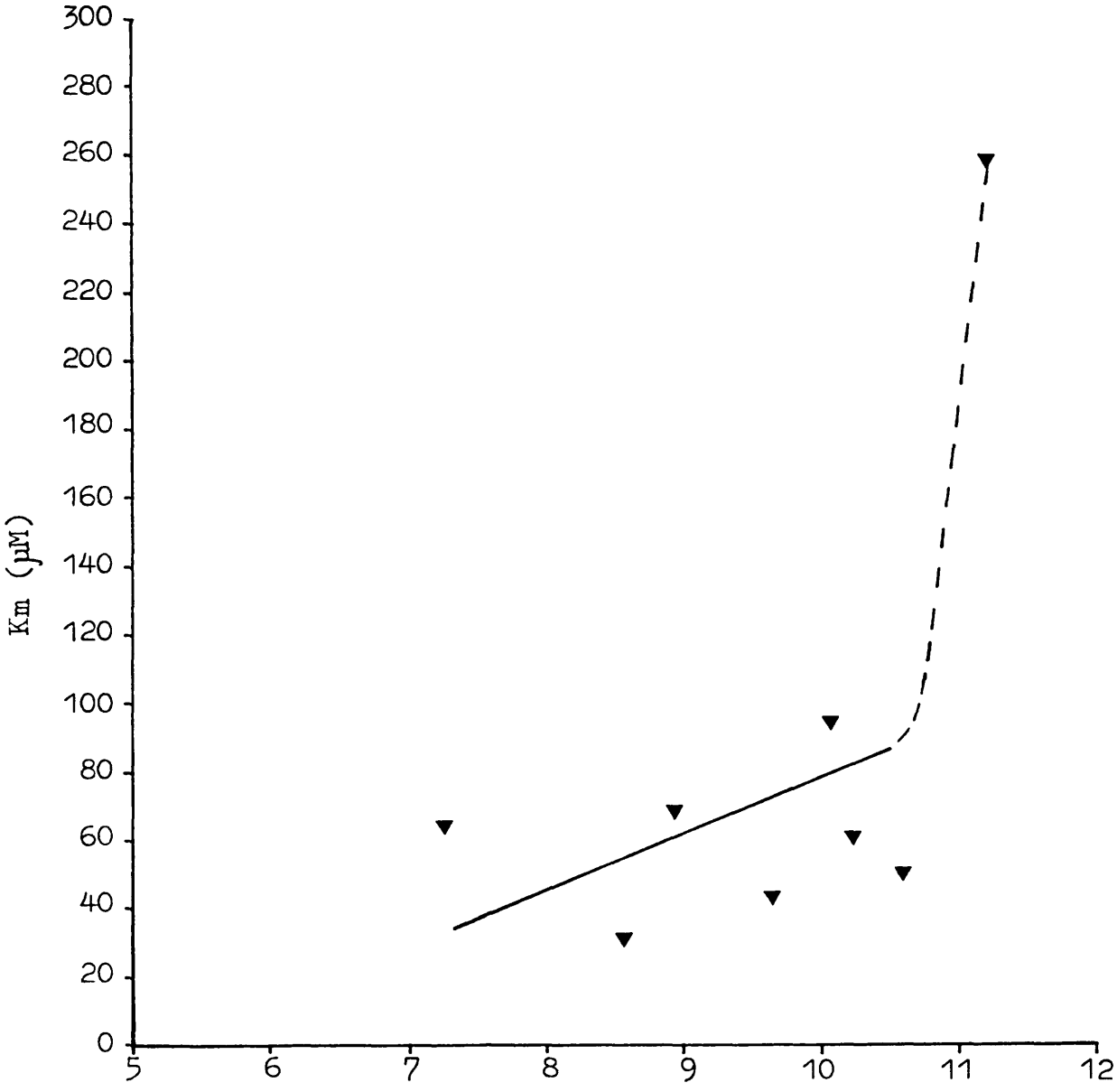
The apparent Km for mannitol was found to be approximately constant at 16.2mM in the range pH6.5 to 10.5. Figure 9 shows the variation of apparent Km for $NADP^+$ with varying pH; the gradient of the straight line in the region of the plot up to pH10.5 was calculated by regression analysis. Once again, the gradient of this straight line is not significantly different from zero when analysed by the "t-test". The steep rise in Km above pH10.5 may have been due to alkaline denaturation. Concentrations of $NADP^+$ above 1.5mM were shown to cause substrate inhibition of the reaction.

3.1.6. Effect of Antagonistic Form of Coenzyme.

Figure 10 shows the effect of added $NADP^+$ on the rate of reduction of fructose at pH7.5 under conditions of a fixed fructose concentration of 800mM and a fixed non-saturating concentration of NADPH (2.2.4.). Added $NADP^+$ inhibited the reaction and an $NADP^+$/NADPH ratio of approximately 38:1 was required for 50% inhibition of the reaction.

Figure 11a shows the effect of added NADPH on the rate

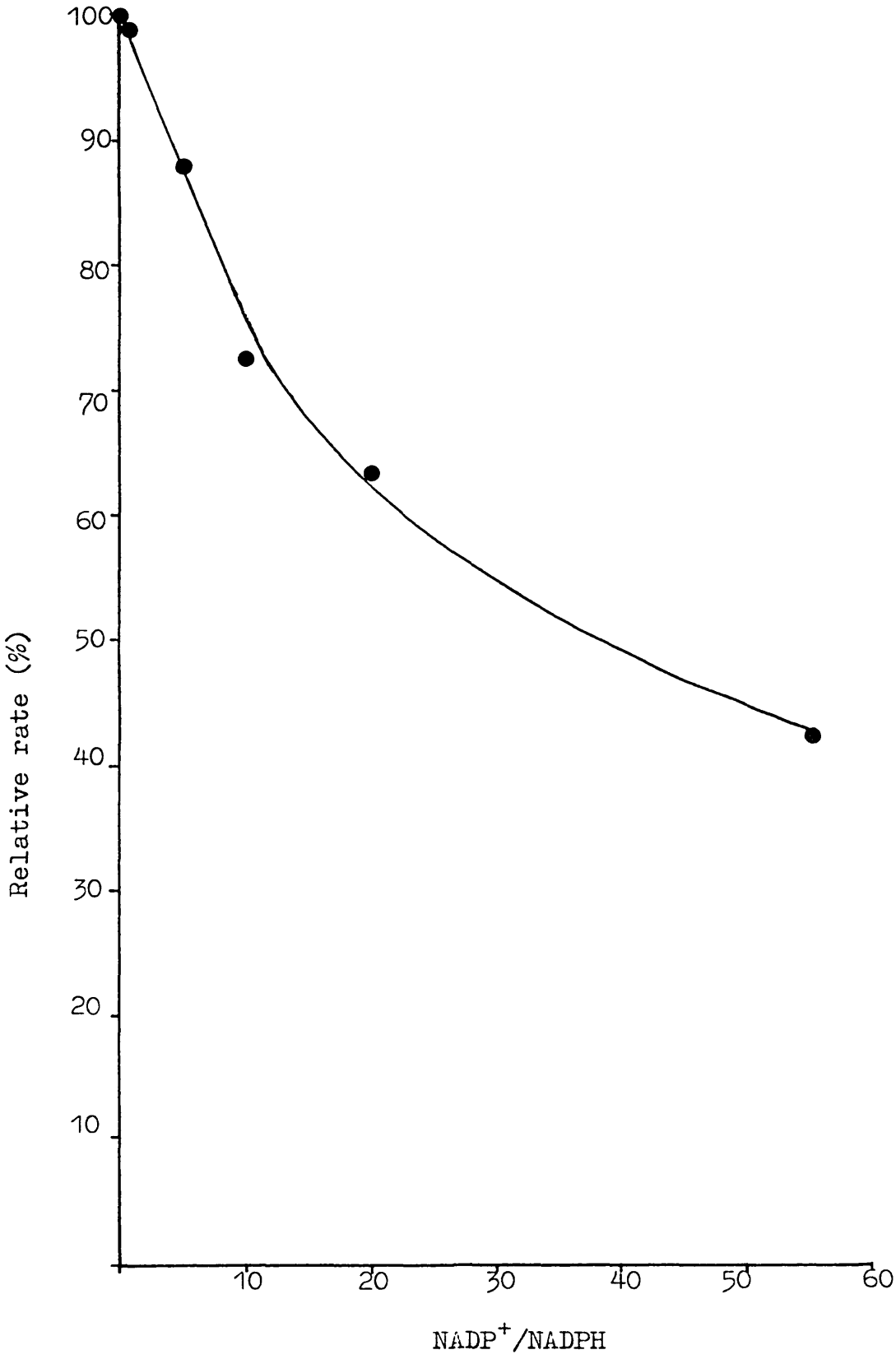
Figure 9: Variation of Km for $NADP^+$ with varying pH.



Values of Km for $NADP^+$ were determined under conditions of mannitol saturation in various 50mM buffers at various pH's. See Methods Section 2.2.4. for details.

The effect of added $NADP^+$ on the rate of fructose reduction with NADPH was determined at pH7.5 with saturating concentrations of fructose (800mM) and non-saturating concentrations of NADPH (6.9μM). See Methods Section 2.2.4. for details. The rates are expressed as percentages relative to the rate of reaction under the same conditions with an initial $NADP^+$ concentration of zero.

Figure 10: Effect of added $NADP^+$ on rate of fructose reduction.



of oxidation of mannitol at pH10.4 (near the optimum pH for this reaction) under conditions of saturating mannitol. The graph shows results for the reaction (i) under conditions of saturating $NADP^+$, i.e. the rate of reaction was not limited by the concentration of $NADP^+$, and (ii) under conditions of limiting, non-saturating $NADP^+$ (2.2.4.). The added NADPH inhibited the rate of mannitol oxidation and this effect was much more marked under conditions of $NADP^+$ saturation; under these circumstances 50% inhibition of the reaction was apparent with an NADPH/$NADP^+$ ratio of 0.45:1 whereas this ratio was increased to 4.6:1 when the $NADP^+$ was non-saturating.

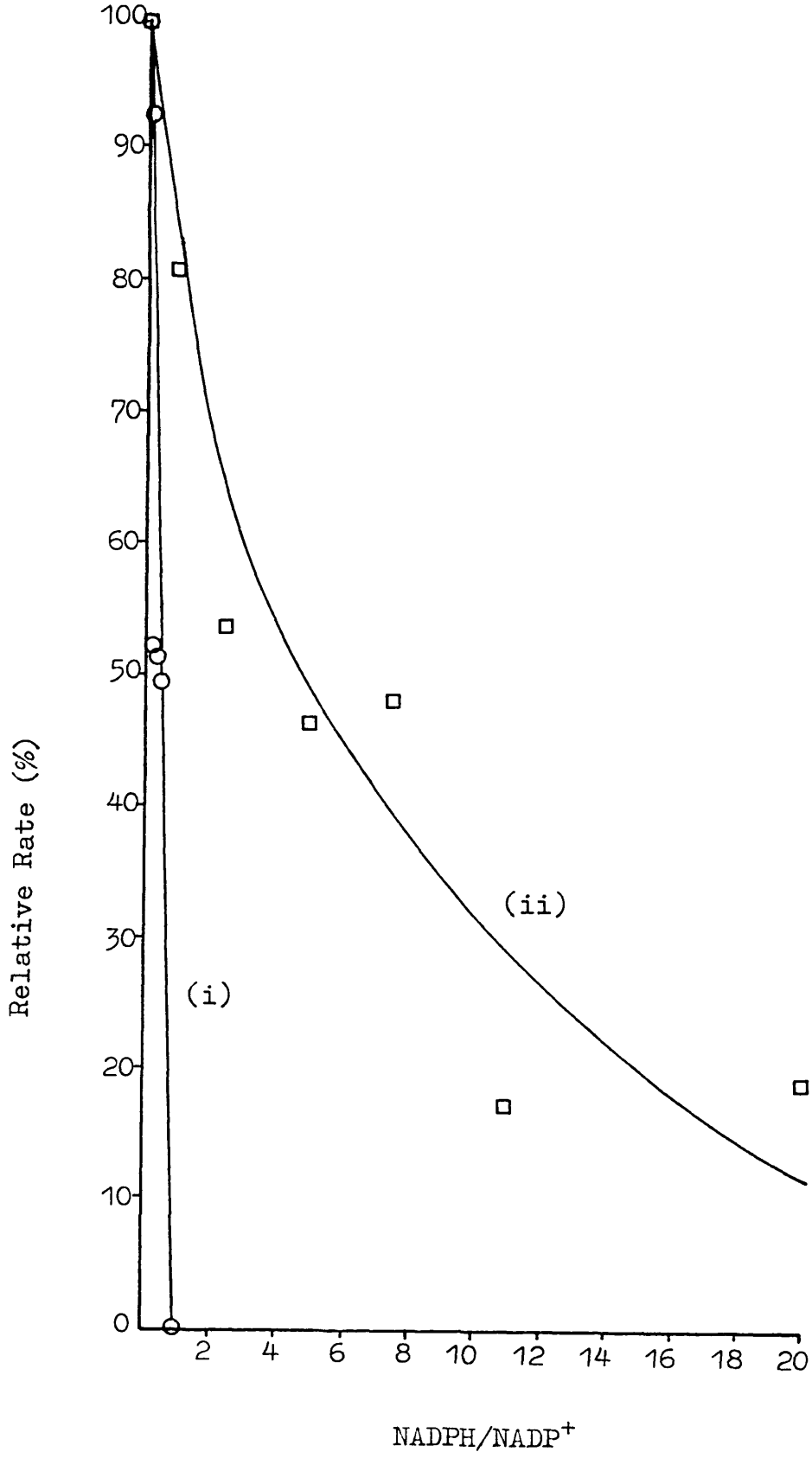
Figure 11b shows the results of the experiment repeated at pH7.5, nearer physiological pH. Similar differences were seen between conditions of saturating (excess) $NADP^+$ and non-saturating (rate limiting) concentrations as seen at pH10.4 (above). However, the inhibitory effect of NADPH was generally greater at the lower pH; an NADPH/$NADP^+$ ratio of 0.1:1 under conditions of $NADP^+$ saturation and of 0.45:1 under conditions of limiting $NADP^+$ concentrations gave 50% inhibition.

3.1.7. Inhibition of Mannitol Dehydrogenase by Iodoacetate and Mercuric Chloride.

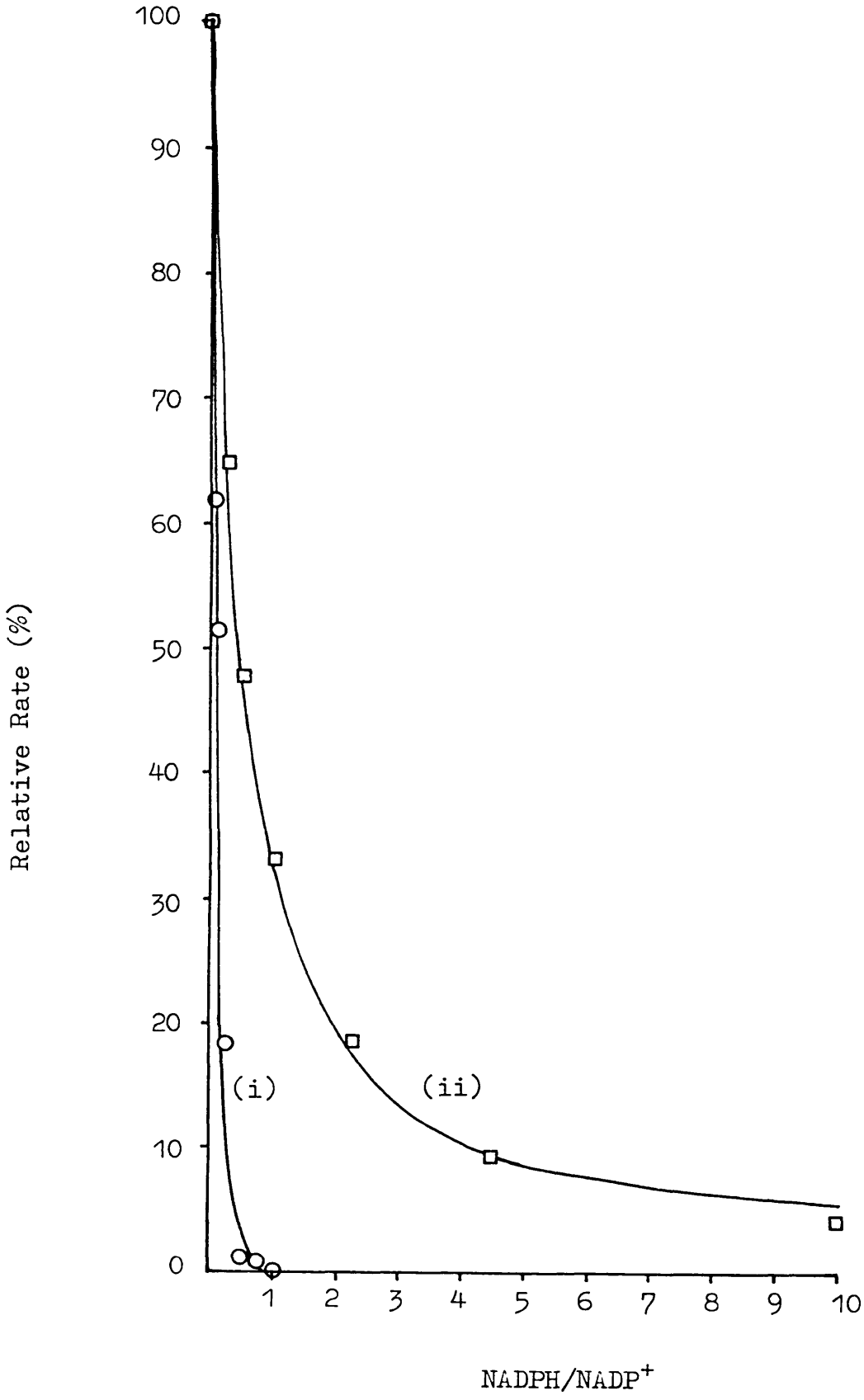
Preincubation of the enzyme for 30 min with either 100mM iodoacetate or 100mM mercuric chloride (2.2.4.) gave at

The effect of added NADPH on the rate of mannitol oxidation with $NADP^+$ was determined at a) pH10.4 and b) pH7.5 with a saturating concentration of mannitol (200mM). Results are plotted of experiments using $NADP^+$ concentrations which were (i) saturating at 1000μM (○) and (ii) non-saturating at 20μM (□). See Methods Section 2.2.4. for details. The rates are expressed as percentages relative to the rate of reaction under the same conditions with an initial NADPH concentration of zero.

Figure 11: Effect of added NADPH on rate of mannitol oxidation.



(a)


100
90
80
70
60
50
40
30
20
10
0
Relative Rate (%)
(i)
(ii)
1
2
3
4
5
6
7
8
9
10
NADPH/NADP+

(b)

least 95% inhibition of the rates of both mannitol oxidation and fructose reduction. The experiment was repeated with iodoacetate at a concentration of 10mM. The rate of fructose reduction by enzyme preincubated in this way, relative to enzyme preincubated in buffer alone, decreased with time as detailed in Table 8. The inhibitory effect of iodoacetate (at either 10mM or 100mM) was not overcome by including fructose (800mM) or NADPH (0.25mM) in the preincubation.

3.1.8. Determination of Keq for Mannitol Dehydrogenase.

Table 9 quotes the initial and final concentrations of reactants and products for the reaction

$$\text{Mannitol} + \text{NADP}^+ \rightleftharpoons \text{Fructose} + \text{NADPH} + \text{H}^+$$

allowed to approach equilibrium as detailed in Methods Section 2.2.5. The final concentrations obtained give a value of 1.99×10^{-11}M for Keq for the reaction.

3.1.9. Determination of Overall Cellular pH.

The pH of the supernatant of crude homogenate (2.5.7.) of sporophores harvested at stages 2-6 (1.3.) was found to be in the range 6.1 to 6.5.

Table 8: Effect of preincubating mannitol dehydrogenase in 10mM iodoacetate on rate of fructose reduction.

Time of preincubation (min)	Rate of fructose reduction (as percent of rate of mannitol dehydrogenase incubated in buffer alone)
0	101
30	97
100	88
300	43
500	34

See text for details.

Table 9: Concentrations of reactants and products used to determine Keq for mannitol dehydrogenase.

Substance	Initial concentration (M)	Final concentration (M)
Mannitol	5.000×10^{-4}	4.696×10^{-4}
$NADP^+$	3.691×10^{-5}	6.52×10^{-6}
Fructose	0	3.039×10^{-5}
NADPH	0	3.039×10^{-5}
H^+	6.607×10^{-11}	6.607×10^{-11}

See Methods Section 2.2.5. for details.

3.2. Discussion.

The purification procedure used was successful in yielding a nearly 100% pure preparation of mannitol dehydrogenase from *A. bisporus* sporophores. It seems likely that inactive enzyme was present in the crude extract from these frozen slices as fresh mushrooms gave a crude extract some 3 to 5 times higher in mannitol dehydrogenase activity on an activity per unit soluble protein basis (6.1.2.); possibly the freezing process had denatured some of the protein but this was removed from the extract during the purification, expecially at the Matrēx Gel Red A stage which acted as an affinity analogue.

The enzyme was found to have a subunit molecular weight of 29,500 and a native molecular weight of 115,000 was estimated by gel filtration. The gel filtration result is consistent with values of 130,000 and 113,500 for the molecular weight of the native enzyme as estimated by rate zonal centrifugation and band sedimentation ultra-centrifugation respectively (see Appendix I). Results therefore suggest the native enzyme is a tetramer with a subunit molecular weight of 29,500; this differs significantly from the value of 40,000 for the molecular weight of the native enzyme found by Ruffner *et al* (1978) using the technique of thin layer gel filtration. At present, it is not possible to account for the observed differences.

Purified mannitol dehydrogenase was shown to be highly specific towards NADP(H) as coenzyme (Tables 6a and b) and this agrees with the findings of Ruffner et al (1978), as does the requirement for D-fructose as hexose (Table 6a). However, there is some difference between results found here and those reported by Ruffner et al for polyol specificity. No detectable activity was found with L-arabitol and activity with D-sorbitol was 7.7% of that with D-mannitol; this contrasts with figures of 2% and 21% for L-arabitol and D-sorbitol respectively found by the above workers. However, since there have been no reports of significant levels of polyols other than mannitol in A. bisporus, it is unlikely that the activity of mannitol dehydrogenase against D-sorbitol is of importance under physiological conditions.

Preincubation of the mannitol dehydrogenase enzyme in buffers below pH6.5 led to inactivation of the enzyme and this inactivation effect increased with decreasing buffer pH (3.1.3.). This was presumably due to irreversible denaturation of the enzyme. There may also have been reversible denaturation in the pH range 6.5 to 7.0, possibly combined with a limited but variable amount of irreversible denaturation. Denaturation is reflected by the dramatic variation in kinetic parameters in this region; viz., a sharp drop in the Km for fructose (Figure 6), a sharp rise in the Km for NADPH (Figure 7) and a sharp drop in the Vmax for fructose as determined with varying

fructose concentrations (graph "b", Figure 8). Further possible denaturation effects were shown at high pH, above 10.5; viz., a rise in Km for $NADP^+$ in this region (Figure 9) and an irregular variation of Vmax for mannitol oxidation with pH such that the fall in Vmax was much sharper above the optimum (10.25) than below it (graph "c", Figure 8).

In the range pH7 to 10.5, it is reasonable to assume pH effects were reversible effects on ionisable groups in the enzyme molecule. In considering these pH effects, it is necessary to discuss the experimental conditions under which the effects were observed. The reaction in either direction requires both coenzyme and carbohydrate reactants. To study the effect of varying concentration of one reactant on the enzymic reaction, and to calculate apparent kinetic parameters for that reactant, the second reactant is normally set at a "saturating" concentration in the incubation mixture. Under such conditions the reaction velocity should be independent of the concentration of the "saturating" reactant, i.e. zero order, and hence show pseudo first order kinetics with respect to the reactant that is being varied in concentration. Abbreviating substrate concentration as [S], Roberts (1977) states that, "When $[S] > 100Km$ the deviation from zero order kinetics is less than 1%; even when $[S] > 10Km$ the deviation from zero order kinetics is only 9%". An approximation to zero order kinetics by this definition was achieved with respect to mannitol in

the study of the effects of varying $NADP^+$ concentration on the rate of mannitol oxidation, with respect to $NADP^+$ in the study of the effects of varying mannitol concentration on the rate of mannitol oxidation and with respect to NADPH in the study of the effects of fructose concentration on the rate of fructose reduction; the relevant data for establishing these approximations are summarised in Table 10. However, Table 10 also shows that the approximation to zero order kinetics as outlined above was not achieved when fructose was held at a constant concentration of 800mM in the study of the effects of NADPH on the rate of fructose reduction. Despite this, each reaction in this series of experiments was apparently first order with respect to NADPH; plots of Log_e [NADPH] against time gave a straight line. Values for initial rate of reaction for each incubation were plotted against concentration on Lineweaver-Burk plots; results fell on a straight line and approximate values of the apparent Km for NADPH and the apparent Vmax for fructose reduction could, therefore, be extrapolated from these plots (3.1.5.). The values obtained for apparent Km for NADPH were confirmed by plotting results on an Eadie-Hoftsee plot; in the region pH7 to 10, apparent Km's were between 14 and 21μM when extrapolated from Lineweaver-Burk plots (Figure 7) and between 10 and 24μM when extrapolated from Eadie-Hoftsee plots (not shown). Apparent values of Vmax for fructose reduction obtained from these experiments with NADPH as the variable reactant (graph "b", Figure 8) are lower than those obtained from experiments

Table 10: Constant experimental concentration and Km of coreactant used in the study of the effect of variable concentration of each reactant on the rate of the mannitol dehydrogenase reaction.

Reactant under study	Coreactant	Concentration of coreactant used in all experiments, mM (x)	Km of coreactant in pH range 7 to 10.5, mM (y)	x/y
$NADP^+$	Mannitol	200	16.2	12.3
Mannitol	$NADP^+$	1.0	0.036-0.086	27.8-11.6
Fructose	NADPH	0.25	0.0173-0.0192	14.5-13.0
NADPH	Fructose	800	1230-120	0.65-6.7

with fructose as the variable reactant (graph "a", Figure 8); the use of "non-saturating" fructose in the former series of experiments must account for this difference. There is, however, a similarity in the shapes of the graphs ("a" and "b", Figure 8) obtained by the two methods; the effects of pH on the reaction are the same under either set of experimental conditions, only the absolute values of Vmax are altered by altering the variable reactant (above).

There is also a similarity in shape between the two graphs above (graphs "a" and "b", Figure 8) and the graph obtained for the variation of Km for fructose with varying pH (Figure 6); the latter shows a rise in Km to a maximum around pH7.5 as the pH falls. The pKa values determined from these graphs are in the range pH8 to 9. A pKa in the same region is obtained from graph "c" in Figure 8 for variation in Vmax for mannitol oxidation with varying pH, but this graph, in contrast to those discussed above, shows a rise with pH from pH7 to an optimum of pH10.25. The similarity of pKa values in the range pH8 to 9 suggests that ionising cysteine groups are involved in the enzyme's mechanism of action. The inhibition by iodoacetate and mercuric chloride (3.1.7.) also indicates that the enzyme may contain essential thiol residue(s). However, it is not possible to conclude firmly that cysteine residue(s) are involved at the catalytic and/or binding sites of the enzyme since protection by either fructose or NADPH from inhibition by iodoacetate is not achieved (3.1.7.). Also

inhibition was slow with a relatively low concentration of iodoacetate (10mM compared to 100mM) indicating that the inhibitor might be attacking group(s) remote from the active site. Studies of other dehydrogenases have frequently failed to confirm that a pKa in the region pH8 to 10 is due to the ionisation of cysteine residue(s) directly involved in coenzyme binding. For example, Dalziel (1975) reported that the affinity for NADH of horse liver alcohol dehydrogenase and dogfish lactate dehydrogenase decreased in the range pH8 to 10 and implicated the involvement of a thiol group. However, this was inconsistent with crystallographic data and so Dalziel proposed that the observed pH effect on coenzyme binding was due to ionisation of Tyrosine 85 in dogfish lactate dehydrogenase and deprotonation of Arginine 47 in horse liver alcohol dehydrogenase. Furthermore, carboxymethylation of the cysteine residues at the active site of liver alcohol dehydrogenase, Cysteines 46 and 174, does not prevent coenzyme binding (Branden et al, 1975). However, these cysteine residues appear to have a role at the active site, namely as zinc chelators (Branden et al, 1975). Sogin and Plapp (1976) found that diazonium-1H-tetrazole (DHT) inhibited horse liver alcohol dehydrogenase and selectively attacked Cysteine 174; these workers suggested inhibition occurred because Cysteine 174 is involved in catalysis, or because modification of this residue by the large negatively charged tetrazole ring of DHT inactivates the enzyme due to steric hinderance of substrate binding.

Yeast alcohol dehydrogenase also contains two especially reactive cysteine residues; the modification of these residues inactivates the enzyme and these cysteines seem analogous to residues 46 and 174 in horse liver alcohol dehydrogenase and are involved in the coordination of a zinc atom (Branden et al, 1975; Klinman, 1975). In contrast modification of Cysteine 165 of pig heart lactate dehydrogenase alters the kinetics of the binding of pyruvate or lactate but this residue is not located at the active site; modification could cause a conformational change in this enzyme which affects the active site structure (Holbrook et al, 1975; Bloxham et al, 1979).

In the present studies, the values of Km for mannitol, $NADP^+$ and NADPH remained approximately constant in the pH region 7 to 10.5. The values of Km for $NADP^+$ and NADPH with changing pH can be considered a constant for purposes of studying the enzyme's mode of action. A constant Km with pH but a varying Vmax is indicative of an enzyme-substrate binding system where the pKa values of any ionisable group(s) on the free enzyme involved in the system are the same as the pKa values of those group(s) in the enzyme-substrate complex; H^+ ions can affect the rate of reaction but not the binding of the substrate and hence act as non-competitive inhibitors of the reaction (Roberts, 1977; see Appendix II). Hydrogen ions act as non-competitive inhibitors of both mannitol oxidation and fructose reduction as shown by the variation of Vmax for each of these reactions with pH with the H^+ ions not competing with mannitol, $NADP^+$ or

NADPH. However, as described above, the Km for fructose does vary with pH and so detailed analysis of the enzyme mechanism becomes more complicated and requires a knowledge of whether the binding of reactants is ordered and hence whether carbohydrate or coenzyme binds first; isotope exchange studies have been used as evidence for the existence of compulsory binding orders and were introduced by Silverstein and Boyer (1964). These workers followed the rate of interconversion of radioactively labelled NAD^+ and NADH and of radioactively labelled lactate and pyruvate for the lactate dehydrogenase reaction at equilibrium;

$$\text{Lactate} + NAD^+ \rightleftharpoons \text{Pyruvate} + NADH + H^+.$$

Silverstein and Boyer found that, with rabbit muscle and bovine heart lactate dehydrogenases under specific conditions of pH, an increase in the lactate-pyruvate concentration while maintaining equilibrium resulted in a continued increase in the interconversion of lactate and pyruvate, but in a rise followed by a fall to near zero in the rate of interconversion of NAD^+ and NADH. This fall was interpreted as indicating the existance of a compulsory binding order with the presence of NAD^+ or NADH essential for the binding of lactate or pyruvate respectively. Other isotope exchange studies have yielded evidence for different mechanisms (Dalziel, 1975); for example, with horse liver and yeast alcohol dehydrogenase at pH7.9, bovine heart lactate dehydrogenase at pH9.7 and malate dehydrogenase

at pH9.0, coenzyme isotope exchange continues at a finite rate at high reactant concentrations but this rate is much less than the reactant exchange rate; this indicates that the mechanism is not totally random and is ordered in at least one direction. Some reactions have been shown to approximate to a random mechanism, e.g. bovine liver glutamate dehydrogenase (Dalziel, 1975).

The values for Km for carbohydrate and coenzyme reactants found here show some difference from those observed by Ruffner *et al* (1975) for the *A. bisporus* enzyme and by Edmundowicz and Wriston (1963) for the *A. campestris* enzyme. The differences are small and within an order of magnitude for the Km for coenzymes; 17μM (Table 6) compared with 16μM (Edmundowicz and Wriston) and 5.25μM (Ruffner *et al*) for NADPH, and 82μM (Table 6) compared with 22μM (Edmundowicz and Wriston) for $NADP^+$. With the technical difficulties involved in working with such low concentrations, these differences in coenzyme Km are not of real significance. The Km for mannitol found here (16.2mM; section 3.1.5.) is approximately half that observed by Ruffner *et al* (34mM) and approximately thirty-five times the value of 0.46mM observed by Edmundowicz and Wriston; the reason for the large difference with the latter result may be related to the use of only partially purified preparations of mannitol dehydrogenase from *A. campestris* by these workers. However, the major difference in values between all three sets of results is in the Km value for fructose; 1.2M determined

here (3.1.5.), 1.4mM reported by Edmundowicz and Wriston and 190mM reported by Ruffner *et al*. The reasons for the differences are difficult to explain but the method of enzyme preparation and degree of purification may again be influential in determining this kinetic parameter. The result observed here with highly purified enzyme is not without precendent for the same enzyme type from other fungi. For example, a similar low affinity for fructose with a Km of 1.0M was reported for the mannitol dehydrogenase of *Cephalosporium chysogenium* (Birken and Pisano, 1976). The high Km for fructose (1.2M) observed here for *A. bisporus* indicates the rate of mannitol production will be directly proportional to the concentration of fructose since its concentration in sporophore cells is of the order 0.5mM (Hammond and Nichols, 1975; see below). Conversely, oxidation will occur at a rate almost independent of mannitol levels in the sporophores since the Km (16.2mM) is considerably lower than the range of concentrations (63-195mM) calculated for mannitol in sporophore cell water (Hammond, 1977). Thus, the physiologically important reaction, namely fructose reduction, is subject to far greater rate control by substrate concentration than the reverse reaction.

Ruffner *et al* (1978) placed emphasis on the sensitivity of the mannitol dehydrogenase reaction to the $NADP^+$/NADPH ratio; they found a ratio of 4:1 gave 50% inhibition of the rate of fructose reduction. Some sensitivity of the

rate of fructose reduction to $NADP^+$ inhibition was observed here though relatively larger amounts of $NADP^+$ were required to give 50% inhibition; the ratio was found to be approximately 38:1 (Figure 10). Comparison of this ratio with the approximate 1:1 ratio estimated to exist *in vivo* in the *A. bisporus* sporophore (Hammond, unpublished data) indicates that the inhibitory ratio is unlikely to occur in the whole sporophore cell though it might occur if compartmentalisation exists. Greater sensitivity was shown by the rate of mannitol oxidation to NADPH concentration. The reaction was inhibited by NADPH but the degree of inhibition depended on the level of $NADP^+$ and on pH (Figures 11a and b). Inhibition was far more marked with an initial concentration of $NADP^+$ which was saturating at 1mM; however, the inhibition at these levels of $NADP^+$ and NADPH is probably due to a total coenzyme effect as $NADP^+$ concentrations above 1.5mM caused inhibition without added NADPH (3.1.5.). Furthermore, the concentrations of both $NADP^+$ and NADPH have been estimated in crude extracts of *A. bisporus* to be in the region 50 to 170μM (Hammond, unpublished data). At an $NADP^+$ concentration of 20μM, the rate of mannitol oxidation was halved by a ratio of NADPH/$NADP^+$ of 4.6:1 at pH10.4 and 0.45:1 at pH7.5; sensitivity will probably increase if $NADP^+$ concentrations are higher than 20μM (above). Excluding any consideration of substantial compartmentalisation of H^+ ions within the *A. bisporus* cell, the higher sensitivity of the enzyme to NADPH at the lower pH is of greater importance as this pH is closer

to the overall physiological pH of the cell, pH6.1 to 6.5 (3.1.9.). The oxidation of mannitol must, therefore, be very sensitive to changes in the redox status of the coenzyme under normal physiological conditions of pH and coenzyme concentration.

The redox status of the coenzyme may be related to the level of operation of the pentose phosphate pathway. Strobel and Kosuge (1965) monitored the mannitol content and the activity of mannitol dehydrogenase and glucose-6-phosphate dehydrogenase during the growth in culture of the grape-rotting fungus, Diplodia viticola. They found that mannitol accumulated in young cultures and then disappeared again as the culture ages. The level of mannitol was more closely related to the activity of glucose-6-phosphate dehydrogenase than to the activity of mannitol dehydrogenase; this suggests that the production of mannitol is limited by the supply of NADPH from the pentose phosphate pathway in this organism. Dütsch and Rast (1972) have shown from tritium labelling experiments that NADPH produced by oxidation of glucose at the C-1 or C-3 position, such as would occur in the pentose phosphate pathway, was reoxidised by the reduction of fructose to mannitol; they concluded that the rate of turnover of the pentose phosphate pathway could be limited by the supply of $NADP^+$ formed in the production of mannitol. Hammond (1977) reported a higher rate of activity of the pentose phosphate pathway in the sporophore than in the mycelium

of A. bisporus; he calculated that the increased activity of this pathway may be just great enough to produce the NADPH required for synthesising the increased level of mannitol in the sporophore. The pentose phosphate pathway in the A. bisporus sporophore may provide sufficient NADPH to promote mannitol synthesis and to inhibit mannitol oxidation. It would only be under conditions of low NADPH, relative to $NADP^+$, that mannitol oxidation could occur at a significant rate. Such conditions might occur after the sporophore has been harvested or during senescence when mannitol levels in the sporophore are known to fall (Hammond and Nichols, 1975; 1976a). However, in the healthy, growing A. bisporus sporophore NADPH levels are relatively high and the enzyme will be active in the fructose reducing direction, producing the mannitol which is know to accumulate in the fruit-body (Hammond and Nichols, 1976a).

If all forms of in vivo limitations and control of mannitol dehydrogenase were removed, the reation would still tend towards the production of mannitol at physiological pH. This is demonstrated by the in vitro value for Keq of the mannitol dehydrogenase reaction (3.1.8.);

$$Keq = \frac{[Fructose]\ [NADPH]\ [H^+]}{[Mannitol]\ [NADP^+]} = 1.99 \times 10^{-11}\ M$$

This value is more than an order of magnitude smaller than the value of 5.0×10^{-10} M found by Edmundowicz and Wriston

(1963) with the partially purified enzyme from A. campestris. However, with either value the reaction is heavily displaced towards mannitol synthesis.

The equilibrium in the above reaction is strongly influenced by the concentration of H^+ ions. This can be illustrated by the following calculation:

$$Keq = \frac{[Fructose]\ [NADPH]\ [H^+]}{[Mannitol]\ [NADP^+]} = 2 \times 10^{-11}\ M$$

$$\therefore [Fructose] = \frac{2 \times 10^{-11}\ [NADP^+]\ [Mannitol]}{[NADPH]\ [H^+]}\ M$$

Assuming $[NADPH] = [NADP^+]$ in the A. bisporus sporophore (Hammond, unpublished data), then:

$$[Fructose] = \frac{2 \times 10^{-11}\ [Mannitol]}{[H^+]}\ M$$

Taking the example of stage 2 pileus tissue where Hammond (1977) has calculated the concentration of mannitol in the cell water to be approximately 0.175M, then:

$$[Fructose] = \frac{2 \times 10^{-11} \times 0.175}{[H^+]}$$

$$= \frac{3.5 \times 10^{-12}}{[H^+]}\ M$$

If a pH of 6.0 is assumed as a mean for the cell (3.1.9.)

then:

$$[\text{Fructose}] = \frac{3.5 \times 10^{-12}}{10^{-6}}$$

$$= 3.5 \times 10^{-6}\text{M}$$

$$= 3.5\mu\text{M}.$$

Alternatively, in the case where the pH in a specific area of the cell is pH10, a value close to the optimum for mannitol oxidation, then:

$$\text{Fructose} = \frac{3.5 \times 10^{-12}}{10^{-10}}$$

$$= 3.5 \times 10^{-2}\ \text{M}$$

$$= 35\text{mM}$$

Local variations in pH would thus affect the relative levels of fructose and mannitol in the cell. The above calculation assumes the reaction is at equilibrium and this may not be the case; reactant concentrations could be continually altered by transport of carbohydrate, coenzyme or H^+ ions; also, other reactions could continually alter the concentrations of the reactants of the mannitol dehydrogenase reaction. However, assuming the state of equilibrium, the pH of the cell in the environment of the enzyme must be considerably less than pH10 since fructose is present at only very low levels in the sporophore; Hammond and Nichols (1976a) did not consistently detect fructose in the growing mycelium or sporophore of A. bisporus and the same authors (1975) detected it at levels below

0.1% (w/v) of dry weight (equivalent to a concentration in the cell below 0.5mM by the conversion of Hammond (1977)) in stored sporophores. Whilst the pH of the enzyme's enviroment is probably much lower than pH10, it seems unlikely that mannitol dehydrogenase would occur in an area of the cell that has a pH similar to the mean for the whole cell (6.0 to 6.5; section 3.1.9.) since this would cause irreversible inactivation (above; 3.1.3.). Mannitol dehydrogenase is thus affected by pH in terms of reaction equilibrium, rates of reaction and irreversible inactivation.

4. Non-specific Acid Phosphatase.

4.1. Results.

4.1.1. Enzyme Purification.

Table 11 summarises the purification of acid phosphatase (2.3.2.) from 240g of frozen sporophore slices harvested at stage 2 of development (1.3.). Polyacrylamide-gel electrophoresis of native samples (2.5.3.1a.) of the final preparation showed several bands when stained for protein (Plate 5a) and similar gels of both the final preparation (Plate 5a) and of crude extract (Plate 5b) showed one diffuse band when stained for acid phosphatase activity (2.5.4.4.). The final preparation contained a number of different proteins and it was not possible to assign the enzyme activity to a specific protein band on the gels as the stain for phosphatase activity gave a very diffuse band (Plate 5a). It is possible that there may have been more than one isoenzyme staining within the diffuse phosphatase band.

4.1.2. Kinetic Properties.

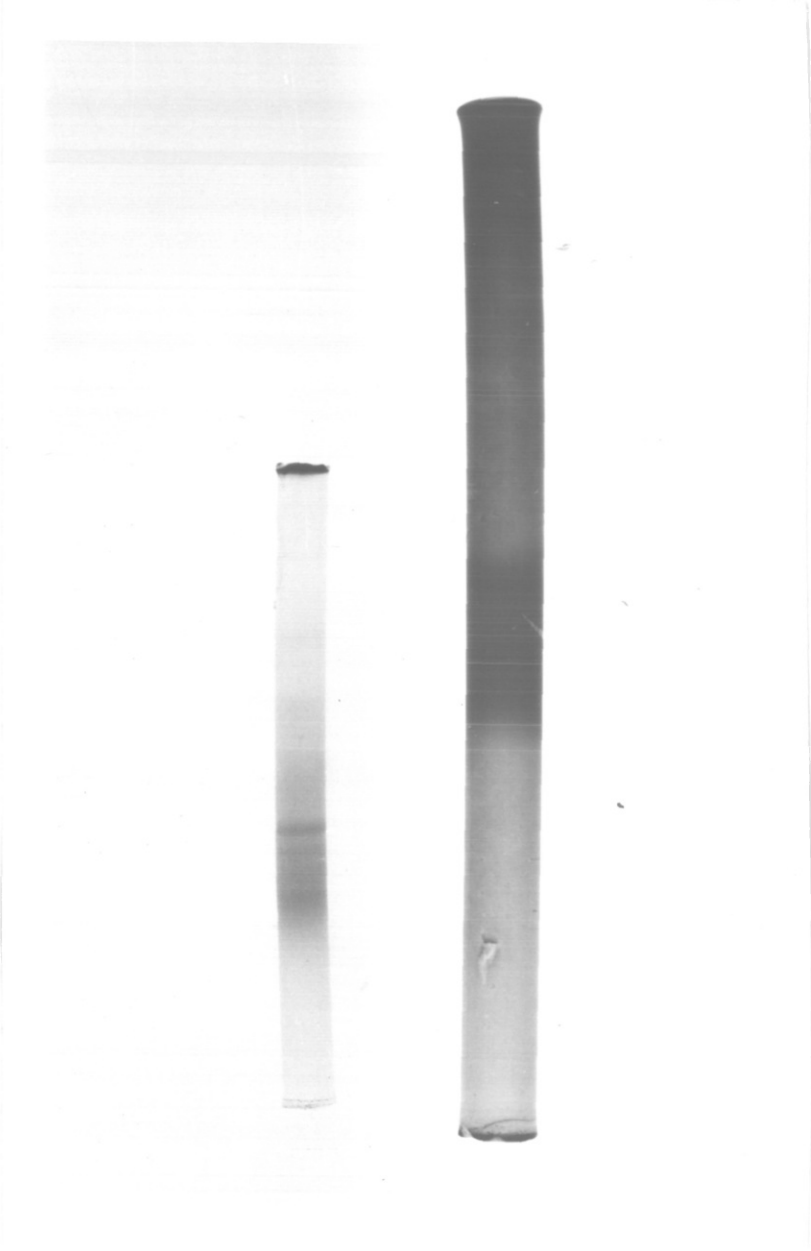
Figure 12 shows the pH activity profile of the enzyme with 10mM *p*-nitrophenylphosphate as substrate (2.2.7.). A pH optimum of approximately 4.65 was observed with a particularly steep fall in activity with decreasing pH below this value;

Table 11: Purification of non-specific acid phosphatase from 240g of frozen A. bisporus sporophore slices.

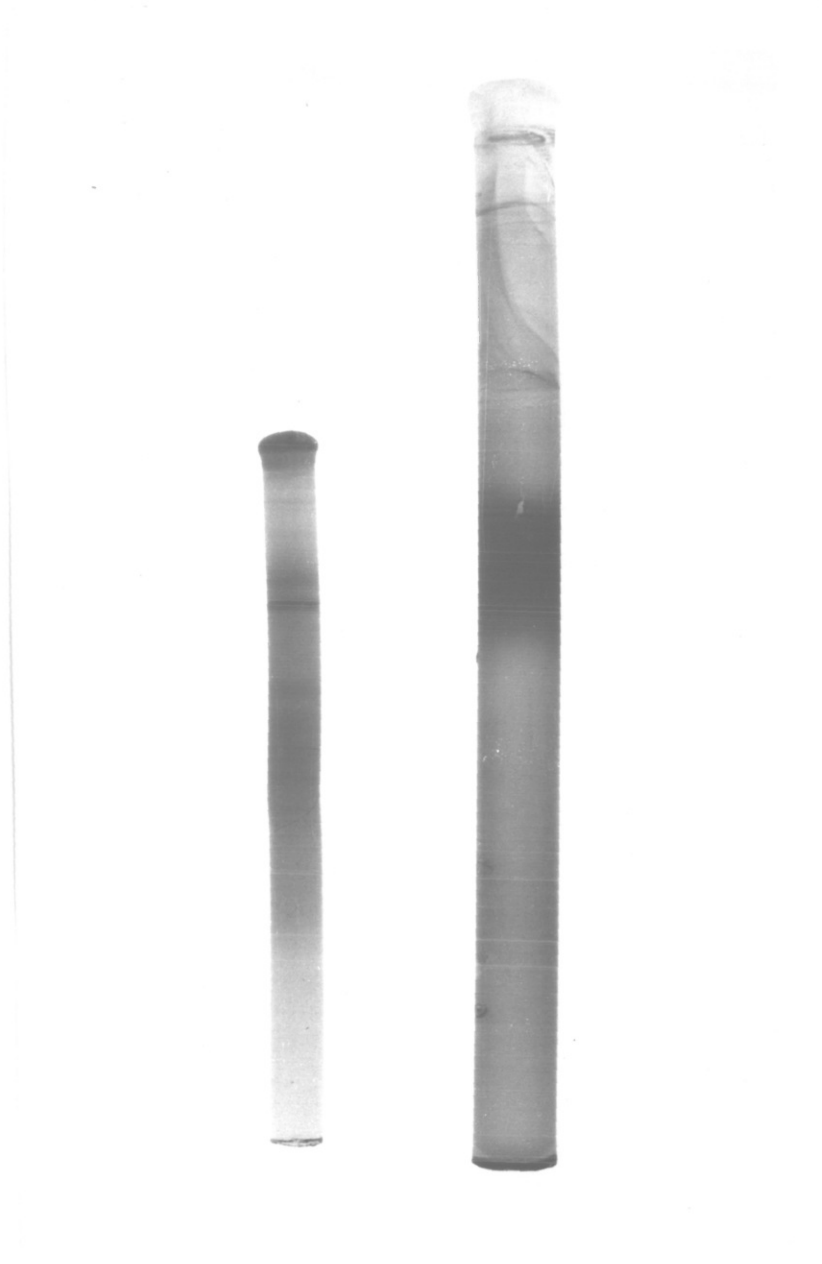
Stage	Volume (ml)	Protein (mg)	Total activity (Units x 10^{-3})	Specific activity (Units mg^{-1} protein)	Purification (Fold)	Yield (%)
Crude extract after centrifugation.	162	5,767	740	128	1	100
Protamine sulphate precipitated extract.	153	2,876	566	197	1.53	76
50%-80% saturated ammonium sulphate precipitate redissolved.	5.5	372	123	331	2.59	16.6
Eluate from Sephadex G100 column bulked and concentrated.	5.8	138	74.3	538	4.20	10.0
Eluate from DEAE-Sephadex A50 column bulked and dialysed.	12.2	20.3	45.4	2,236	17.47	6.14

See text for details. Units are as defined in section 2.2.6.1.

Plate 5: Polyacrylamide-gel electrophoresis of native samples of partially purified acid phosphatase preparation and of crude extract.



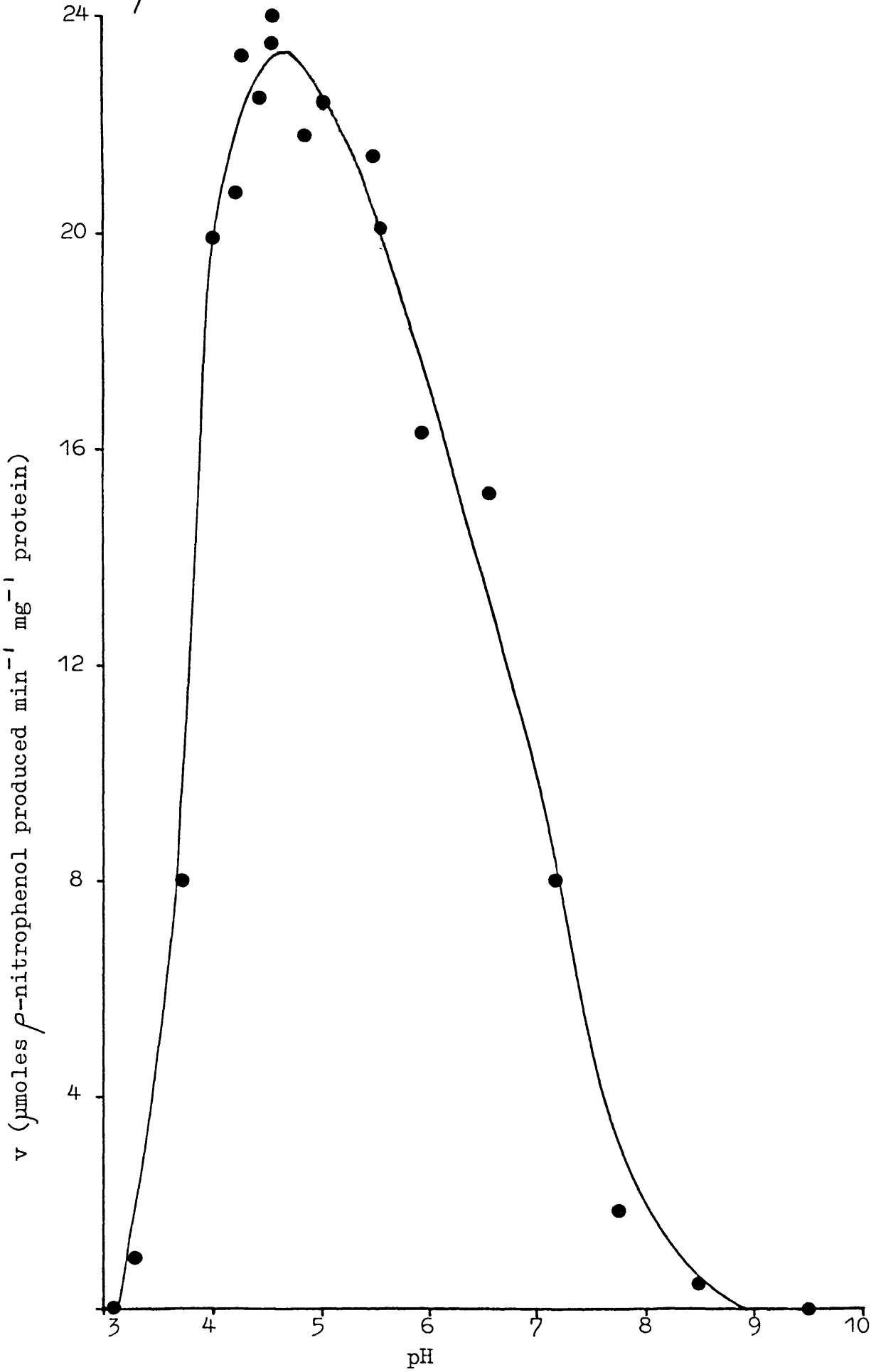
(a)



(b)

Partially purified acid phosphatase preparation (a) and crude extract (b) were subjected to electrophoresis at pH8.0 on 8% (w/v) polyacrylamide-gels (2.5.3.1a.). Gels were stained for protein (left) and acid phosphatase activity against fructose-6-phosphate with a 15 min incubation (right) as described in section 2.5.4.

Figure 12: Variation of acid phosphatase activity against ρ-nitrophenylphosphate with varying pH.



See text for details.

this may have been due to acid denaturation of the enzyme. The observed optimum pH was used to estimate the Km and maximum rate of reaction (Vmax) of the preparation with *p*-nitrophenylphosphate as substrate (2.2.8.). Preliminary experiments for assessing activity against sugar phosphates at various pH's were carried out using the routine assay of section 2.2.6.2. but incubating in the series of 50mM buffers used in section 2.2.7.; these indicated a pH optimum for activity against sugar phsophates of approximately 6.5. This observed optimum pH was used to determine values of Km and Vmax of the preparation with each of the following substrates; D-fructose-6-phosphate, D-fructose-1-phosphate, D-fructose-1,6-bisphosphate and D-glucose-6-phosphate (2.2.8.). Results are quoted in Table 12 and indicate a broad specificity for enzyme substrate.

Table 12: Kinetic constants of acid phosphatase preparation.

Substrate	Vmax (μmoles phosphate produced min^{-1} mg^{-1} protein)	Km (mM)
p-Nitrophenylphosphate	2.40	1.72
D-Fructose-6-phosphate	2.80	40.0
D-Fructose-1-phosphate	4.80	80.0
D-Fructose-1,6- bisphosphate	4.14	57.1
D-Glucose-6-phosphate	1.13	26.7

See text for details.

4.2. Discussion.

The enzyme had no high specificity for any sugar phosphate (Table 12) and must, therefore, be broadly classified as a non-specific acid phosphatase. It is unlikely that an enzyme with low specificity for fructose-6-phosphate would be regulated by cell metabolites, but the enzyme could be involved in the control of mannitol biosynthesis if it was not constitutively produced. Repressible acid phosphatases have been observed in *Aspergillus nidulans* (Dorn and Riviera, 1966) and *Neurospora crassa* (Nyc, 1967) but production of these enzymes was shown to be responsive to phosphate in the growth medium rather than a developmental stage in the life-cycle; this latter response is necessary for regulation at the phosphatase production level of mannitol synthesis in *Agaricus bisporus*. *Coprinus lagopus*, an agaric fungus closely related taxonomically to *A. bisporus*, produces an alkaline phosphatase which is repressible in the mycelium but not the sporophore (North and Lewis, 1971). Evidence is presented below (6.1.2.) which indicates that the levels of non-specific acid phosphatase in the *A. bisporus* cell do not vary significantly during sporophore development and that other fructose-6-phosphatase enzymes detected in the cell are more likely to have a specific role in mannitol biosynthesis; the broadly specified acid phosphatase enzyme doubtless has a more general role in the *A. bisporus* sporophore cell.

5. Fructokinase.

5.1. Results and Discussion.

Considerable difficulty was experienced in assaying for fructokinase activity in extracts from A. bisporus. No activity with crude extract could be detected using the assay linked to cresol red (2.2.9.3.; Darrow and Colowick, 1962). This method relies on a change of colour produced by changes in H^+ concentrations following ATP consumption, and the assay may have been unsuccessful for a number of reasons; low activity, interference from other coloured substances in the extract, interference from other reactions involving H^+ ions produced by fructokinase activity or a combination of these effects. Activity could be detected in crude extracts using the assay linked to phosphoglucose isomerase and glucose-6-phosphate dehydrogenase (2.2.9.1.) but a strong background reaction was found with ATP in the reaction mixture and with fructose omitted. The assay system involves the following series of reactions;

$$\text{Fructose} + \text{ATP} \xrightarrow{\text{Fructokinase}} \text{Fructose-6-phosphate} \xrightarrow[\text{isomerase}]{\text{Phosphoglucose}}$$

$$\text{Glucose-6-phosphate} \xrightarrow[\text{dehydrogenase}]{\text{Glucose-6-phosphate}} \text{6-phosphogluconate}.$$

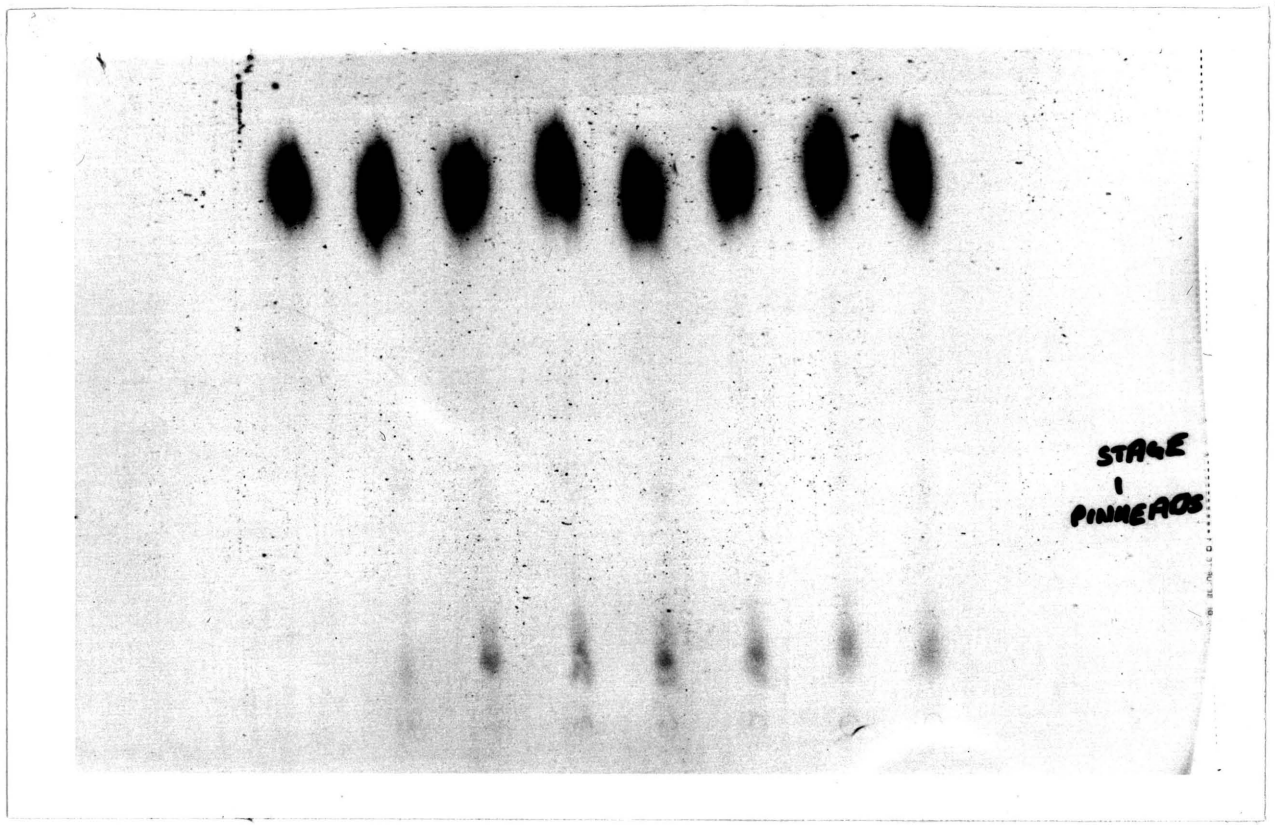
$$\text{NADP}^+ \longrightarrow \text{NADPH} + \text{H}^+$$

The assay was monitored by the detection of NADPH produced. The strong background reaction observed may have been due to the production of glucose-6-phosphate from glucose in the crude extract and the ATP added. This would require the presence of a hexokinase in the crude extract and kinase activity against glucose was detected in all preparations tested by the use of glucose in place of fructose in the above assay system and by the use of isotopically labelled glucose (2.2.9.2.). Any glucose-6-phosphate produced would be utilised in the reaction pathway above and oxidised to 6-phosphogluconate by the glucose-6-phosphate dehydrogenase enzyme. This would produce NADPH which is then measured spectrophotometrically. There are other possible reactions in the complex crude extract which could produce NADPH or NADH in response to the addition of ATP and result in the strong background reaction observed before the addition of fructose to the assay system. There was, however, a difference in the rate of NADPH production recorded as a background level and that recorded with either fructose or glucose added; this indicates that both fructokinase and glucokinase (above) activities were present in crude extracts. Nevertheless, the assay system was too complex for reliable use with crude extracts. The background reaction was not so significant in preparations after passage through a Sephadex G100 column, probably because the substrate(s) for the reaction had been removed by gel filtration. It was not possible to study the fructokinase reaction after dialysis to remove

substrate(s) responsible for the background reaction since dialysis against buffer for even a short period (4h) caused the loss of all detectable fructokinase activity. The background reaction may also have been removed by gel filtration because interfering enzymes had not copurified with the fructokinase. The glucose-6-phosphate dehydrogenase assay was used for assaying semi-purified preparations. The use of ^{14}C-labelled fructose gave the only conclusive demonstration of fructokinase activity in crude extracts (2.2.9.2.). Plate 6 shows an autoradiogram of chromatographed samples of an incubation of crude extract with ^{14}C-fructose, ATP and magnesium ions. Two samples were taken before the addition of ATP; one before the addition of crude extract and one 15 min later. The autoradiogram showed a background reaction by this method too, though of a different type to that described above; there was observable conversion of fructose to fructose-6-phosphate (as identified by markers; 2.5.6b.), possibly utilising ATP extracted from the sporophore and present in the undialysed extract. However, on addition of further ATP to the incubation there was more rapid phosphorylation of fructose. This could be quantified by liquid scintillation counting of the radioactive areas of the chromatogram and examples of such results are quoted in section 6.1.4. This method of assaying fructokinase was lengthy to perform but was probably the most reliable method of quantifying activity in crude extracts.

Plate 6: Autoradiogram of chromatographed samples of a fructokinase incubation.

D-U- ^{14}C-Fructose was incubated with crude extract of A. bisporus sporophores and samples were taken 0 (1) and 15 (2) min after addition of the extract. ATP was added and samples were taken after 1, 2, 4, 8, 20 and 40 min (3-8 respectively); details in section 2.2.9.2. The positions of fructose, fructose-6-phosphate and glucose-6-phosphate were located by markers (2.5.6b.) shown.



Fructose

Fructose-6-phosphate
Glucose-6-phosphate

The more rapid method of detecting activity, using the detection of NADPH linked to glucose-6-phosphate dehydrogenase (2.2.9.1.), was used for routine attempts at purifying the fructokinase. Despite the problems with this assay, results were conclusive enough to demonstrate that the fructokinase enzyme was highly labile and more than 70% of the activity was lost on storage at $4^{o}C$ overnight. Not surprisingly, this loss of activity was increased at room temperature. All activity was lost on dialysis (above) and on freezing the extract. Little or no activity could be detected in crude extracts of frozen sporophore slices (2.1.); it was essential to use fresh mushrooms (2.3.3.). The enzyme was not stabilised by addition of Mg^{2+}, ATP or fructose and was slightly less stable if mercaptoethanol was removed from extraction buffers.

The most successful attempt at purifying the enzyme (2.3.3.) is summarised in Table 13. Because of the instability of the enzyme the purification was completed as quickly as possible, hence the use of a batch process rather than a column process for ion exchange on Sephadex A50. However, activity was lost at each stage and this is shown by the specific activity of the bulked fractions from the void volume of the G200 column which were lower than the specific activity of the extract at the previous stage in the purification. The table shows that this specific activity was taken after the fractions had been bulked and stored at $4^{o}C$ overnight; the net specific activity

Table 13: Purification of fructokinase from 200g of fresh sporophores harvested at stage 2 of development.

Stage	Volume (ml)	Protein (mg)	Total Activity (units)	Specific activity (units mg^{-1} protein)	Purification (Fold)	Yield (%)
Crude extract after centrifugation	147	3258	63.9	0.020	1	100
Protamine sulphate precipitated extract	129	3491	50.6	0.015	0.75	79
55% saturated ammonium sulphate fraction.	7.0	620	25.6	0.041	2.05	40
Void volume fractions from Sephadex G100 column bulked and stored overnight.	40	285	8.71	0.031	1.55	13.6
2.0M sodium chloride fraction from DEAE-Sephadex A50 after concentration by ultrafiltration and addition of glycerol.	17.4	9.22	0.767	0.083	4.15	1.20

See text for details.

Units as defined in section 2.2.9.1.

of the active fractions prior to bulking and storage overnight was approximately 50% higher than the figure given in the table. This gives some indication of the rate at which activity was lost in a partially purified preparation at 4°C. The final storage medium for the enzyme contained 1.0M sodium chloride and 50% (v/v) glycerol in addition to 25mM mercaptoethanol and 0.125mM PMSF in 5mM tris/HCl buffer, pH9.0, and the preparation was stored at -20°C; this medium for storage was the best that could be found for stabilising the enzyme but the rate of reaction under standard conditions dropped significantly (> 10%) on storage for more than a day; there was a similar and simultaneous increase in Km for fructose on storage as shown by preliminary kinetic experiments. The enzyme preparation in this form reacted with glucose at approximately the same rate as with fructose.

The work on this enzyme, although difficult, clearly established that a fructokinase does exist in the A. bisporus sporophore. The activity was shown to be dependent on ATP and magnesium ions and to involve the conversion of fructose to fructose-6-phosphate. In the sporophore, although probably only under certain conditions (see section 3.2.), mannitol may be oxidised to fructose. The fructokinase activity demonstrated here could phosphorylate the fructose and thus activate it for further metabolism, e.g. by the Embden-Myerhof pathway. The partially purified preparation of fructokinase enzyme isolated here also

contained glucokinase activity. The activities observed may have been due to the presence of one or more general hexokinases in the preparation. Alternatively, there may have been specific enzymes for each hexose substrate in the preparation similar to the specific fructokinase reported in green pea seeds (Medina and Sols, 1956); however it seems unlikely that activity against the two substrates resided in different enzymes as results indicated that activity against glucose was lost from the preparation at approximately the same rate as that against fructose. Further work is necessary to characterise the nature of the enzyme(s) studied here and to investigate the possibility that other enzymes with fructokinase activity may be produced by the A. bisporus sporophore.

6. Changes in Enzyme Levels with Sporophore Development.

6.1. Results (all experimental details in section 2.4.).

6.1.1. Soluble Protein Levels.

Figure 13 shows the change in soluble protein content measured as mg protein per g of dry weight with development of the sporophore. After an initial steady rise there was a drop in protein content of both stipe tissue and pileus plus gill tissue. The level in the stipe dropped between day 3 and day 6, remained roughly constant between day 6 and day 10 and then rose again. In the pileus plus gill tissue the drop in soluble protein level also occurred between days 3 and 6 but was followed by an immediate rise with further development.

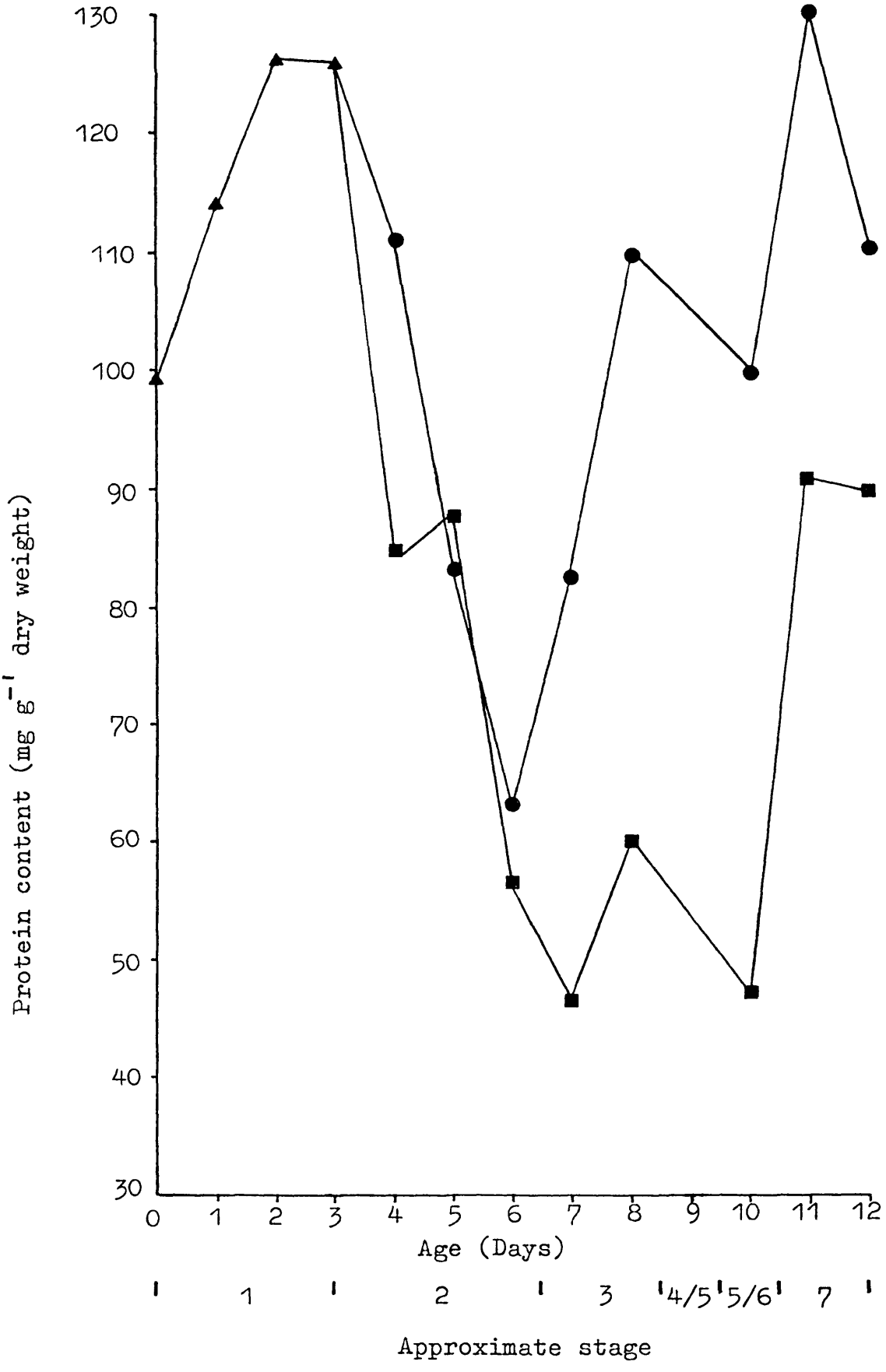
6.1.2. Mannitol Dehydrogenase Levels.

The changes with development in mannitol dehydrogenase activity, per mg of soluble protein in sporophore extracts, are shown in Figure 14; the rates of both mannitol oxidation and fructose reduction were assayed in all extracts. Both followed similar patterns with a steep rise in activity up to day 2 followed by a steady decline towards zero by day 12. The similar pattern of the two activities is emphasised by Figure 15a which shows an approximately constant ratio of mannitol oxidation activity : fructose

Protein concentrations in crude extracts were calculated relative to the estimated dry weight of tissue extracted from sporophores of known age. Results are means of 2 or 3 values showing less than 10% variation for whole pinheads (▲), stipe (■) and pileus plus gill tissues (●).

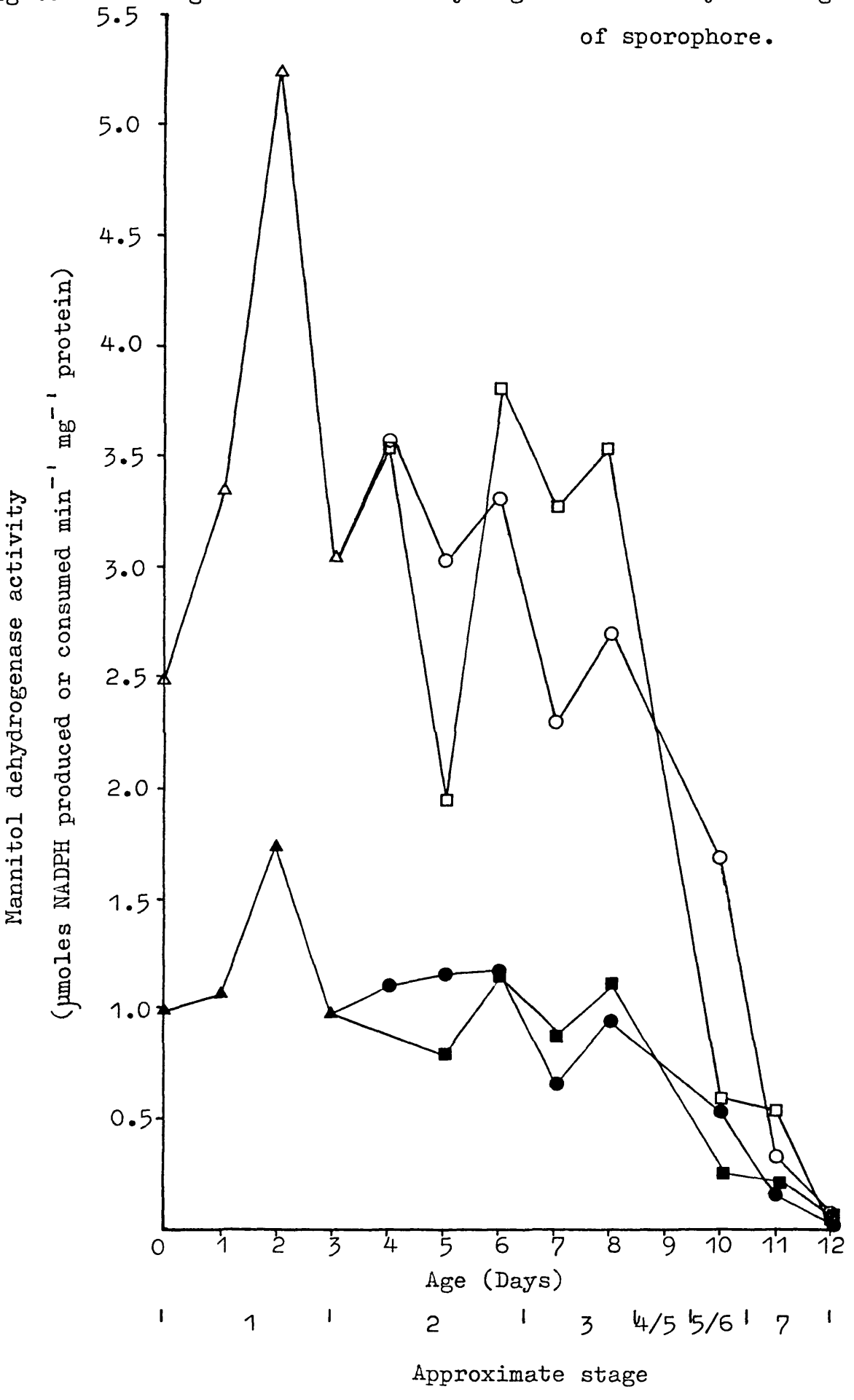
For experimental details see section 2.4.

Figure 13: Changes in soluble protein content with age of sporophore.



Mannitol dehydrogenase activity in crude extracts of sporophore tissue of known age was assayed per mg of soluble protein in the extract. For experimental details see section 2.4. Activity was measured as a rate of mannitol oxidation in whole pinheads (△), stipe tissue (□) and pileus plus gill tissue (○) and as a rate of fructose reduction in whole pinheads (▲), stipe tissue (■) and pileus plus gill tissue (●). Results are means of 2 or 3 separate determinations showing less than 15% variation.

Figure 14: Changes in mannitol dehydrogenase activity with age of sporophore.



reduction activity at each stage of development; a slight decrease in the ratio with development of both stipe tissue and pileus plus gill tissue was calculated by regression analysis, but the gradients of the lines thus derived (Figure 15a) approximate closely to zero and do not show statistically significant variation from zero. The constant ratio was roughly maintained in samples assayed after dialysis (Figure 15b); once again the ratio in both tissue types decreased slightly with development as determined by regression analysis especially in the stipe, but the gradients of the lines obtained (Figure 15b) are not significantly different from zero by the "t-test". In general, the ratio of mannitol oxidation activity : fructose reduction activity was slightly greater in dialysed than undialysed extract but again this may not be significant.

Plate 7 shows that the mannitol dehydrogenase enzyme appeared as a single discrete band after polyacrylamide-gel electrophoresis at pH5.0 of extracts from stipe tissue or pileus plus gill tissue at all stages of development. This band was related to a band on gels stained for protein by staining sample gels for both enzyme activity and protein (2.5.4.3.). With the band identified, changes in the relative intensity of the mannitol dehydrogenase protein stain on the gels were observed (Plate 8). The polyacrylamide-gels stained for protein were scanned and Plate 9 shows a typical scan. The relative intensity of the stained band per μg of soluble protein in the sample

The ratio of mannitol oxidation activity : fructose reduction activity was determined for a) undialysed extracts and b) dialysed extracts of whole pinheads (▲), stipe tissue (-■-) and pileus plus gill tissue (-●-) at various stages of development. Each point represents a single determination and lines were calculated be regression analysis. Pinhead results were included in the regression analysis of both stipe and pileus plus gill tissues. For experimental details see section 2.4.

Figure 15: Changes in ratio of mannitol oxidation activity: fructose reduction activity with age of sporophore.

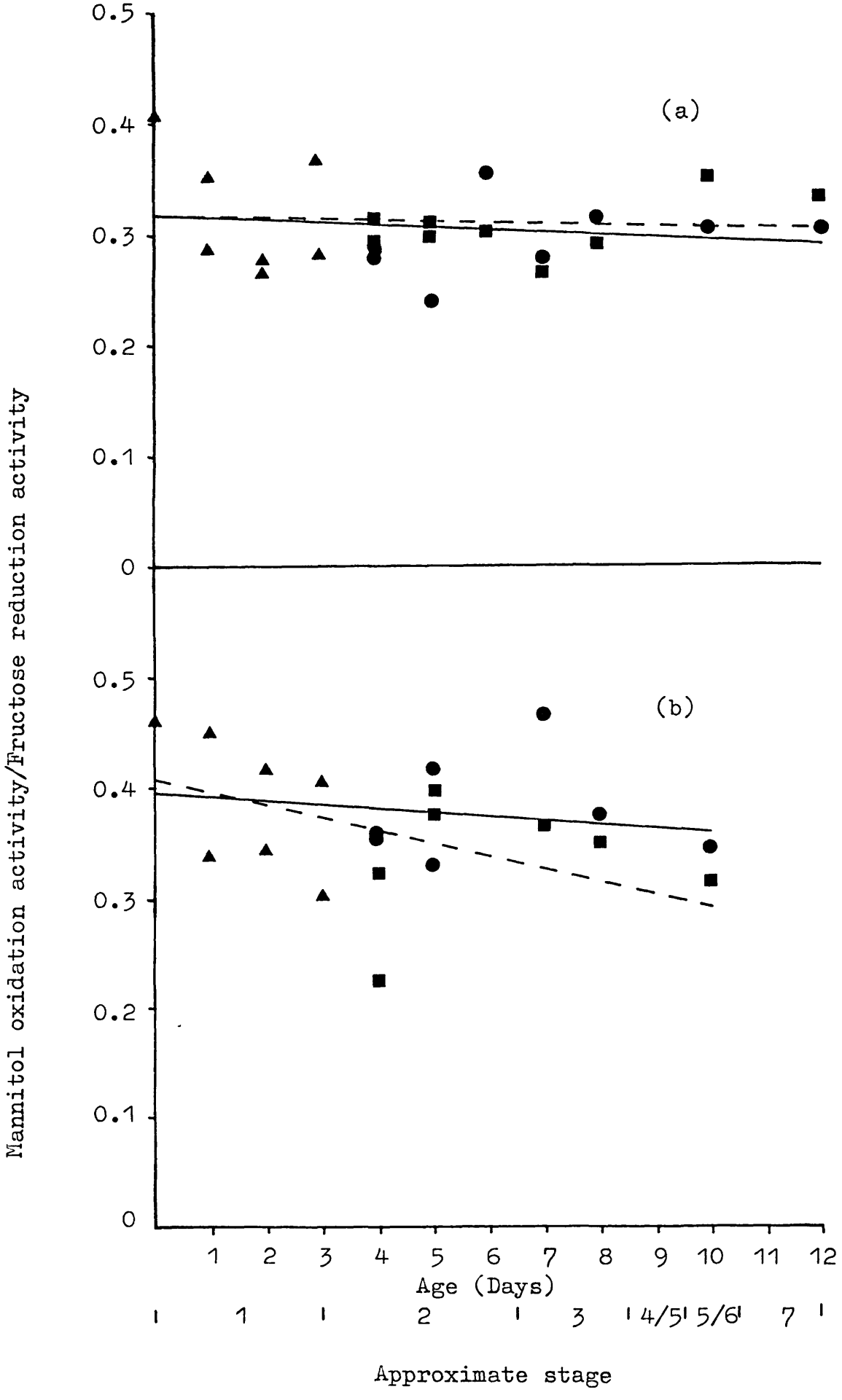
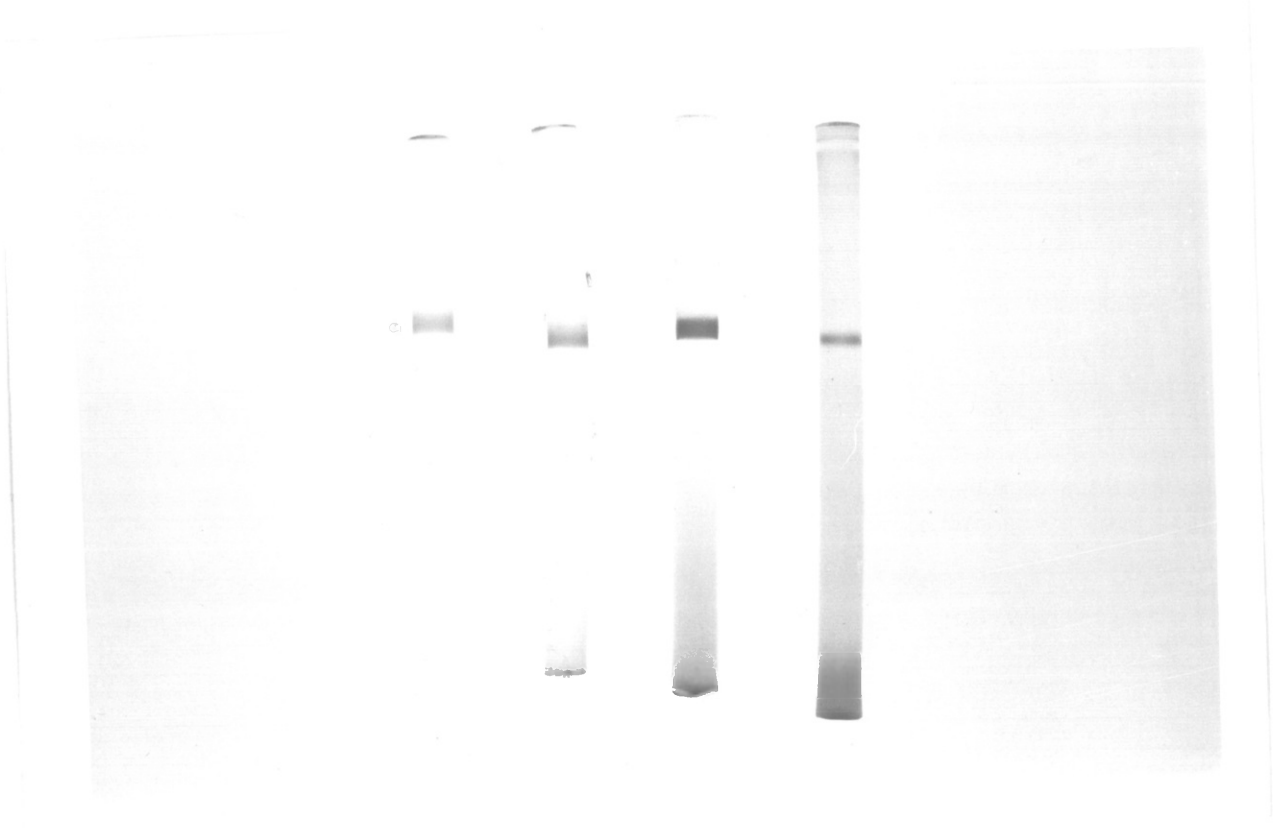


Plate 7: Polyacrylamide-gels stained for mannitol dehydrogenase activity at various stages of development.



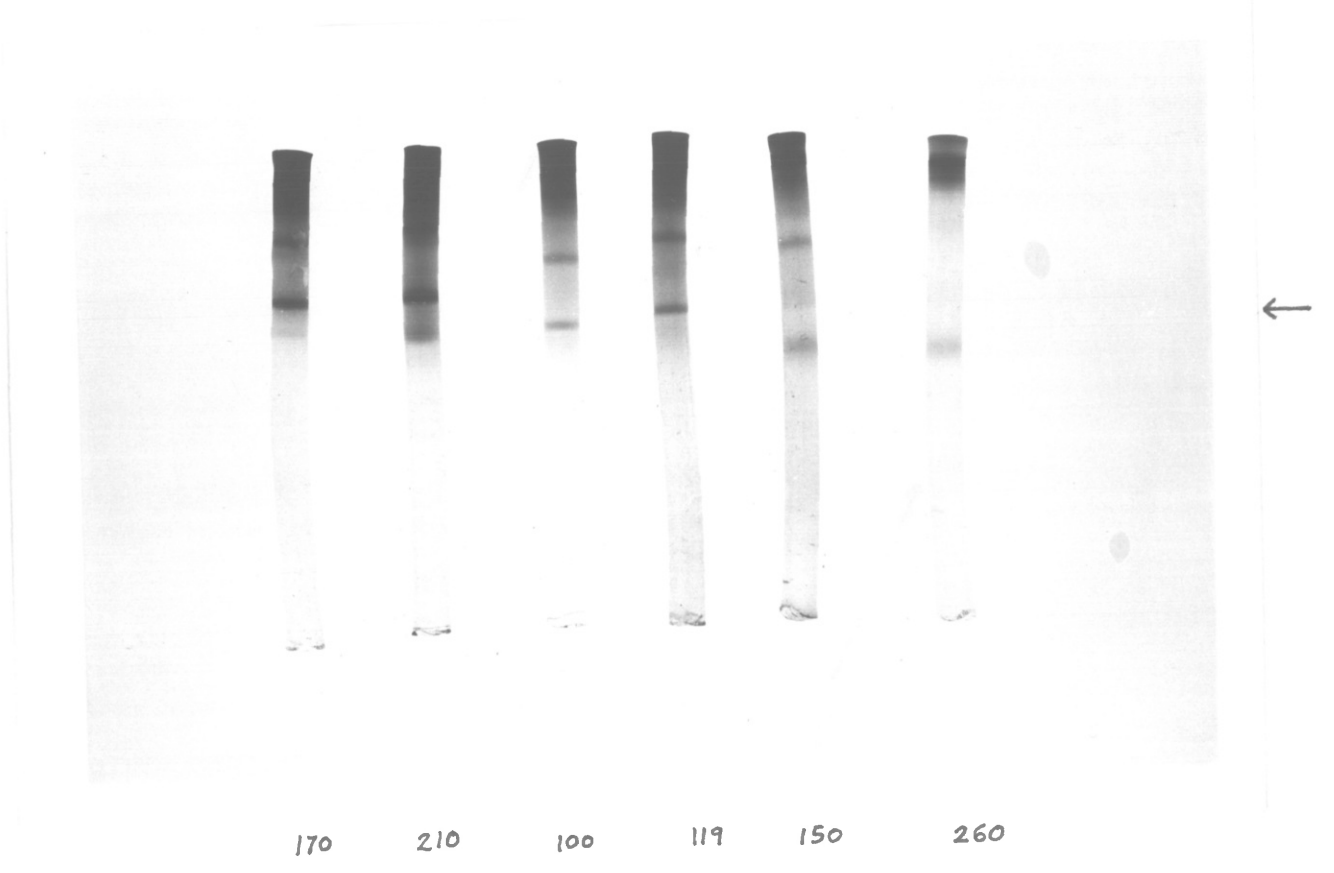
a



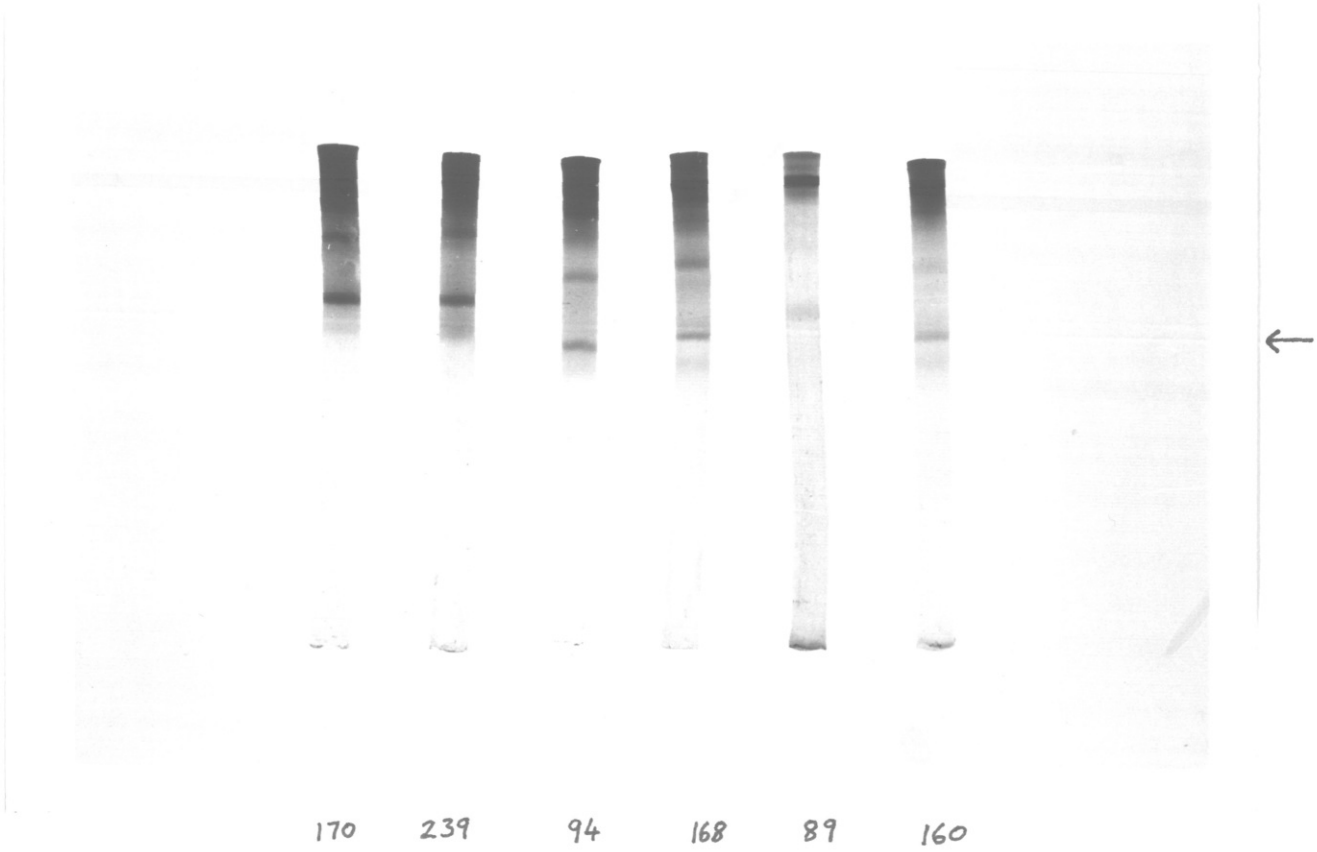
b

Samples were subjected to electrophoresis in 8% (w/v) polyacrylamide-gels at pH5.0 and stained for enzyme activity; experimental details are given in section 2.4. The samples were taken (from left to right) at day 2, 5, 7 and 10 from a) stipe tissue (whole pinheads at day 2) and b) pileus plus gill tissue (whole pinheads at day 2).

Plate 8: Polyacrylamide-gels stained for protein at various stages of development.



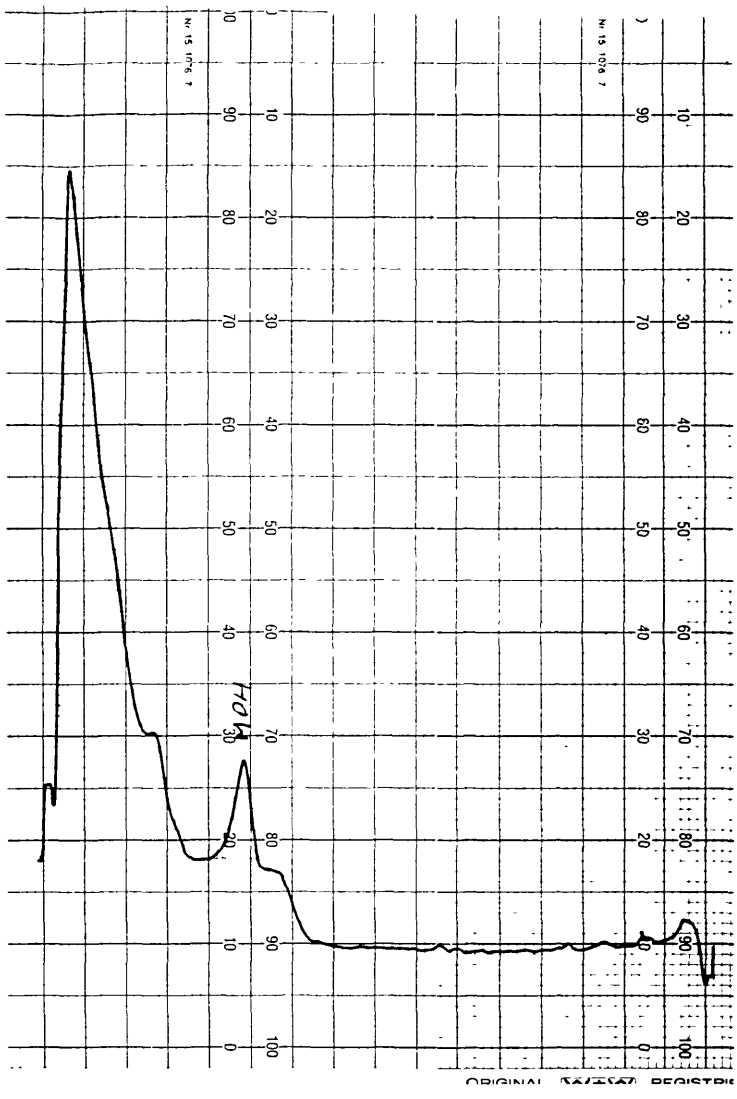
a



b

Samples were subjected to electrophoresis in 8% (w/v) polyacrylamide-gels at pH5.0 and stained for protein; experimental details are given in section 2.4. The samples were taken (from left to right) at day 2, 4, 6, 7, 10 and 11 from a) stipe tissue (whole pinheads at day 2) and b) pileus plus gill tissue (whole pinheads at day 2). The amount of soluble protein applied (µg) is shown below each gel. The arrow indicates the position of the mannitol dehydrogenase band.

Plate 9: Scan of polyacrylamide-gel stained for protein.



Each gel shown in Plate 8 was scanned at 550nm and a typical scan corresponding to the gel of a sample taken at the day 2 pinhead stage is shown here. See section 2.4. for experimental details.

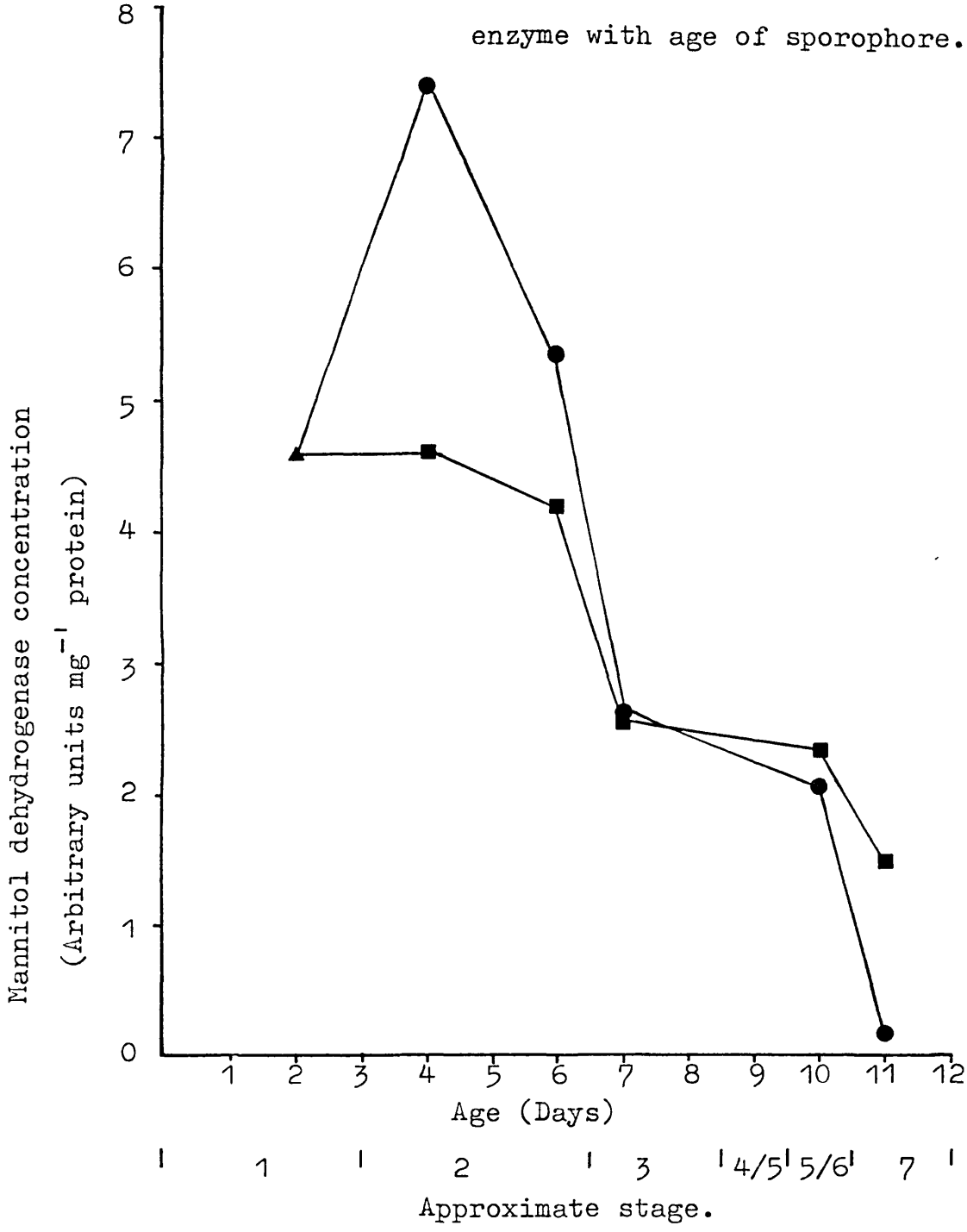
of extract applied was calculated and is plotted for various stages of development (Figure 16). This shows a steady fall in the relative amount of enzyme present in crude extracts with development from day 2 to day 11 in the stipe and an apparent rise between day 2 and day 4 followed by a similar fall in the pileus plus gill.

6.1.3. Phosphatases.

The changes in levels of phosphatase activity, per mg of soluble proteins in extracts, with development of the sporophore are shown in Figures 17a, b and c; substrates used were a) ρ-nitrophenylphosphate, b) glucose-6-phosphate and c) fructose-6-phosphate, each at pH4.5 and pH6.5. For the two sugar phosphates a peak of activity was apparent at about day 6 in all tissues; the stipe showed a drop in activity between day 2 (pinheads) and day 5 prior to this peak. With ρ-nitrophenylphosphate as substrate, there was a general rise in activity up to day 8 followed by a drop.

Samples of the extracts at various stages of development were subjected to electrophoresis at pH8.5 on polyacrylamide-gels. Table 14 schematically summarises the results as shown by the plates. Plate 10 (a-d) shows the change in electrophoretic pattern of fructose-6-phosphatase activity with age in stipe tissue and pileus plus gill tissue; in addition to the non-specific acid phosphatase studied in section 4

Figure 16: Changes in relative amounts of mannitol dehydrogenase enzyme with age of sporophore.

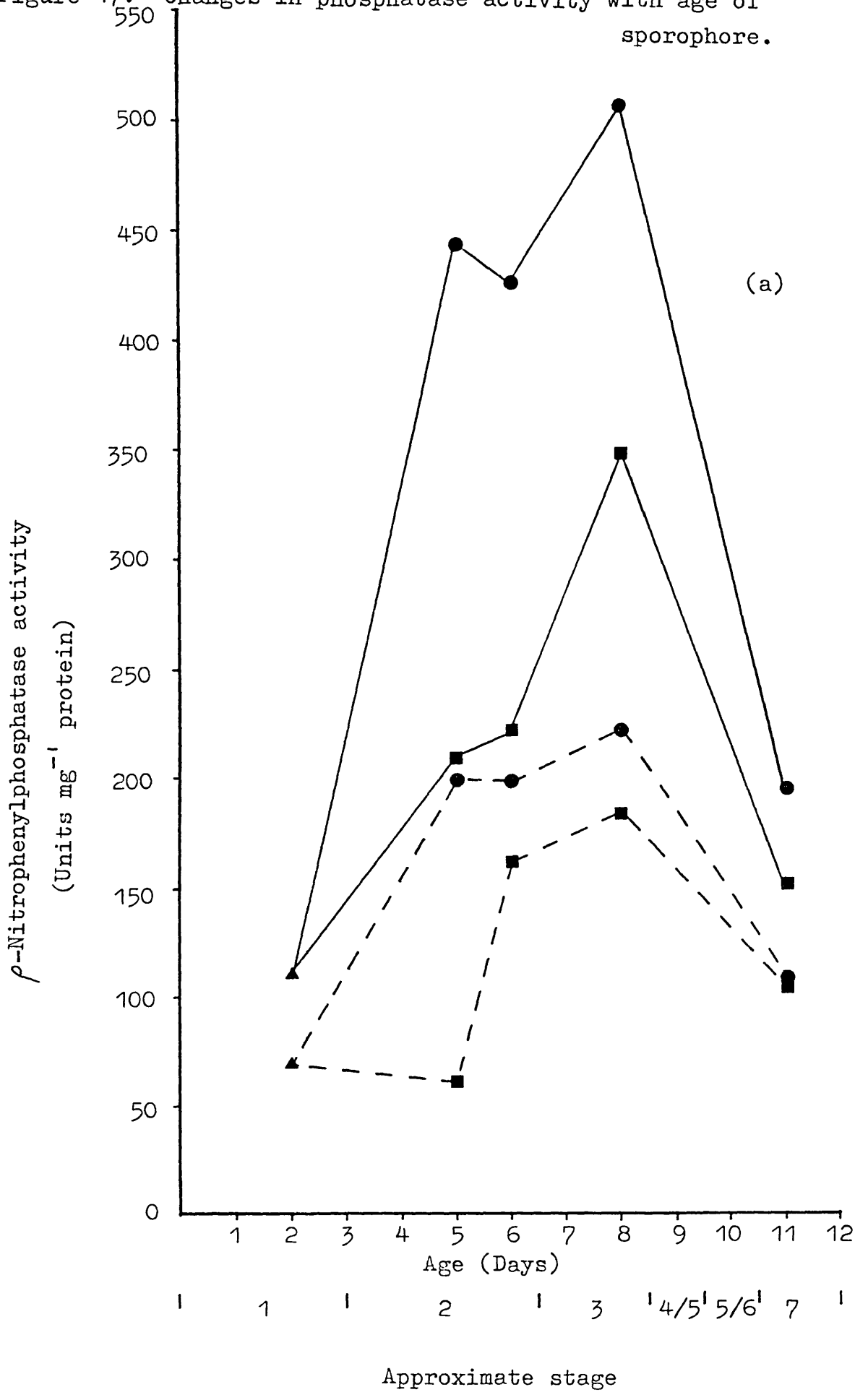


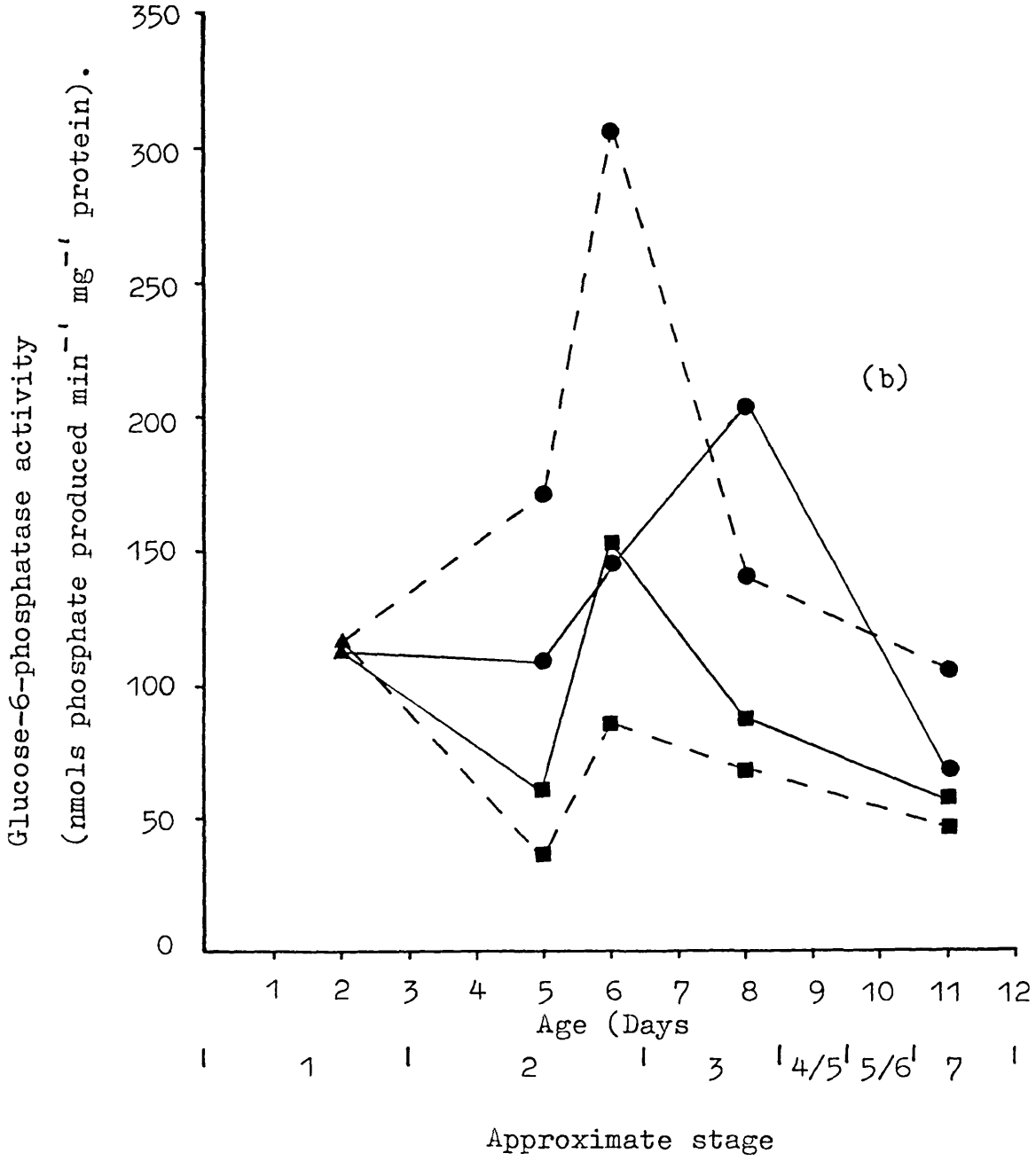
Polyacrylamide-gels were subjected to electrophoresis at pH5.0 and stained for protein. The protein band corresponding to mannitol dehydrogenase was identified. The gels were scanned at 550nm and the relative intensity of the mannitol dehydrogenase band per μg of soluble protein in the sample applied was calculated. Experimental details are given

in section 2.4. The relative intensity μg^{-1} protein is plotted here for samples from whole pinheads (▲), stipe tissue (■) and pileus plus gill tissue (●). Each point represents a single determination.

Phosphatase activity in whole pinheads (▲), stipe tissue (■) and pileus plus gill tissue (●) was assayed with a) ρ-nitrophenylphosphate, b) glucose-6-phosphate and c) fructose-6-phosphate as substrate at pH4.5 (solid line) and pH6.5 (broken line) at various stages of development. Results are means of two determinations showing less than 10% variation. Experimental details are given in section 2.4.

Figure 17: Changes in phosphatase activity with age of sporophore.




Glucose-6-phosphatase activity
(nmols phosphate produced min⁻¹ mg⁻¹ protein).
350
300
250
200
150
100
50
0
(b)
1 2 3 4 5 6 7 8 9 10 11 12
Age (Days
1 2 3 4/5 5/6 7
Approximate stage

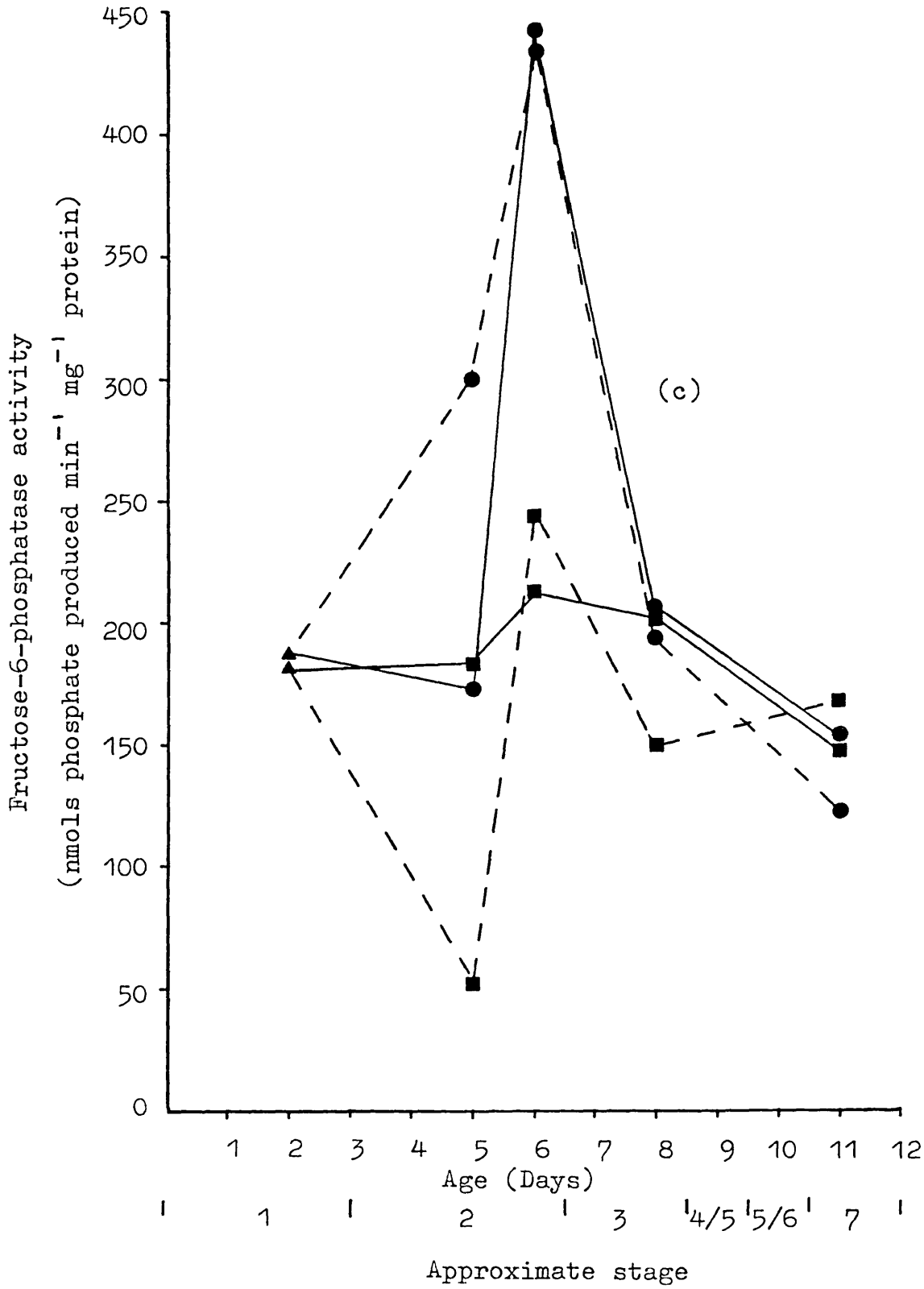
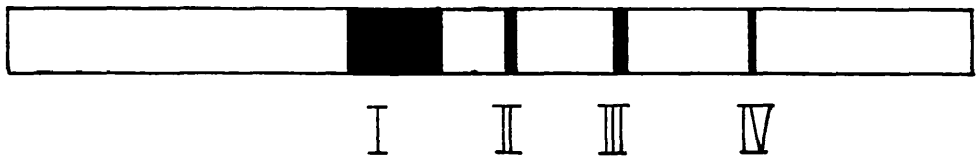

Fructose-6-phosphatase activity
(nmols phosphate produced min⁻¹ mg⁻¹ protein)
450
400
350
300
250
200
150
100
50
0
(c)
1 2 3 4 5 6 7 8 9 10 11 12
Age (Days)
1 2 3 4/5 5/6 7
Approximate stage

Table 14: Summary of intensity of phosphatase activity bands stained on polyacrylamide-gels.



A "schematic" gel is shown above indicating the position of the four enzymes revealed by staining polyacrylamide-gels for phosphatase activity; details in text and on Plates 8 to 12. The intensity of staining of each plate is summarised in the table below; visual estimates are +++++, most intense, to -, absent.

Plate 10) Stained for fructose-6-phosphatase activity at various stages of development.

	I	II	III	IV
a) Stipe tissue, 2 min incubation with fructose-6-phosphate				
Day 0	+++++	-	++	-
Day 3	+++++	+	++	-
Day 5	++++	++	++	-
Day 8	+++++	++	++	-
Day 12	++++	-	++	-

b) Stipe tissue, 20 min incubation with fructose-6-phosphate

Day 0	+++++	-	-	-
Day 3	+++++	-	-	-
Day 5	+++++	-	-	-
Day 8	+++++	-	-	-
Day 12	+++++	-	-	-

c) Pileus/gill tissue, 2 min incubation with fructose-6-phosphate

Day 0	+++++	-	++	-
Day 3	+++++	+	++	-
Day 5	++++	++	++	-
Day 8	+++++	+	++	-
Day 12	++++	++	+	-

d) Pileus/gill tissue, 20 min incubation with fructose-6-phosphate

Day 0	+++++	-	-	-
Day 3	+++++	-	-	-
Day 5	+++++	-	-	-
Day 8	+++++	-	-	-
Day 12	+++++	-	-	-

Plate 11) Changes in band intensity with length of incubation with fructose-6-phosphate.

Length of incubation (min)	Intensity of band			
	I	II	III	IV
2	+++++	+	++	-
5	+++++	+	+	-
20	+++++	-	-	-

Plate 12) Stained for glucose-6-phosphatase activity at various stages of development.

	Intensity of band			
	I	II	III	IV
a) Stipe tissue, 2 min incubation with glucose-6-phosphate				
Day 0	+++++	-	-	-
Day 3	+++++	+	+	-
Day 5	+++++	+	+	-
Day 8	+++++	-	-	-
Day 12	+++++	-	-	-
b) Stipe tissue, 20 min incubation with glucose-6-phosphate				
Day 0	+++++	-	-	-
Day 3	+++++	-	-	-

Day 5	+++++	-	-	-
Day 8	+++++	-	-	-
Day 12	+++++	-	-	-

c) Pileus/gill tissue,
2 min incubation with
glucose-6-phosphate

Day 0	+++++	-	-	-
Day 3	+++++	+	+	-
Day 5	+++++	+	+	-
Day 8	+++++	-	-	-
Day 12	+++++	-	-	-

d) Pileus/gill tissue,
20 min incubation with
glucose-6-phosphate

Day 0	+++++	-	-	-
Day 3	+++++	-	-	-
Day 5	+++++	-	-	-
Day 8	+++++	-	-	-
Day 12	+++++	-	-	-

Plate 13) Stained for phosphatase activity with various substrates.

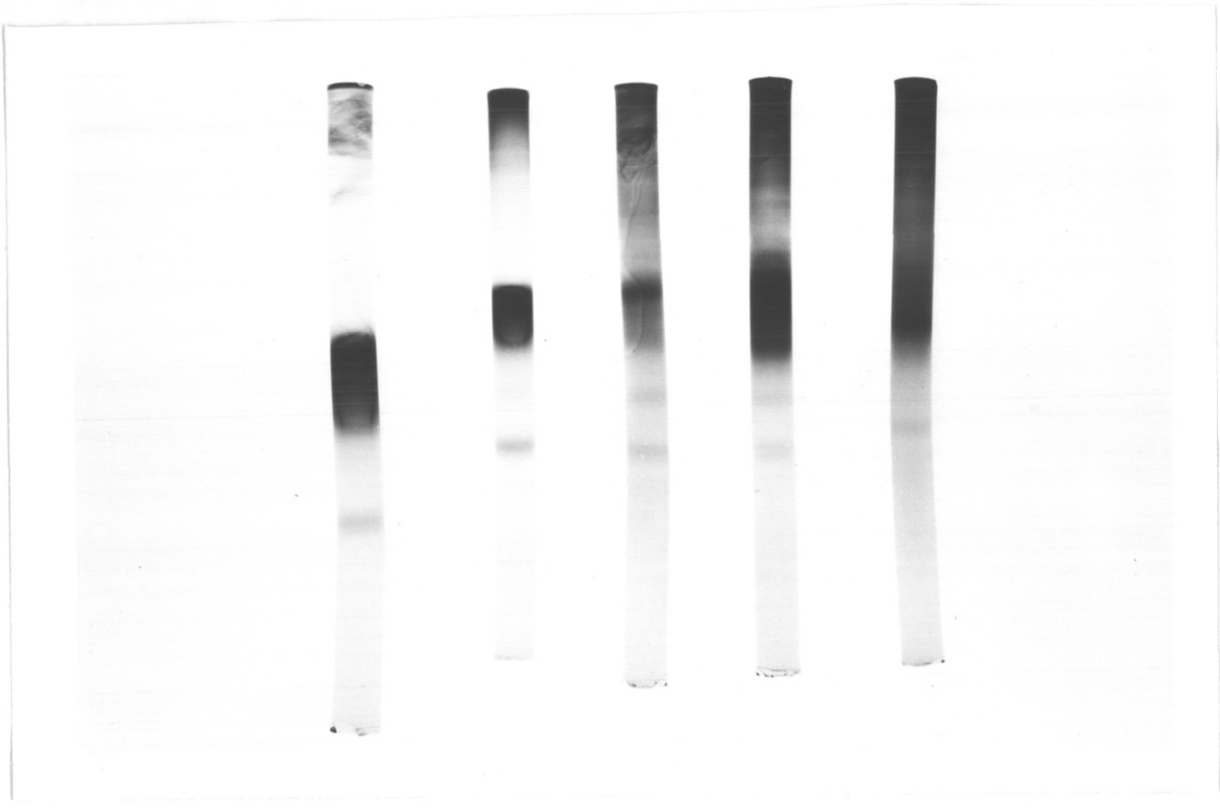
Substrate	Intensity of band			
	I	II	III	IV
D-Fructose-6-phosphate	+++++	+	++	-
D-Glucose-6-phosphate	+++++	+	+	-

D-Fructose-1-phosphate	+++++	-	++	+?
D-Fructose-1,6-diphosphate	+++	-	-	-
p-Nitrophenylphosphate	+++++	++	+++	+

Plate 14) Stained for fructose-6-phosphatase activity at acid and alkaline pH.

pH	Intensity of band			
	I	II	III	IV
4.5	+++++	+	++	-
8.5	++	+	+	-

Plate 10: Polyacrylamide-gels stained for fructose-6-phosphatase activity at various stages of development.



a



b



c



d

Samples were subjected to electrophoresis in 8% (w/v) polyacrylamide-gels at pH8.5 and gels were stained for fructose-6-phosphatase activity by incubating for 2 min (a and c) or 20 min (b and d); experimental details are given in section 2.4. Plates a and b; samples of extracts from (from left to right) whole pinheads (day 0, day 3) and stipe tissue (day 5, day 8, day 12). Plates c and d; samples of extracts from (from left to right) whole pinheads (day 0, day 3) and pileus plus gill tissue (day 5, day 8, day 12).

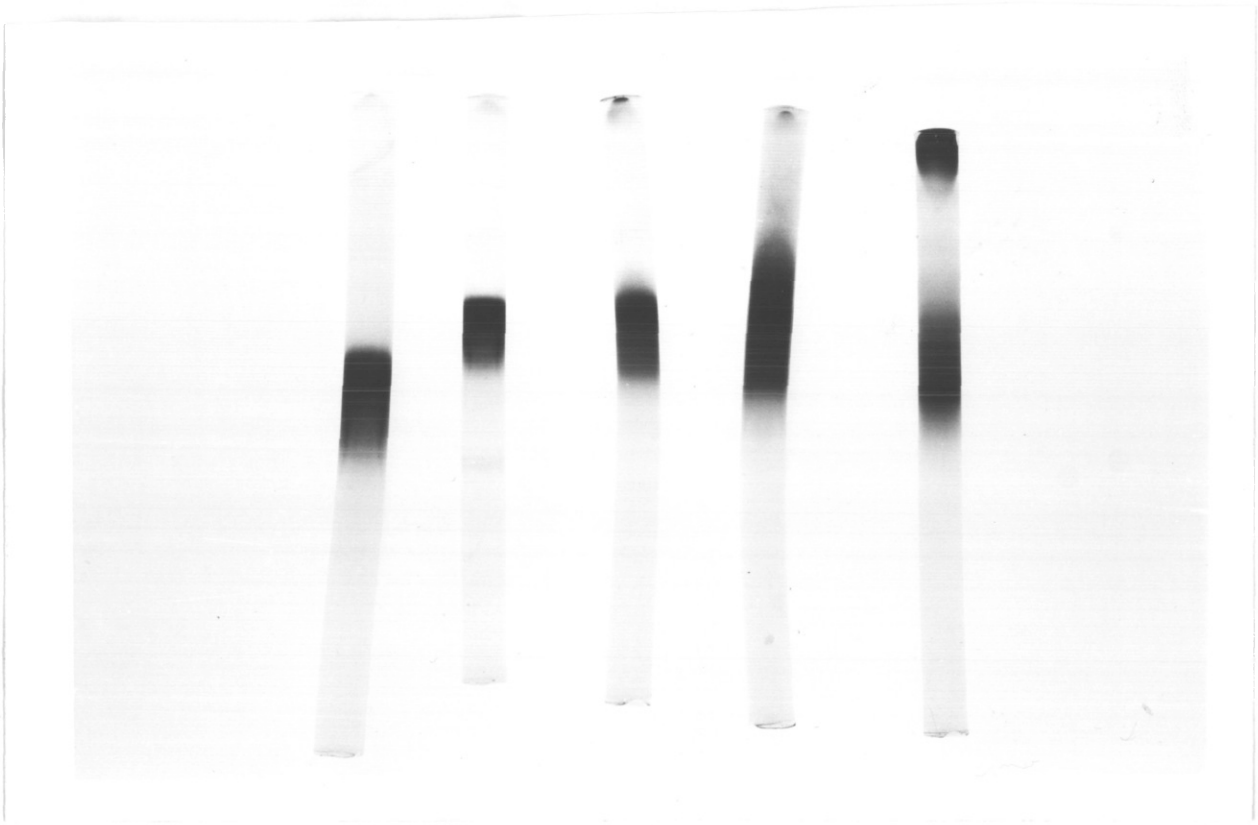
(Phosphatase I) two other bands of fructose-6-phosphatase activity were observed on the gels (Phosphatases II and III), each of which was more mobile in the gels than the non-specific enzyme. Phosphatases II and III stained strongly on gels incubated with fructose-6-phosphate for only 2 min and were not at all apparent on gels incubated with the substrate for 20 min. To examine this time effect, a gel was incubated with fructose-6-phosphate for 5 min and this gave band intensities intermediate between the 2 min and 20 min incubations (Plate 11). The most mobile of these phosphatase activities in the gels, Phosphatase III, was present throughout sporophore development. Phosphatase II was not detectable at day 0 but was clearly present at day 3. Both activity bands faded in intensity late in development. The substrate specificity of the enzymes was qualitatively examined by incubating the gels with glucose-6-phosphate in place of fructose-6-phosphate (Plate 12, a-d). Phosphatase III appeared as a faint band when incubated with this substrate for 2 min and Phosphatase II was barely detectable by this staining procedure. Polyacrylamide-gels of the samples were also incubated for 2 min with other phosphate compounds replacing fructose-6-phosphate and stained by the normal procedure; results are shown in Plate 13. Phosphatase I gave a strong staining reaction with fructose-1-phosphate and fructose-1,6-bisphosphate. Phosphatase III gave a reaction with fructose-1-phosphate and no reaction with fructose-1,6-bisphosphate. No reaction could be detected between Phosphatase II and

Plate 11: Changes in intensity of phosphatase stains with length of incubation of gels with fructose-6-phosphatase.

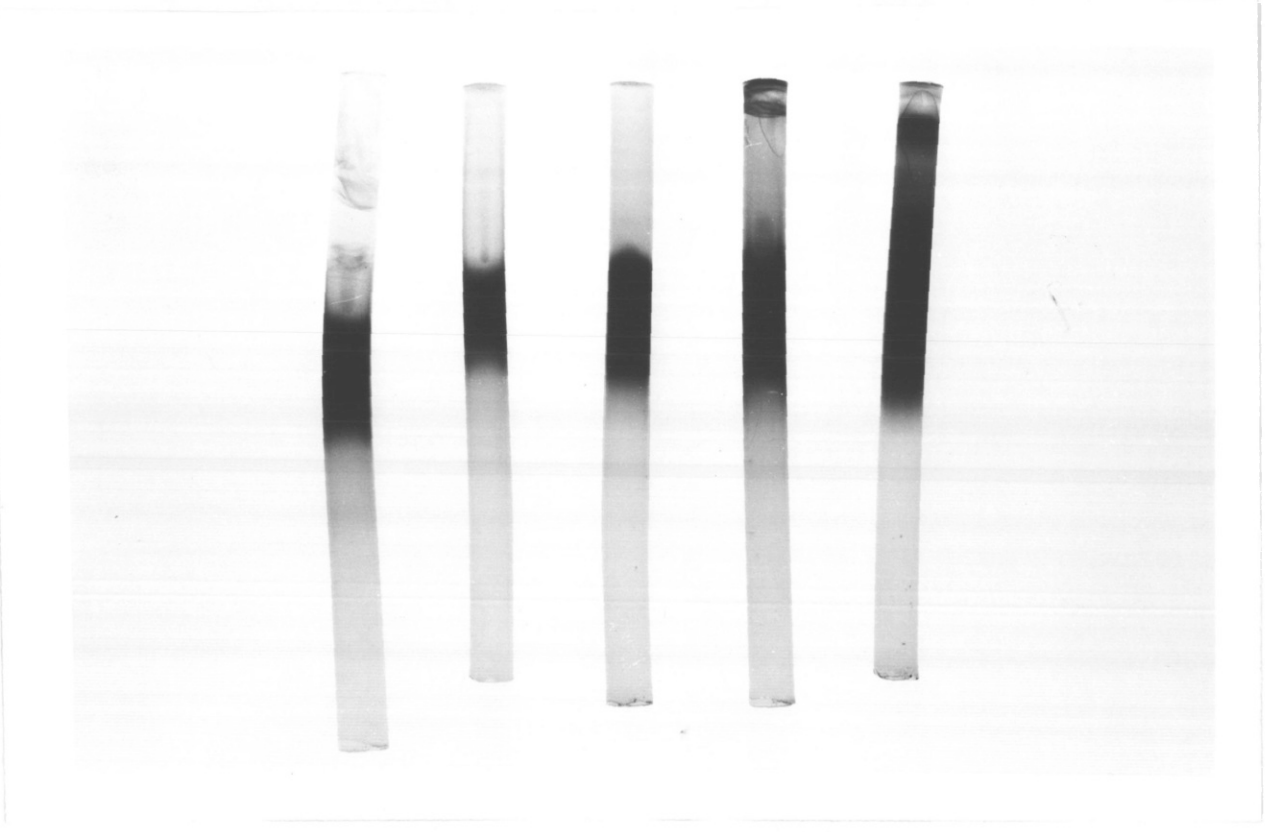


Samples of extracts from whole pinheads (day 3) were subjected to electrophoresis in 8% (w/v)polyacrylamide-gels at pH8.5 and the gels incubated with fructose-6-phosphate for (from left to right) 2 min, 5 min and 20 min and stained by the normal procedure; experimental details are given in section 2.4.

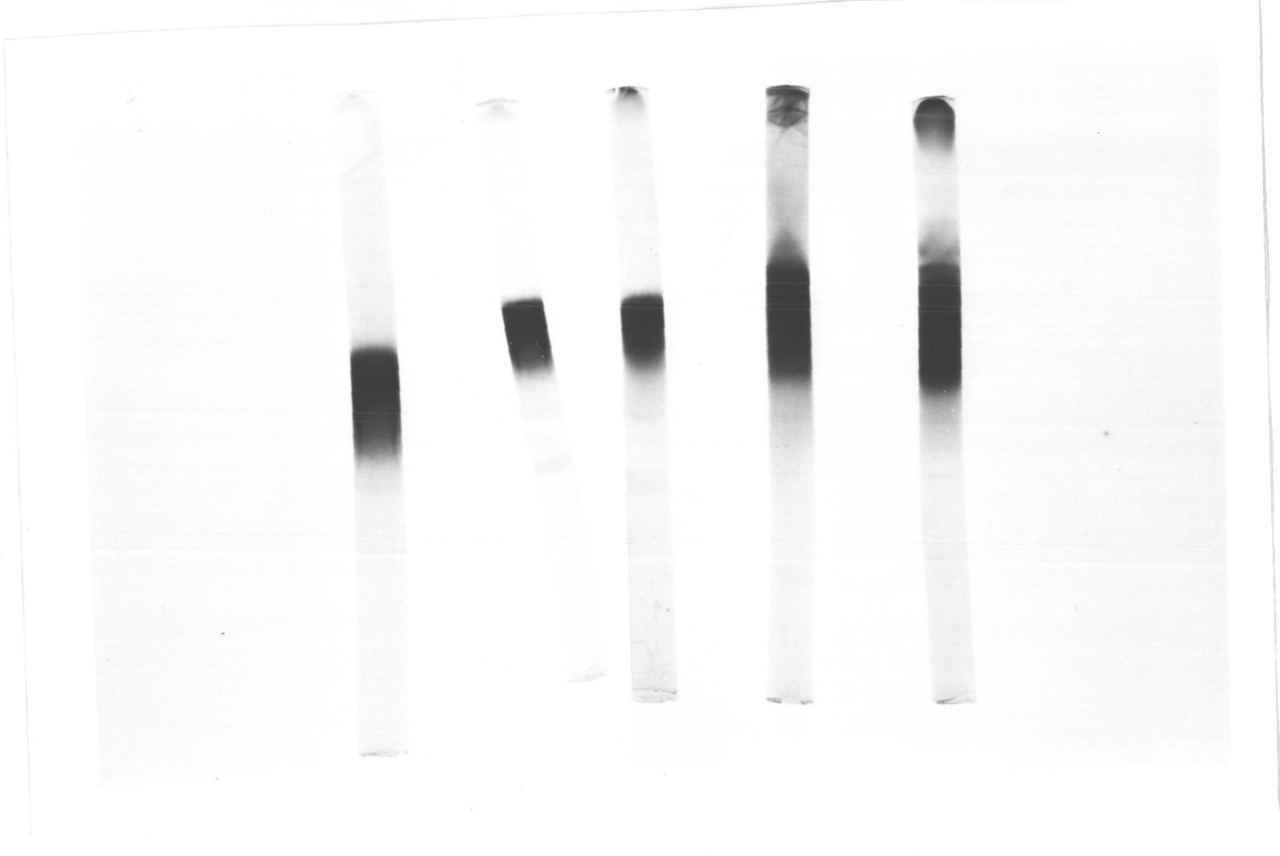
Plate 12: Polyacrylamide-gels stained for glucose-6-phosphatase activity at various stages of development.



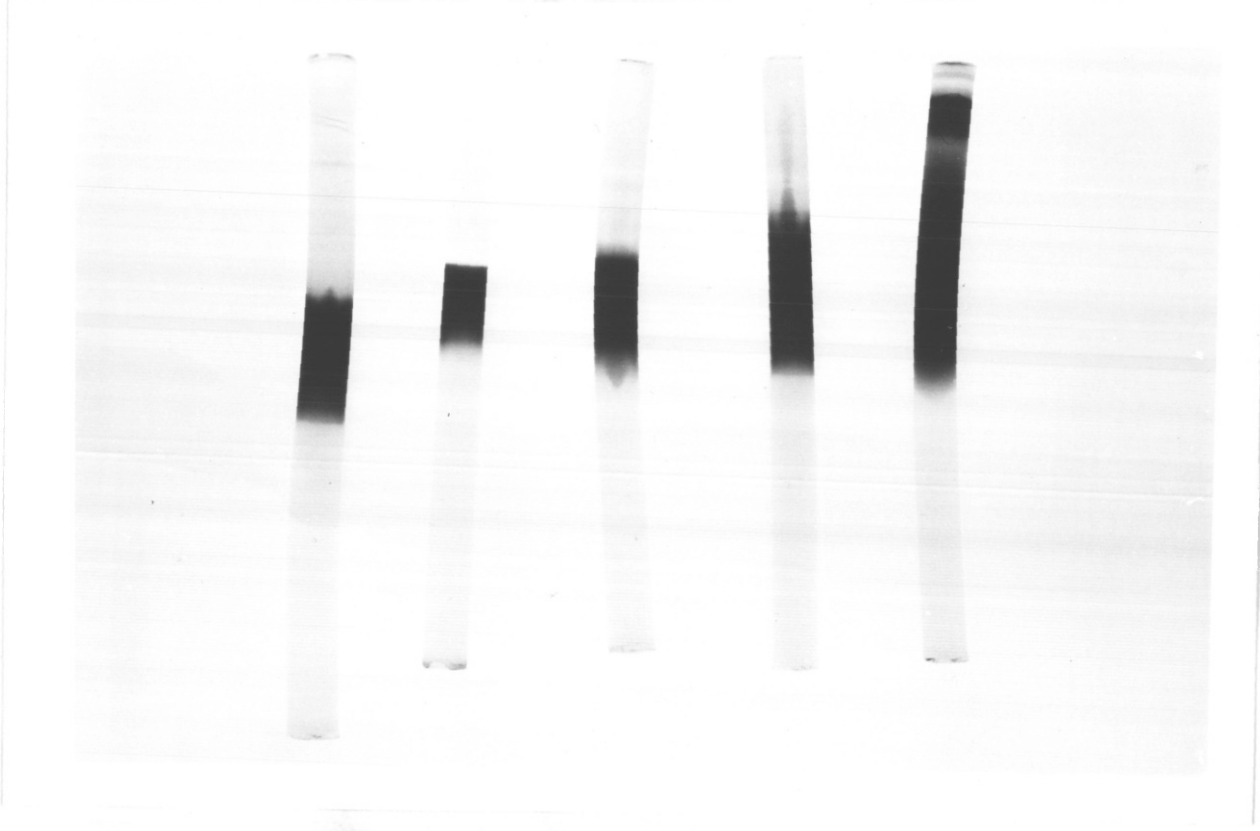
a



b



c



d

Samples were subjected to electrophoresis in 8% (w/v) polyacrylamide-gels at pH8.5 and gels were stained for glucose-6-phosphatase activity by incubating for 2 min (a and c) or 20 min (b and d); experimental details are given in section 2.4. Plates a and b; samples of extracts from (from left to right) whole pinheads (day 0, day 3) and stipe tissue (day 5, day 8, day 12). Plates c and d; samples of extracts from (from left to right) whole pinheads (day 0, day 3) and pileus plus gill tissue (day 5, day 8, day 12).

Plate 13: Polyacrylamide-gels of samples of day 3 pinhead extracts stained for phosphatase activity with various substrates.



Samples of extracts from day 3 pinheads were subjected to electrophoresis in 8% ( ) polyacrylamide-gels at pH8.5 and gels were stained for phosphatase activity by incubating for 2 min with (from left to right) D-fructose-6-phosphate, D-glucose-6-phosphate, D-fructose-1-phosphate, D-fructose-1, 6-bisphosphate and ρ- nitrophenylphosphate; experimental details are given in section 2.4. Incubation with some of the substrates has given a high background stain but bands are discernible.

either fructose-1-phosphate or fructose-1,6-bisphosphate. Phosphatases II and III appeared, by this method, to show a limited degree of specificity for fructose-6-phosphate. All three phosphatases above were stained after incubation with *p*-nitrophenylphosphate and an additional phosphatase, Phosphatase IV, was revealed by incubation with this substrate. Phosphatase IV was more mobile than the other phosphatases in gels under these conditions. Polyacrylamide-gels of extracts incubated with fructose-6-phosphate at pH8.5 instead of pH4.5 gave the same number of bands as at the lower pH but all appeared fainter (Plate 14); all the phosphatases thus seemed to be of the acid type.

In addition to the discrete bands, the gels also showed diffuse staining to various extents at the top of the gel when incubated for 2 min with fructose-6-phosphate (for example Plate 10 a, c). This staining was not so intense when gels were incubated for longer periods nor did it appear on gels incubated with glucose-6-phosphate. This staining may have been due to denatured protein or enzyme in "loose" association with proteins or other polymers present in the crude extract applied to the gels.

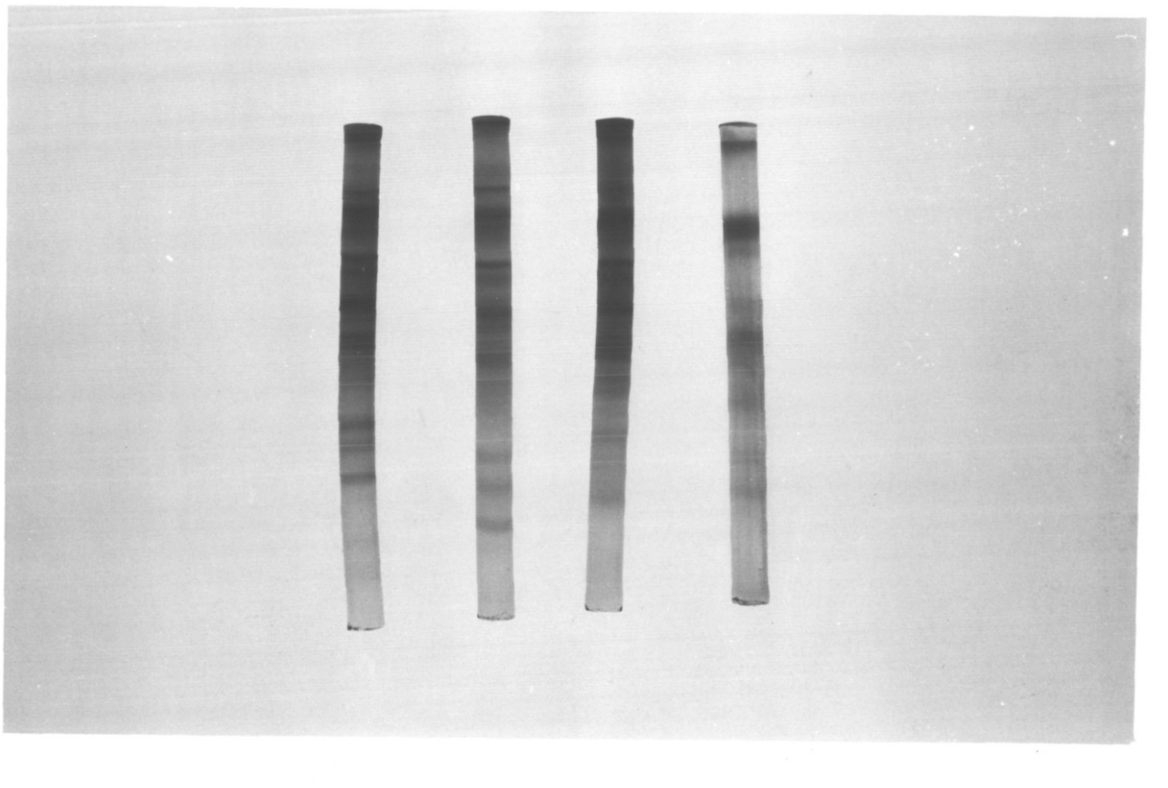
The changes with development patterns of gels stained for protein are shown in Plate 15.

Plate 14: Changes in intensity of phosphatase stains with pH of incubation with fructose-6-phosphate.

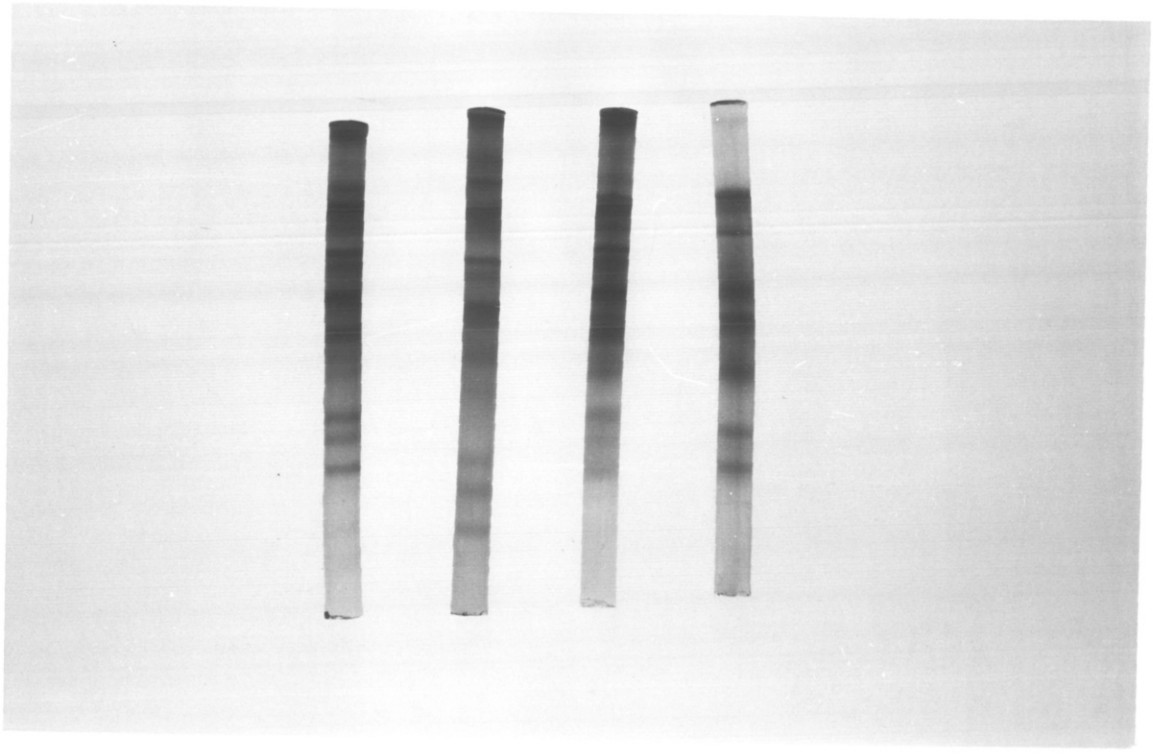


Samples of extracts from whole pinheads (day 3) were subjected to electrophoresis in 8% (w/v) polyacrylamide-gels and the gels were incubated with fructose-6-phosphate for 2 min at pH4.5 (left) and pH8.5 (right) and stained by the normal procedure; experimental details are given in section 2.4.

Plate 15: Polyacrylamide-gels stained for protein at various stages of development.



(a)



(b)

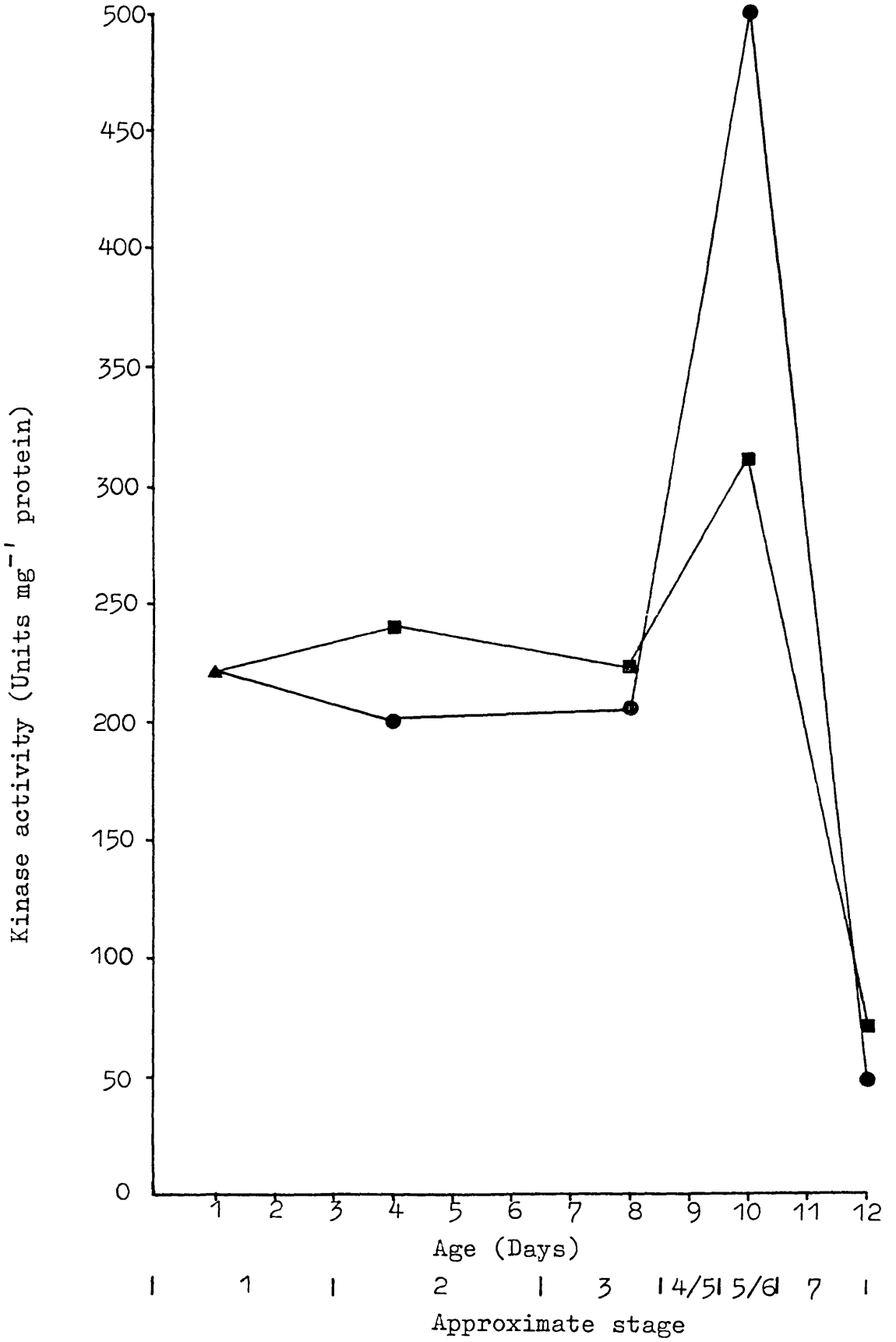
Samples of extracts were subjected to electrophoresis in 8% (w/v) polyacrylamide-gels and gels were stained for protein; experimental details are given in section 2.4. Plate a; samples of extracts from (from left to right) whole pinheads (day 2) and stipe tissue (day 5, day 8, day 12). Plate b; samples of extracts from (from left to right) whole pinheads (day 2) and pileus plus gill tissue (day 5, day 8, day 12).

6.1.4. Fructokinase.

The changes in levels of fructokinase activity, per mg of soluble protein in extracts, with development of the sporophore are shown in Figure 18. The results presented are for single determinations only and because of this and the problems involved with the instability of this enzyme (5.1.), these changes can only be considered as general trends. However, it seems the level of activity remained approximately constant between day 2 and day 8, then rose to a maximum at day 10 and finally fell off sharply to a low level at day 12. The pattern was the same for both stipe tissue and pileus plus gill tissue.

Fructokinase activity in whole pinheads (▲), stipe tissue (■) and pileus plus gill tissue (●) was assayed at various stages of development. Each point represents a single determination. Experimental details are given in section 2.4.

Figure 18: Changes in fructokinase activity with age of sporophore.



6.2. Discussion.

The pattern of soluble protein levels relative to tissue dry weight (Figure 13) is similar to that found by Hammond and Nichols (1976a). The values recorded here were approximately half those reported by Hammond and Nichols on a dry weight basis; this may be due to a different method of extraction, a different method of estimation, the use of dialysed extracts (here) against undialysed extracts (Hammond and Nichols) or any combination of these. However, the differences in actual levels are less important than the comparative aspects of changes in protein levels in this study.

The variation in levels of mannitol dehydrogenase activity (Figure 14) follows expected patterns if the enzyme is acting mainly in the direction of mannitol synthesis. The level in pinheads rises from day 0 to day 2 when mannitol production may be at its highest rate as the difference in mannitol content between mycelium and sporophore (Hammond and Nichols, 1976a) is established. This level drops at day 3 and remains approximately constant up to day 8. However, enzyme levels are still high and enough to account for mannitol synthesis over this period; a change of mannitol content from approximately 30% to approximately 50% of the dry weight occurs between stages 1 and 5 (Hammond and Nichols, 1976a), which corresponds to days 3 to 8, and a hypothetical value for the time needed to synthesise this mannitol can be estimated as follows:

the level of mannitol dehydrogenase activity during the period day 3 to day 8 is approximately 3μmoles NADPH consumed min^{-1} mg^{-1} soluble protein (Figure 14). This is equivalent to the synthesis of 3μmoles mannitol min^{-1} mg^{-1} soluble protein. Assuming a value for soluble protein content of 80mg g^{-1} dry weight during this phase (Figure 13), the rate of mannitol synthesis can now be given as 240μmoles mannitol min^{-1} g^{-1} dry weight. This is approximately equivalent to 44mg mannitol min^{-1} g^{-1} dry weight or to a change in mannitol content in the sporophore of 4.4% of the dry weight min^{-1}. On this basis, the hypothetical time required for the 20% increase in mannitol content over the five day period (above) is only of the order of $4\frac{1}{2}$ min. This rate of synthesis is hypothetical as it assumes optimum pH, a temperature of 25°C and concentrations of fructose and NADPH of 800mM and 250μM respectively; such conditions are unlikely in the A. bisporus cell, especially the reactant concentrations. The hypothetical rate indicates excess mannitol sythesising capacity during this phase of development which is probably limited by fructose or NADPH concentration and/or the possible control mechanisms discussed in section 3.2.

After day 8 the level of mannitol dehydrogenase activity declines. This might not be expected if the enzyme's main physiological role was to oxidise mannitol, rather than the biosynthetic role considered above, as it is at the late stages of sporophore development that mannitol levels

fall (Hammond and Nichols, 1976a). Nevertheless, taking a value of 0.2μmoles NADPH produced or mannitol oxidised min^{-1} mg^{-1} soluble protein (Figure 14) and a value for soluble protein content in the sporophore at day 11 of 100mg g^{-1} dry weight (Figure 13), the enzyme could reduce the content of mannitol at a rate of approximately 0.4% of the dry weight min^{-1} when calculated as above. At this rate of turnover all the mannitol in the sporophore at stage 7 (approximately 46% of the dry weight; Hammond and Nichols, 1976a) could be oxidised in approximately 2h. Again this time is a hypothetical value for the same reasons as those abovefor the earlier phase of development. The difference in pH between the *in vitro* pH of 10.4 used in the esimation of enzyme activity and the pH likely to occur *in vivo* may be particularly important in limiting mannitol oxidation. Furthermore, unless the *in vivo* pH is high and/or fructose is rapidly removed from the environment of the enzyme, the reaction equilibrium will still favour mannitol production (see section 3.2.). However, the calculation above does indicate that, under the right conditions, the mannitol dehydrogenase content at day 11 is sufficient to be responsible for significant turnover of mannitol.

The levels of fructose reducing activity reported here for stage 1 and stage 2 sporophores on a per mg of soluble protein basis (Figure 14) are approximately 100 fold those reported by Hammond (1981) and 500 fold those reported by

Hammond (1977). The main reasons for this difference are: first, the above worker used a fructose concentration below 4mM for his enzyme assays, whereas the highest rate of reaction observed here was with a fructose concentration of 800mM (3.1.5.), and, second, the sporophores were frozen before extraction and the extract dialysed before assaying for enzyme - both these treatments were found here to lead to a loss of activity (3.2.).

The pattern of mannitol dehydrogenase activity throughout the whole development period was roughly the same when assayed for rate of mannitol oxidation as for rate of fructose reduction. The ratio of the two activities was approximately constant (Figure 15) and very similar to that found with the purified preparation (3.1.5.). This indicates that the enzyme is probably not affected by any regulatory molecules bound to it in the cell and still adhering in the crude extract; any such molecules would be expected to promote or inhibit the reaction in one direction or the other and thus alter the ratio of the two activities. This was confirmed by assaying dialysed extract. Although this treatment led to a loss in absolute activity, the ratio of the activities of mannitol oxidation and fructose reduction remained the same (Figure 15). If anything, there was a slight increase in the relative level of mannitol oxidising activity which may have been due to inhibition by high concentrations of mannitol present in the crude, but not the dialysed, extract.

If there is no regulation of mannitol dehydrogenase in crude extracts, as suggested by the data above, the variations in levels of *in vitro* activity will reflect variations in the amount of mannitol dehydrogenase in the cell. The variations in amount of enzyme present per µg of soluble protein extracted from the cell were assessed by measuring the intensity of staining of the protein corresponding to mannitol dehydrogenase on polyacrylamide-gels (Figure 16). Allowing for the fact that only one determination was performed, the data for the relative amount of enzyme closely matches that for enzyme activity levels (Figure 14), certainly in the later stages of development.

Figure 16 shows only the relative amount of mannitol dehydrogenase present in the extracts on an arbitrary unit basis. An approximate value for the absolute number of copies of the enzyme subunit present in crude extracts can be estimated as follows: assuming the purified preparation of mannitol dehydrogenase (section 3) to be 100% active enzyme, the specific activity of the enzyme is 195 µmoles NADPH consumed min^{-1} mg^{-1} protein (3.1.1.). The molecular weight of the enzyme subunit is 29,500 (3.2.1.); therefore the specific activity of mannitol dehydrogenase is 5.56×10^{9} µmoles NADPH consumed min^{-1} $mole^{-1}$ subunits. Taking day 2 pinheads as an example, mannitol dehydrogenase activity in crude extracts is 5.22 µmoles NADPH consumed min^{-1} mg^{-1} protein (Figure 14).

Therefore, amount of active enzyme present in this extract = 5.22/5.56 x 10^9 moles subunits mg^{-1} protein = 0.94 nmoles subunits mg^{-1} protein, equivalent to 117.5 nmoles g^{-1} dry weight.

It is difficult to place any great significance on the changes in phosphatase activity with age of sporophore. The activity was monitored with different substrates and at two different pH's and the only consistent patterns appear to be a rise in activity in crude extracts from sporophores of age 6-8 days and a subsequent drop to a low level of activity in extracts of 11 day fruit-bodies (Figure 17). The drop in activity in older sporophores is probably due to senescence as sythesis of new enzyme decreases but turnover of existing enzyme continues; the changes in activity at this stage are probably not of great significance in the life-cycle of the mushroom. The rises in activity around day 6 occur after the bulk of mannitol accumulation has taken place (above) and are unlikely to be related to mannitol biosynthesis.

The polyacrylamide-gels stained for phosphatase activity (6.1.2.) showed the non-specific acid phosphatase studied in section 4 to be dominant and it is likely that assays of phosphatase activity in extracts (above) reflect only the level of this enzyme. However, the polyacrylamide-gels indicated other phosphatase enzymes, active against fructose-6-phosphate, are present in the sporophore (Table 14). The level of two of these phosphatases (II and III)

increased from day 0 to day 3 as judged by the intensity of activity stain on the gels and it is during this period that substantial mannitol synthesis occurs in the sporophore (above). It is possible that one or both of these enzymes has a specific physiological role in producing fructose for mannitol synthesis.

If such phosphatase enzymes are involved in mannitol synthesis, they could be subject to metabolic control and they could act in conjunction with a kinase. The results presented here show there is fructokinase activity in crude extracts throughout sporophore development but give no indication as to the number or nature of kinase enzyme(s) involved. There is a drop in fructokinase activity late in development around day 12 (Figure 18). This, again, could be due to the onset of senescence and is probably not important in the life-cycle of the mushroom since it occurs after sporulation. There was an apparent peak in fructokinase activity at day 10 and, whilst one set of results cannot be conclusive, it is possible that fructose produced by mannitol oxidation at this stage in sporophore development is being phosphorylated for catabolism and energy production; mannitol levels in the sporophore are known to fall at this late stage of development (Hammond and Nichols, 1976a). The fructokinase activity is at a similar level to the mannitol dehydrogenase activity around days 10 and 11 (approximately 0.2μmoles substrate consumed $min^{-1} mg^{-1}$ soluble protein; Figures 14

and 18) and, as calculated above for the dehydrogenase, could therefore phosphorylate fructose produced from the entire mannitol pool in approximately 2h. However, it has been suggested above that mannitol oxidation is not an important *in vivo* role for mannitol dehydrogenase, and the kinase may similarly not be of importance in activating fructose for further metabolism in 10 day old sporophores. The fructokinase enzyme(s) assayed here have not been shown to be specific. The enzyme(s) may be non-specific hexokinases mobilising hexoses in general, rather than fructose in particular.

7. Summary and Conclusions

Mannitol dehydrogenase from Agaricus bisporus has been purified to homogeneity (3.1.1.). The native enzyme is a tetramer of subunit molecular weight 29,500 (3.1.2.). After electrophoresis on polyacrylamide-gels of crude extracts of A. bisporus sporophores at any stage of development, mannitol dehydrogenase activity appears to be associated with a single protein band (6.1.2.). The enzyme shows a high degree of specificity for NADP(H) as coenzyme and fructose as hexose substrate. Specificity towards the polyol substrate is less complete as mannitol dehydrogenase shows a limited amount of activity against sorbitol (3.1.4.).

Mannitol dehydrogenase from A. bisporus catalyses the interconversion of mannitol and fructose but its physiologically important role is the reduction of fructose to mannitol in the developing sporophore (sections 3 and 6).

The reaction equilibrium is strongly influenced by pH (3.2.). The rates of fructose reduction and mannitol oxidation and the Km for fructose all show significant variation with pH in vitro (3.1.5.). Mannitol dehydrogenase has a high Km for fructose (3.1.5.), especially in relation to physiological levels of this hexose, indicating the supply of fructose to the enzyme could limit mannitol synthesis in the sporophore (3.2.). The mannitol

dehydrogenase reaction is sensitive to the $NADP^+$/NADPH ratio *in vitro*, especially in the direction of mannitol oxidation (3.1.6.). The effects of compartmentalisation of mannitol dehydrogenase and reactants have not been studied here but are a potential form of regulation of the reaction. Although mannitol dehydrogenase does not exhibit any allosteric properties (3.1.5.), any of the effects of pH, limiting fructose supply, $NADP^+$/NADPH ratio and compartmentalisation could individually or synergistically be of importance in controlling the rate and amount of mannitol biosynthesis *in vivo* in the *A. bisporus* fruit-body.

Fructokinase activity was detected in extracts of *A. bisporus* sporophores but the enzyme(s) responsible for this activity were difficult to purify and characterise (section 5).

Fructose-6-phosphatase activity was detected in extracts of *A. bisporus* sporophores (4.1.; 6.1.3.). The presence of this activity along with fructokinase and mannitol dehydrogenase activities permits mannitol metabolism to be integrated into a general scheme for carbohydrate metabolism in *A. bisporus* as shown in Figure 19.

At least three phosphatases, active against fructose-6-phosphate, are present in *A. bisporus* sporophores at various stages of development. One of these enzymes, phosphatase I, is more active than the other phosphatases,

Figure 19: A generalised scheme for carbohydrate metabolism in A. bisporus with particular reference to mannitol synthesis and turnover.

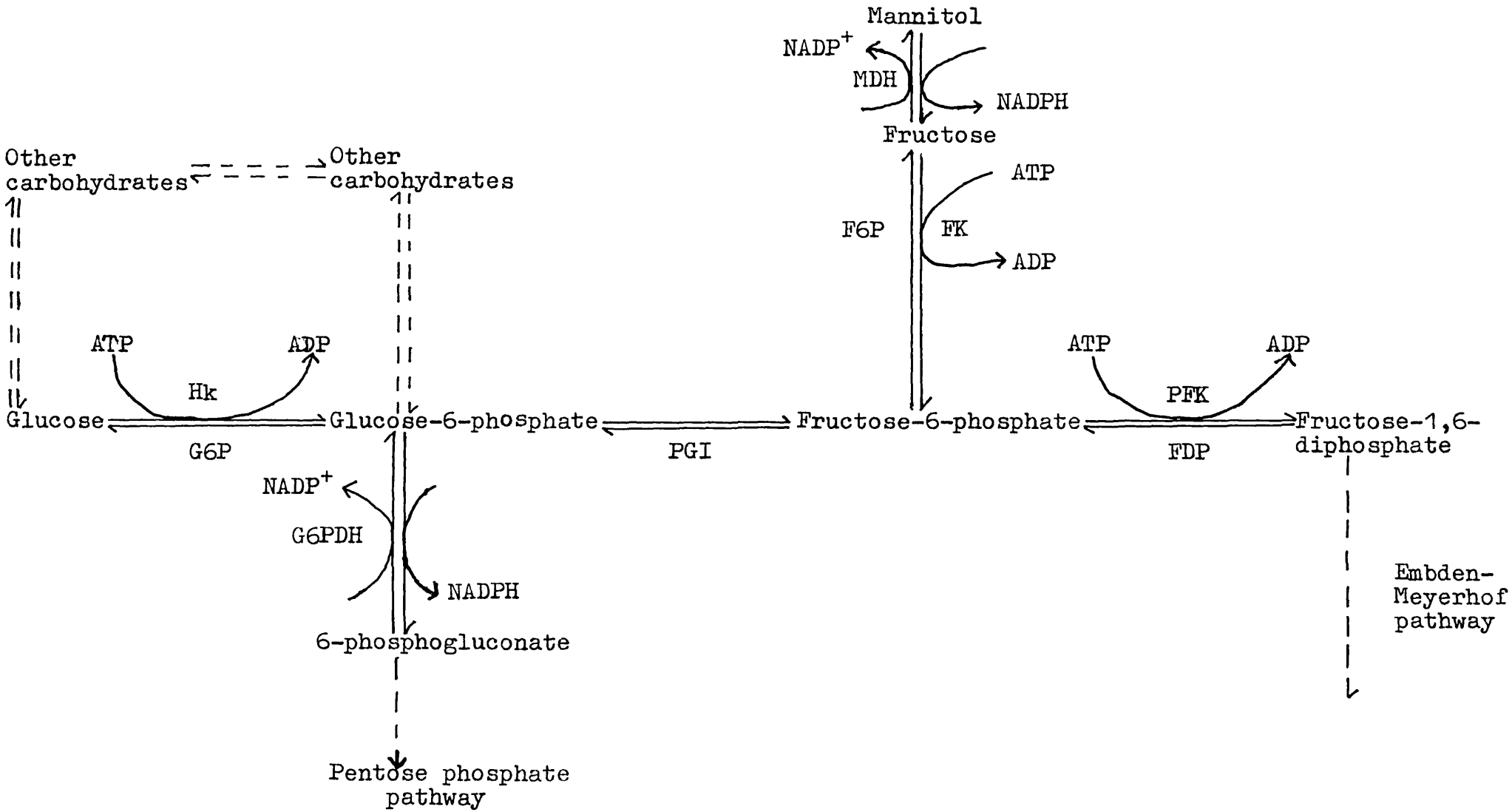
Hk - Hexokinase G6P - Glucose-6-phosphatase

G6PDH - Glucose-6-phosphate dehydrogenase

PHI - Phosphoglucose isomerase F6P - Fructose-6-phosphatase

Fk - Fructokinase MDH - Mannitol dehydrogenase

PFk - Phosphofructokinase FDP - Fructose diphosphatase


Mannitol
NADP+
MDH
NADPH
Fructose
Other carbohydrates
Other carbohydrates
ATP
F6P
FK
ADP
ATP
ADP
Hk
ATP
ADP
PFK
Glucose
Glucose-6-phosphate
Fructose-6-phosphate
Fructose-1,6-diphosphate
G6P
PGI
FDP
NADP+
G6PDH
NADPH
6-phosphogluconate
Embden-Meyerhof pathway
Pentose phosphate pathway

as judged by the intensity of activity staining on polyacrylamide-gels (6.1.3.). Phosphatase I has a low specificity and affinity for fructose-6-phosphate and no regulatory properties were observed (section 4). The remaining phosphatases (II and III) are, at least slightly, more specific for fructose-6-phosphate and could have a role in the synthetic pathway leading to mannitol. If this is the case, the phosphatase(s) may be subject to metabolic regulation. Any such control of the phosphatase reaction would possibly function in conjunction with control of the reverse, namely kinase, reaction.

Other enzymes involved in the scheme shown in Figure 19 could also be involved in the regulation of mannitol production. Hammond and Nichols (1977) noted that there may be a restriction on the glucosephosphate isomerase reaction together with competition for fructose-6-phosphate between the phosphofructokinase and fructose-6-phosphatase enzymes. Under these circumstances, they suggested that the relative proportion of fructose-6-phosphate entering the Embden-Meyerhof pathway compared to that used in mannitol synthesis would depend, at least partially, on the concentration of ATP available to the phosphofructokinase enzyme.

The production of mannitol is regulated by the synthesis of mannitol dehydrogenase and possibly other enzymes in the *A. bisporus* sporophore. The level of mannitol

dehydrogenase activity is highest in extracts of sporophores in which mannitol synthesis is at its highest (6.2.). The level of mannitol dehydrogenase activity falls beyond sporophore stage 2, as would be expected for an enzyme which is active in mannitol synthesis. The changes in mannitol dehydrogenase activity levels roughly correspond to changes in levels of the enzyme itself (6.1.2.). However, mannitol dehydrogenase activity (as calculated in section 6.2.) is higher than that required to account for all changes in sporophore mannitol content throughout development. During the five day period of development from stage 1 to stage 5 there is a 20% increase in dry weight mannitol content but sufficient enzyme activity for this synthesis to occur in approximately 5 min. In addition, enzyme activity levels are high enough in the stage 7 sporophore to oxidise the entire mannitol content in approximately 2h (6.2.). Changes in levels of activity of phosphatase (6.1.3.) and kinase (6.1.4.) enzymes are less easy to interpret in terms of their significance in the development of the sporophore. However, synthesis of specific phosphatase enzymes may be of importance in controlling mannitol production; polyacrylamide-gel electrophoresis indicates increases in the levels of phosphatases II and III at the stage of development associated with the greatest mannitol biosynthesis in the A. bisporus sporophore (6.1.3.; 6.2.).

Appendix I: Determination of Native Molecular Weight of Mannitol Dehydrogenase by Centrifugation Techniques.

The native molecular weight of mannitol dehydrogenase was estimated by centrifugation techniques by Dr A. A. Brunt at the Glasshouse Crops Research Institute, Littlehampton.

a) Analytical Ultracentrifugation (Eason and Campbell, 1978).

Samples of purified mannitol dehydrogenase were dissolved in water to a concentration of 5mg ml^{-1} and loaded into a synthetic boundary cell. The sample was spun at 40,000 rpm at 20°C. The position of the protein boundary was monitored by Schlieren optics every 16 min for 80 min. A sedimentation coefficient ($S_{20,w}$) of 5.95 was calculated by the graphical method of Markham (1960). From this an approximate value for the molecular weight of 113,574 was estimated.

b) Rate Zonal Centrifugation (Martin and Ames, 1961).

Partially purified preparations of mannitol dehydrogenase were mixed with bovine serum albumin and horse liver alcohol dehydrogenase and applied to the top of a centrifuge tube containing a 20 to 50% (w/v) sucrose gradient. This was spun for 22h at 50,000 rpm and 5°C in a "Beckman L8-55" preparative ultracentrifuge with

an SW60 titanium rotor. At the end of this period, the tube was unloaded into a fraction collector. Fractions were assayed for mannitol dehydrogenase activity and optical absorbance at 280nm. Two determinations were carried out; the molecular weight was estimated as 129,000 and 131,000 by the method of Martin and Ames (1961).

Appendix II: Considerations of the Effect of pH on Enzyme-Catalysed Reaction Rates (after Roberts, 1977).

Alterations in pH will affect the state of ionisable groups in the enzyme. The following is a simplified account of the possible resultant effects on the enzyme's kinetic parameters.

a) The ionisation effect may occur in the free enzyme such that the enzyme has two ionisable groups and the substrate only binds to one of the three ionisable forms. This can be summarised thus:

$$\begin{array}{ccccccc} & & E^- & & & & \\ & K_{2e} & \Vert & & & & \\ & & EH & + S \rightleftharpoons EHS & \xrightarrow{k_2} & EH + P & \\ & K_{1e} & \Vert & & & & \\ & & EH_2^+ & & & & \end{array}$$

where E = enzyme, S = substrate and P = product. The dissociation constant, K_S, is given by k_{-1}/k_1. From steady-state equilibria and the conservation equation for the enzyme species ($[E_0] = [E^-] + [EH] + [EHS] + [EH_2^+]$), an equation for the rate of reaction, "v", can be derived:

$$v = \frac{k_2 [E_0] [S]}{[S] + K_S \{1 + (K_{2e}/[H^+] + [H^+]/K_{1e})\}}$$

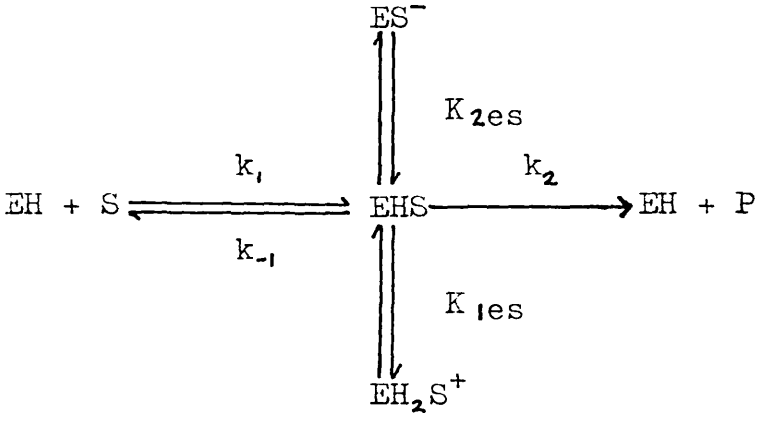
Hence, $Km = K_S (1 + K_{2e}/[H^+] + [H^+] / K_{1e})$ and $Vmax = k_2[E_O]$.

In this system, the equation for "v" at low pH where $[H^+] \gg K_{2e}$ approximates to

$$v = \frac{k_2 [E_O] [S]}{[S] + K_S \{1 + ([H^+]/K_{1e})\}}$$

which has the form of an equation for inhibition with hydrogen ions acting as competitive inhibitors. Also in this system Vmax is not affected by hydrogen ion concentration but Km will vary with pH; in the hypothetical case a plot of pKm against pH will have a slope of +1 below pK_{1e} and of -1 above pK_{2e}, and pKm will be a maximum (and hence Km a minimum) at a pH equal to $(pK_{1e} + pK_{2e})/2$.

b) The ion ionisation effect may occur in the enzyme-substrate complex and not in the free enzyme. Using the same format as above, this can be represented by:



From steady-state equilibria and the conservation equation

for the enzyme species ($[E_o] = [EH] + [ES^-] + [EHS] + [EH_2S^+]$), an equation for the rate of reaction, "v" can again be derived:

$$v = \frac{k_2 [E_o][S]}{K_S + [S]\left\{1 + ([H^+]/K_{1es}) + (K_{2es}/[H^+])\right\}}$$

$$\text{Hence, } Km = \frac{K_S}{\left\{1 + ([H^+]/K_{1es}) + (K_{2es}/[H^+])\right\}}$$

$$\text{and } Vmax = \frac{k_2 [E_o]}{\left\{1 + ([H^+]/K_{1es}) + (K_{2es}/[H^+])\right\}}$$

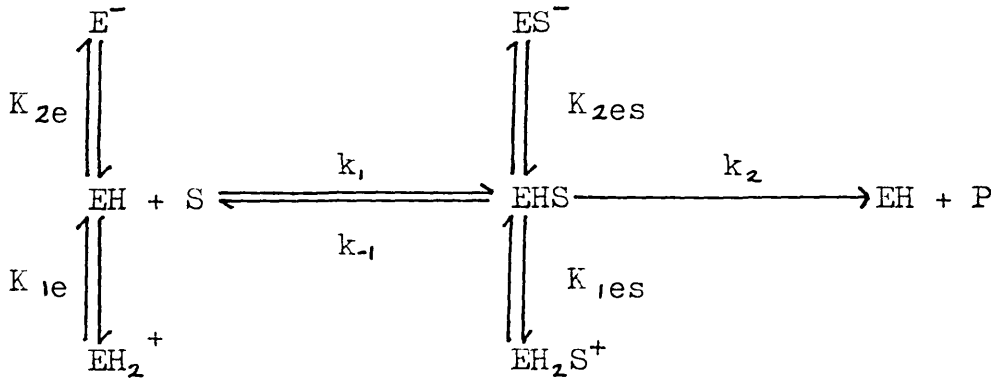
In this system, the equation for "v" at low pH where $[H^+] \gg K_{2es}$ approximates to

$$v = \frac{k_2 [E_o][S]}{K_S + [S]\left\{1 + ([H^+]/K_{1es})\right\}}$$

which has the form of an equation for inhibition with hydrogen ions acting as uncompetitive inhibitors. In this system both Vmax and Km vary with pH; in the hypothetical case, a plot of Log Vmax against pH will have a slope of +1 below pK_{1es} and of -1 above pK_{2es} and Log Vmax (and hence Vmax) will be a maximum at a pH equal to $(pK_{1es} + pK_{2es})/2$; also a plot of pKm against pH will have a slope of -1 below pK_{1es} and of +1 above pK_{2es}, and pKm will be a minimum (and hence Km a maximum) at a pH equal to $(pK_{1es} + pK_{2es})/2$.

c) The two schemes above can be combined with ionisation occurring in the free enzyme and the enzyme-substrate

complex, thus:



From steady-state equilibria and the conservation equation for the enzyme species ($[E_o] = [E^-] + [EH] + [EH_2^+] + [ES^-] + [EHS] + [EH_2S^+]$), an equation for the rate of reaction, "v", can again be derived.

$$v = \frac{k_2 [E_o][S]}{\left\langle K_S \left\{1 + ([H^+]/K_{1e}) + (K_{2e}/[H^+])\right\} + [S]\left\{1 + ([H^+]/K_{1es}) + (K_{2es}/[H^+])\right\}\right\rangle}$$

Hence $Km = \dfrac{K_S \left\{1 + ([H^+]/K_{1e}) + (K_{2e}/[H^+])\right\}}{\left\{1 + ([H^+]/K_{1es}) + (K_{2es}/[H^+])\right\}}$

and $Vmax = \dfrac{k_2[E_o]}{\left\{1 + ([H^+]/K_{1es}) + (K_{2es}/[H^+])\right\}}$

In this system there will be little or no variation in Km with pH. Variation will occur when the pKa values of

the two groups in the enzyme are altered upon binding the substrate, i.e. $K_{1e} \neq K_{1es}$ and/or $K_{2e} \neq K_{2es}$. The degree of variation of Km with pH below K_{1e} will depend on the degree of perturbation in pK_{1e} upon binding of the substrate, and the degree of variation of Km with pH above K_{2e} will similarly depend on the perturbation in K_{2e} upon binding of the substrate. No variation in Km with pH will be observed if the pKa values of the ionisable groups are unchanged by the binding of the substrate, i.e. $K_{1e} = K_{1es}$ and $K_{2e} = K_{2es}$. In this case the equation for "v" becomes

$$v = \frac{k_2 [E_o] [S]}{\left\langle (K_s + [S]) \left\{ 1 + ([H^+]/K_{1e}) + (K_{2e}/[H^+]) \right\} \right\rangle}$$

In this system, the equation for "v" at low pH where $[H^+] \gg K_{2e}$ approximates to

$$v = \frac{k_2 [E_o][S]}{\left\langle (K_s + [S]) \left\{ 1 + ([H^+]/K_{1e}) \right\} \right\rangle}$$

which has the form of an equation for inhibition with hydrogen ions acting as non-competive inhibitors. In this system, Vmax will vary with pH as described in section b above. Mannitol dehydrogenase appeared to fit such a scheme with a variation in Vmax but no variation in Km for $NADP^+$ or NADPH with pH (3.2.).

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