STUDIES ON SORBITOL DEHYDROGENASE IN ACETOBACTER SUBOXYDANS

Ahmed Lotf Al-Amari

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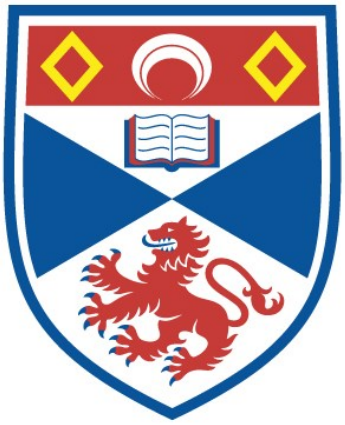

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by

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submitted to the University of St. Andrews in application

for the degree of Doctor of Philosophy.

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ABSTRACT

Studies on Sorbitol Dehydrogenase in Acetobacter Suboxydans

Ahmed Lotf Al-Amari

A chemically defined medium containing no amino acids supported cellular growth of A. suboxydans NCIB 9108. The effectiveness of selected carbon sources for the growth of the organism in a carbohydrate / salts medium was investigated. Growth was, in general, stimulated by the addition of vitamins to a sorbitol / salts medium. The role of yeast extract as a supplier of growth factors is described. The product of sorbitol oxidation by whole growing cells of A. suboxydans was found to be sorbose. G.L.C. analysis showed that sorbitol was completely oxidised to sorbose.
Induction of sorbitol dehydrogenase activity by A. suboxydans in the presence of various carbon sources was investigated. Production of sorbitol dehydrogenase during the growth of A. suboxydans on all the carbon sources investigated was found to be maximal in the late log phase.

Sorbitol dehydrogenase from A. suboxydans was purified by ammonium sulphate fractionation, chromatography on DEAE - Cellulose and bioaffinity chromatography on 5' AMP - Sepharose 4B. The purification of the partially purified enzyme increased 17 fold (after DEAE - Cellulose chromatography) and 80 fold (after 5' AMP - Sepharose 4B

bioaffinity chromatography) compared with the crude extract.

The enzyme has a pH optimum in the range of 6.5 - 7.0 with a maximum velocity of 5.35 U. mg^{-1}. The K_m was found to be 176 mM for fructose and 0.028 mM for NADH.

Inhibition studies involving sorbitol, mannitol, sorbose, nicotinamide, salicylic acid, P - chloromercuribenzoate and o - iodosobenzoate demonstrated competitive inhibition with all inhibitors studied except sorbose which exhibited uncompetitive inhibition. The K_i values were found to be 19 mM, 600 mM, 13 mM, 5 mM, 10.4 mM, 1.3 mM and 3.6 mM respectively.

DECLARATION.

I hereby declare that the following thesis is based on work carried out by me, that the thesis is my own composition, and that no part of it has been presented previously for a higher degree.

The research was carried out in the Department of Biochemistry and Microbiology, Faculty of Science, University of St. Andrews, under the supervision of Dr. W. M. Ledingham.

(Ahmed L. Al-Amari)

CERTIFICATE

I hereby certify that Ahmed Lotf Al-Amari has spent twelve terms engaged in research work under my direction and that he has fulfilled the conditions of Ordinance General No.12 and the Resolutions of the University Court 1967, No.1 and that he is qualified to submit this thesis for the degree of Doctor of Philosophy.

(Dr. W. M. Ledingham)

CAREER

I first matriculated as a research student in the Faculty of Science (Department of Biochemistry and Microbiology), University of St. Andrews in October 1975 to work under the supervision of Dr. W.M. Ledingham, following a fifteen-year professional career in Medical Laboratory Technology; including a three-year World Health Organisation Fellowship in the Department of Microbiology, Royal Free Hospital, School of Medicine, London.

I was admitted to the Associateship of the Institute of Medical Laboratory Sciences, London (AIMLS) in August, 1969. I later took up employment in the Health Service in the Yemen Arab Republic as a clinical microbiology laboratory scientific officer.

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to Dr. W. M. Ledingham for his continued encouragement and guidance throughout this work and to Maria do Socorro Santos Ferreira, Abu Bakar Bin Salleh and Dr. R. Griffiths for the many helpful discussions.

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Finally, I wish to thank also Professor G.R.Tristram and all members of his staff for the opportunity of working in his department, to the World Health Organisation for the grant during the first seven terms and to my father-in-law for his encouragement and support.

ABBREVIATIONS

Unless otherwise stated the unit of temperature used is in degrees centigrade, all other abbreviations in this thesis are those recommended by the IUPAC-IUB commission on Biochemical Nomenclature as published in the Biochemical Journal Instruction to Authors (revised) 1975 (1).

C O N T E N T S

1. INTRODUCTION

Acetobacter suboxydans (A. suboxydans)(Gluconobacter suboxydans (2)), a small gram-negative highly aerobic rod-shaped organism, is one of the more important members of the group commonly known as the acetic acid bacteria. Its discovery by Kluyver and Leeuw (3), did much to broaden the use of the acetic acid bacteria in industrial processes. Their ability to oxidise ethanol to acetic acid, the basis of vinegar manufacture (4), is one particular example. In addition to this acetic acid bacteria oxidise numerous carbohydrates to compounds which are of industrial or biochemical interest (5). Oxidation carried out by the acetic acid bacteria occurs at specific groupings of the substrate molecule and the rates of oxidation are high with the reaction frequently proceeding to completion. These advantages over tedious chemical oxidation procedures resulted in widespread industrial applications in the late 1950s. Newer synthetic chemical syntheses, based on petroleum feedstocks, later replaced many microbial processes but the latter may yet become important again in times of dwindling fossil fuel reserves.

1.1 Metabolism of Carbohydrate

The carbohydrate metabolism of A. suboxydans has been studied extensively. There is, however, one other feature of metabolism worth noting. Cheldelin (6), William and Rainbow (7), reported that A. suboxydans lacks a fully operative Tricarboxylic acid cycle (TCA). King and Cheldelin (8,9), Kondo and Ameyma (10), reported that

acetate, α-ketoglutarate, malate, succinate, fumarate and citrate are oxidised by washed cells or cell-free extracts of A. suboxydans while pyruvate is only oxidised to the acetate stage.

Stouthamer (11), also noted the non-oxidisability of succinate, fumarate, DL-malate and α-ketoglutarate in two strains of A. suboxydans. Oxidative decarboxylation of α-ketoglutarate to succinate was shown not to occur by Rao (12), According to King and Cheldelin (13), resting cells of A. suboxydans failed to oxidise citrate, α-ketoglutarate, succinate or fumarate even in the presence of glycerol as a potential "sparker". Soluble and insoluble fractions of the cells also failed to oxidise TCA cycle members even in the presence of a hot water extract of the organism or methylene blue, or both. Acetate and oxaloacetate were not condensed to citrate even in the presence of Co-enzyme A and Adenosine 5' diphosphate.

From these experiments it appears that neither the TCA cycle nor the C_4-dicarboxylic acid cycle participates in the oxidative metabolism of A. suboxydans, yet two pathways have been suggested by Sekizawa et al. (14), for glutamate synthesis from acetate catalysed by cell extracts. The first is a partial TCA cycle following an initial condensation of oxaloacetate and acetyl Co-enzyme A; the second is a citramalate-mesaconate pathway following an initial condensation of pyruvate and acetyl Co-enzyme A. In order to determine which pathway functions in growing cells (15), acetate-1-^{14}C was added to a culture growing in minimal medium. The experiments established that growing

cells synthesised glutamate via a partial TCA cycle.

A. suboxydans has the capacity for limited oxidation of a large number of polyols, and for excretion of the oxidation products into the growth medium. If one assumes that these oxidations provide the cell with energy for growth, then this obligate aerobe would have little need for an oxidative TCA cycle. The partial TCA cycle found in this bacterium then would appear to function primarily for glutamate, aspartate and succinate biosynthesis.

Edson and McCorkindale (16), showed that the polyol dehydrogenase of rat liver reacted specifically with diphosphopyridine nucleotide (DPN)(NAD) and polyhydric alcohols. Arcus and Edson (17), demonstrated that A. suboxydans contains at least two polyol dehydrogenases:

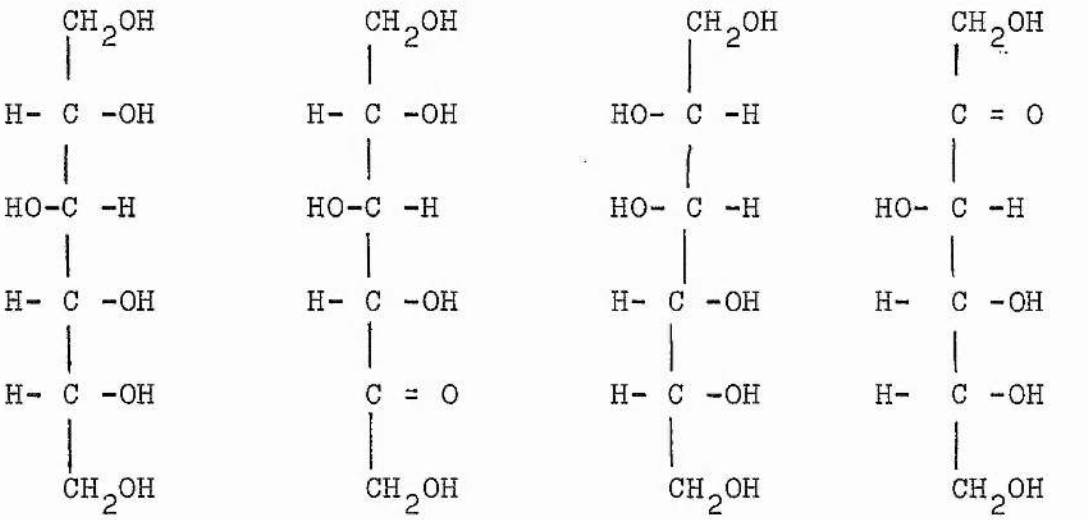
a) a particulate dehydrogenase which catalyses the oxidation of polyols at pH 5.0 without addition of nicotinamide-adenine dinucleotide (NAD). The enzyme was associated with particles containing cytochrome and was named cytochrome linked D-mannitol dehydrogenase.

b) a soluble NAD-linked polyol dehydrogenase with activity best developed at pH 8.0. The enzyme oxidised mannitol vigorously and was named NAD-linked D-mannitol dehydrogenase.

Bertrand (18), working with Acetobacter xylinum, and Hudson (19), working with A. suboxydans, stipulated the structural requirements of the polyol which would allow the organism to oxidise the substrate.

The Bertrand-Hudson rule states that *Acetobacter species* oxidise polyols which have *cis* hydroxyl groups adjacent to a primary (or secondary) alcohol, and the carbon immediately adjacent to the alcohol is the position which is oxidised.

A. suboxydans is somewhat more specific in that the *cis* hydroxyls must be of the D-configuration



D-sorbitol L-sorbose D-mannitol D-fructose

Cummins *et al.* (20), found that cell free preparations of *A. suboxydans* possess three pathways for the oxidation of sorbitol

a) a particulate dehydrogenase which catalyses a one-step oxidation of sorbitol (presumably to hexose)

b) a soluble dehydrogenase which catalyses sorbitol to sorbose in the presence of nicotinamide-adenine dinucleotide phosphate ($NADP^+$).

c) a soluble dehydrogenase which catalyses sorbitol to fructose in the presence of NAD^+.

The pyridine nucleotide thus appear to determine which of the pathways in the soluble fraction will be employed. Subsequent studies of Shaw and Bygrave (20), have shown that A. suboxydans has several NAD^+ and $NADP^+$-linked soluble polyol dehydrogenases, which can be divided into the following two categories:

a) particulate

D-mannitol : cytochrome oxidoreductase

b) soluble

NAD^+-linked D-mannitol dehydrogenase

NAD^+-linked sorbitol dehydrogenase

$NADP^+$-linked D-mannitol dehydrogenase

NAD^+-linked D-erythro dehydrogenase

$NADP^+$-linked xylitol dehydrogenase

With particular reference to the metabolism of sorbitol, investigations began when Embden and Griesbach (21), discovered that sorbitol was converted into fermentable reducing sugars from which the only identifiable osazone prepared was phenylglucosazone. On the basis of changes in optical rotation, these authors considered that the primary product of sorbitol oxidation was fructose, which was subsequently transformed into glucose. Additional evidence consistent with this view was provided by Anschel (23), who observed a large increase in the excretion of fructose following administration of sorbitol to a patient suffering from fructosuria.

The work of Embden and Griesbach (22), prompted the suggestion that sorbitol might prove a useful carbohydrate source in diabetes mellitus (24); but attempts to assess the utilisation of sorbitol in diabetics by measurement of respiratory quotients and blood-sugar levels led to much controversy.

Blakley (25), decided to try and obtain a more precise definition of the initial steps in sorbitol metabolism. Such information was desirable in view of the antiketogenic property of sorbitol described by Edson (26), who found that sorbitol was more effective than glucose or fructose in supressing spontaneous ketogenesis in the liver slices of starved rats. Blakley (25), found that sorbitol was rapidly oxidised by rat liver whether the animal had been well fed or starved. In agreement with Edson (26), he found that 0.01 M sorbitol caused a marked decrease (25-50%) in acetoacetate formed endogenously by liver slices from starved rats. Blakley (25), stated that the oxidation of D-sorbitol in rat liver slices and homogenates is catalysed by a dehydrogenase using Co-enzyme I, and that the oxidation of sorbitol to fructose is a reversible reaction with a $K_{eq} = 0.24 \pm 0.013$.

1.2 Nutritional requirements of A. suboxydans

1.2.1 Assimilation of ammonium salts as nitrogen source

Hoyer (27), reported on the ability of certain acetic acid bacteria to utilise ammonium salts as a sole source of nitrogen when either ethanol or acetic acid was used as the carbon source. Hoyer's solution was proposed as a

synthetic medium for such organisms. It consists of $(NH_4)_2SO_4$ 1 g, $K_3PO_4.H_2O$ 1 g, $MgSO_4.7H_2O$ 1 g, $CH_3.COONa$ 1 g, ethanol 30 ml, and distilled water 1 l. Frateur (28), modified Hoyer's solution to the following composition: $(NH_4)_2SO_4$ 1 g, KH_2PO_4 0.9 g, K_2HPO_4 0.1 g, $MgSO_4.7H_2O$ 0.25 g, 95% ethanol 30 ml and 0.5 ml of an aqueous solution (1% w/v) of $FeCl_2.6H_2O$ in distilled water to make 1 l.

Shimwell (29), reported that Acetobacter xylinum grew well in a modified Hoyer's glucose medium (containing 2% glucose instead of ethanol).

Gray and Tatum (30), reported that a strain of Acetobacter melanogenus utilised ammonium nitrogen in the presence of thiamine, pantothenic acid, nicotinic acid and p-aminobenzoic acid.

In this respect, Rao and Stokes (31), made an interesting observation, namely that the ability to use ammonium nitrogen was more widespread among the acetic acid bacteria than had previously been suspected, they found many strains of A. suboxydans and Acetobacter melanogenus to have this ability, however, according to their finding, most strains of A. suboxydans required the addition of pantothenic acid, nicotinic acid and p-aminobenzoic acid to the medium as growth factors, and most strains of Acetobacter melanogenus needed thiamine in addition. Carbon sources suitable for the growth of these organisms were glucose, arabinose, mannitol, sorbitol and glycerol.

Rao and Stokes (32), observed that those strains of A. suboxydans and Acetobacter melanogenus incapable of growing in a chemically defined medium with ethanol as a sole carbon source, did grow with the addition of a small amount of yeast autolysate, peptone or liver extract. They suggested that the growth-promoting activity of these biological materials was due to some reducing sugars in them and in fact, glucose, fructose, mannitol or glycerol were found capable of replacing yeast autolysate. These reducing sugars were believed to be necessary for initiating growth of the bacteria, which then would utilise ethanol as an additional source of carbon and energy and oxidise it to acetic acid. However, while there seems to be no reports of the mechanism by which ammonia is assimilated, Rainbow (33), suggested that, the initial step in the organism may be the synthesis, catalysed by glutamic dehydrogenase, of glutamate from ammonia and α-ketoglutarate generated by the partial Tricarboxylic acid cycle (TCA cycle), L-aspartate is readily formed from L-glutamate by enzymatic transamination with oxaloacetate while the formation of α-alanine is by transamination between glutamate and pyruvate.

1.2.2 Utilization of carbon source in relation to nitrogen source.

Rainbow and Mitson (34), examined various carbohydrates for their effectiveness as energy sources, using semi-defined medium. The results showed lactate to be the best source of carbon for the growth of A. suboxydans. Sugars and sugar alcohols promoted relatively poor growth or no growth. Glycerol promoted good growth after an initial lag phase.

Fewster (35), also examined various carbohydrates for their effectiveness as an energy source for growth of A. suboxydans NCIB 621 using a complex medium (containing peptone and yeast extract) and a semi-defined medium (containing casein acid hydrolysate, amino acids, minerals and growth factors). Maximum growth was obtained with D-mannitol and D-sorbitol, and good growth with glycerol and $CaCO_3$. Calcium D-gluconate, L-sorbose, D-mannose, D-galactose, D-xylose, L-arabinose and sucrose were reported to give only negligible growth.

According to King and Cheldelin (36), intact cells of A. suboxydans NCIB 621 could oxidise sorbose, lactic acid and pyruvic acid, dihydroxyacetone and acetaldehyde, but could not use these compounds as carbon sources for growth.

1.2.3 GROWTH FACTOR REQUIREMENT

1.2.3.1 Vitamin requirement

In 1942 and 1943, Peterson's group (37, 38), carried out a series of experiments on the specific essential nutrients necessary for the growth of acetic acid bacteria, especially of A. suboxydans. Lampen et al. (37), and Underkolfler et al. (38), found that A. suboxydans NCIB 621, when cultivated in a medium containing casein hydrolysate as a nitrogen source and glycerol as a carbon source, required pantothenic acid, p-aminobenzoic acid and nicotinic acid, while the studies of Landy and Dicken (39), and Landy and Streightoff (40), initially showed that A. suboxydans NCIB 621 could be used as a test organism for the microbiological

assay of p-aminobenzoic acid.

Marshall and Postage (41), reported on an A. suboxydans strain which did not require p-aminobenzoic acid and was resistant to inhibition by sulphonamide.

Biotin was reported by Litsky et al. (42), to be essential for the growth of A. suboxydans, but Karabinoos and Dicken (43), attributed the phenomenon to the possible contamination of biotin with nicotinic acid. According to Hall et al. (44), however, biotin showed good effect on the growth of Acetobacter pasteurianus, Acetobacter ascendens and Acetobacter acidum-mucosum. Nicotinic acid, required for the growth of A. suboxydans, was necessary according to Sarett and Cheldelin (45), for the synthesis of pyridine nucleotide enzymes. Pantothenic acid, was required for growth of Acetobacter ascedas, Acetobacter capsulatus, Acetobacter pasteurianus, Acetobacter turbidans (44), and Acetobacter gluconicus (34).

1.2.3.2 Amino acid requirement

Stokes and Larsen (46), reported on the replacement of vitamin-free casein hydrolysate by a mixture of six amino acids in the growth of a strain of A. suboxydans. Valine, isoleucine and alanine were, according to their findings, the essential amino acids, but with these amino acids alone growth did not take place. Addition of histidine gave a slight growth and addition of cystine or methionine supported a fairly good growth. With further addition of proline, growth increased to the level of that on casein hydrolysate. Development, however, was not so rapid and somewhat less extensive than with yeast extracts. It was also observed that ammonium sulphate was not adequate

for growth, but that at suboptimal concentrations of the required amino acids, it stimulated growth.

In contrast to the results of Stokes and Larsen (46), Hall et al. (44), found that the amino acid requirement of A. suboxydans NCIB 621 was strongly dependent on the pH of the medium. In a glucose medium these were most complex at lower pH values. In a pH range 5.0 - 5.3 the alanine requirement changed from essential to merely stimulatory, and in a glycerol medium the valine requirement changed from non-essential to essential. Valine was not required in a glucose medium, but in a glycerol medium it was required at pH value above 5.3, suggesting a function of glucose in valine synthesis not possessed by glycerol.

According to Kerwar et al. (47), extracts of A. suboxydans NCIB 621 could synthesise valine and isoleucine via acetolactate and acetohydroxybutyrate, respectively. Excess valine in the synthetic medium inhibited growth, the inhibition was reversed by the addition of isoleucine.

The amino acid requirement of A. suboxydans was investigated by Yamada et al. (48), using glucose, fructose, sorbitol or ethanol as independent carbon source. All strains tested (40 in all) had some requirement for glutamic acid.

Belly and Claus (49), decided to clarify the role of amino acids in the nutrition of A. suboxydans NCIB 621 and thereby assess this organism's relative capacity for amino acids biosynthesis. The results obtained demonstrated detailed evidence for the growth of A. suboxydans in the absence of amino acids; which agreed with Kluver and

Leeuw's (3), original description of this species.

Although A. suboxydans was capable of growing slowly without added amino acids, the addition of either L-glutamate, L-glutamine, α-ketoglutarate, L-histidine or L-proline to the basal medium greatly stimulated growth. The addition of either glycine, hydroxy-L-proline, or mesaconate also stimulated growth, but to a lesser extent. Therefore the relationship of the above compounds to the nutrition of A. suboxydans should be considered "stimulatory" or "accessory" rather than "required" as in the case of vitamins (50), or ammonium ions (51).

Each of the compounds that greatly stimulated growth is known to be metabolically converted to glutamate in other cells. This growth-stimulating effect of glutamate and related intermediates seems well correlated with the low activities reported for the acetate-activating complex (12), citrate synthetase (52) and isocitrate dehydrogenase (53). They found that growth-stimulating quantities of amino acids would not replace these requirements. Both these observations suggest that direct amination reactions are essential for growth.

1.3 Sorbitol dehydrogenase [L-iditol: NAD 5-oxido-reductase, EC 1.1.1.14]

Sorbitol dehydrogenase (SDH) is classified with the polyol dehydrogenases. The enzyme was first partially purified by Blakley (25). Since then SDH has been demonstrated in various human, animal and plant tissues. Banks and William-Ashman (54), purified SDH as much as 70 fold

from rat liver, and approximately 10 fold from the rat seminal vesicle and coagulating gland. They found that in addition to the hexitols, D-sorbitol and L-iditol, the pentitols L-arbitol and ribitol were also oxidised by the enzyme. They therefore suggested that due to the wide specificity of the enzyme, its designation as "sorbitol dehydrogenase" was inappropriate, and that the term "ketose reductase" described its properties more adequately. The properties of the enzyme isolated from the seminal vesicle and coagulating gland were found to be similar to those of the liver enzyme.

Banks and William-Ashman (55), measured the relative rates of oxidation of different polyols, using a seminal vesicle preparation of SDH from guinea pig. Identical properties were by similar preparations derived from the seminal vesicle of the rat.

According to the studies of Cummins et al. (20), the cell-free extracts of A. suboxydans contained the enzymes participating (already described) in three pathways of sorbitol oxidation. In addition to a particulate dehydrogenase (56), catalysing one-step oxidation of sorbitol (presumably to hexose), there are two alternative enzyme systems in soluble portions of the cell. One forms sorbose in the presence of $NADP^+$ and the other fructose in the presence of NAD^+.

Fructose is phosphorylated with adenosine-5'-triphosphate (ATP) and is further oxidised via the pentose cycle. This was presumed from the identification of oxidation products

in the presence of NAD^+. Sorbose, however, cannot be further phosphorylated or oxidised with cell-free extract and can be assimilated only in whole cells in the presence of an energy source.

Cummins et al. (20), purified the NAD^+-linked enzyme of the two dehydrogenases about 16 fold and studied its characteristics. The $NADP^+$-linked enzyme was so labile that it was destroyed almost completely during fractionation. The NAD^+-linked sorbitol dehydrogenase was resistant against heat denaturation in the presence of substrate and pyridine nucleotides.

The optimum pH of both enzymes was about the same (8.0 - 8.5), but the activity range for the NAD^+-linked enzyme was much broader. Mg^{++} or Mn^{++} stimulated the activity of both enzymes, Zn^{++} or Ca^{++} did not. Sulphydryl reagents at high concentration inhibited the purified NAD^+linked sorbitol dehydrogenase. Inhibition by p-chloromercuribenzoate and $HgCl_2$ in particular required high concentration. The purified NAD^+-linked sorbitol dehydrogenase was specific for sorbitol and did not oxidise mannitol, ribitol, dulcitol, glycerol, ethanol, acetaldehyde or 2-butene-1-4-diol.

Smith (57), crystalised SDH from sheep liver, and found that the specific activity of the crystalised enzyme was 350 - 400 fold that of the crude extract. Smith (57), also found that the rate of sorbitol oxidation to fructose in the presence of Co-factor NAD^+ had a pH optimum 10.0, and the rate of reduction of fructose to sorbitol in the presence of NADH had a pH optimum 7.0.

Chida et al. (58), purified SDH from rat liver and

investigated its localization using both fluorescence-labelled and peroxidase-labelled antibody methods. Their results showed that activity of the purified enzyme was 350 fold that of the crude extract. The substrate specificity of the purified enzyme was very low; the K_m value was 4.5×10^{-4}M for sorbitol, 2.6×10^{-4}M for xylitol, 2.4×10^{-3}M for ribitol and 3.1×10^{-4}M for NAD^+, respectively. These results accorded with those reported by McCorkindale and Edson (59), on the substrate specificity of partially purified enzyme preparation obtained from rat liver. They reported that the antigen was recognised concentrated around the nucleus and in lower concentrations in the other parts of the cytoplasm of the liver cells in a granular profile. The positive deposits in the electron microscopy were observed in the reticulated amorphous part of the cytoplasm of parenchymal cells, which might correspond to the glycogen area. They also found that the purified SDH showed the same R_f value in disc electrophoresis as that of an enzyme protein which oxidased xylitol in the rat liver homogenate and concluded the possibility that the enzymatic activity to oxidise sorbitol or xylitol in the liver might be derived from the function of single enzyme protein SDH, at least from the histochemical point of view. In other words, the histochemical reaction deposits in the amorphous cytoplasm might be produced by the activity of SDH but not by the xylose dehydrogenase whether the substrate used was sorbitol or xylitol. The results of their work generally coincided with those of Cohen, (60), Chida *et al.* (61), in which the proper enzyme was demonstrated by using a histochemical technique in light and electron microscopy.

Sasajima and Isono (62), investigated the polyol dehydrogenase system using the soluble and particulate fraction of cells of Acetobacter melanogenus in sorbitol medium. NAD^+-linked mannitol dehydrogenase, $NADP^+$-linked mannitol dehydrogenase and NAD^+-linked sorbitol dehydrogenase were obtained from the soluble fractions. They found that the specific activity of these purified enzymes were increased 12 fold, 8 fold and 88 fold respectively, over that of the crude extracts.

NAD^+-linked mannitol dehydrogenase was specific for the interconversion between D-mannitol and D-fructose and was very unstable in alkaline conditions (inactivated at pH $>$ 8.5), while its optimum pH was 8.9.

$NADP^+$-linked mannitol dehydrogenase reduced 5-keto-D-fructose to fructose in the presence of $NADPH_2$. This enzyme was stable against heat and the activity did not decrease even after the incubation period of 30 min at 50^o, while that of the NAD^+-linked enzyme decreased considerably in the course of heat treatment.

NAD^+-linked sorbitol dehydrogenase reduced 5-keto-D-fructose to L-sorbose in the presence of $NADH_2$. The pH optimum was 9.4 for both NAD^+-linked sorbitol dehydrogenase and $NADP^+$-linked D-mannitol dehydrogenase. Cummins et al. (20), reported that $NADP^+$-linked D-mannitol dehydrogenase was less stable than NAD^+-linked sorbitol dehydrogenase. Reduction of D-ribulose found in the soluble fraction of A. suboxydans was reported by Kersters et al. (63), to be NAD^+-linked ribitol dehydrogenase.

Englard and Avigad (64), reported that 5-keto-D-fructose was reduced to L-sorbose by the action of NAD^+-linked SDH while

Sasajima and Isono (62), found that both $NADP^+$-linked mannitol dehydrogenase and NAD^+-linked sorbitol dehydrogenase took part in the reduction of 5-keto-D-fructose which brings about a complicated situation in the metabolism of 5-keto-D-fructose in acetic acid bacteria, although further purification and characterisation of these dehydrogenases would be necessary to obtain a definite conclusion.

The particulate fraction contains the oxidative enzyme system responsible for the oxidation of mannitol, sorbitol, ribitol, D-arabitol, erythritol and glycerol. These oxidations may well be catalysed by one non-specific enzyme.

King (65), showed that not only were ram spermatozoa able to oxidise sorbitol, but, they are also capable of reducing fructose to sorbitol both aerobically and anaerobically. The partially purified SDH prepared from spermatozoa appeared to be similar in its mode of action to the enzyme previously obtained from the rat liver and male accessory glands.

The studies of Herr (66), showed that the fructose secreting seminal vesicle of the sheep contains, in addition to a NAD^+-linked ketose reductase, an aldose reductase which catalyses the reduction of D-glucose to sorbitol with $NADP^+$ as the electron donor. Herr (66) provided strong evidence that fructose formation from glucose in this tissue was a two-step mechanism represented as follows

Aldose-reductase: Glucose + $NADPH+H^+$ $\longleftrightarrow$ sorbitol + $NADP^+$

Ketose-reductase: Sorbitol + NAD^+ $\longleftrightarrow$ fructose + $NADH+H^+$

overall reaction : Glucose + NADPH + NAD^+ $\longleftrightarrow$ fructose + $NADP^+$ + NADH

The K_m for sorbitol of the SDH purified from liver is 0.7 mM according to Blakley (25), compared with 0.45 mM obtained by Chida et al. (58). Myers and Jakoby (67), reported the K_m for fructose to be 300 mM and was increased to 1.43 M in the presence of 30% glycerol and suggested that caution was necessary in interpreting data from protein systems in which polyhydric compounds had been added as stabilizing agents.

SDH is inhibited by borate, cyanide, monoiodoacetate and diaminoethane tetraacetic acid (EDTA), while 0.5 mM $AgNO_3$ or $HgCl_2$ caused 100% inhibition, 0.2 mM 2,3-dimercaptoethanol caused 91% inhibition (68).

Charkavorty et al (69), reported that extracts of Candida utilis contained an NAD^+-linked polyol dehydrogenase which catalyses the reversible oxidation of a number of polyols to the corresponding ketoses. They purified the enzyme about 35 fold from these extracts. The purified enzyme preparations catalyse the oxidation of xylitol to D-xylulose, D-sorbitol and D-mannitol to D-fructose and ribitol to D-ribulose. They found also that at neutral pH theequilibrium strongly favours the formation of the polyol, and that the enzyme was irreversibly inactivated by incubation with EDTA and that NADH but NAD^+ protected against this effect.

Leon and Marr (70), studied a soluble inducible enzyme and reported that sorbitol dehydrogenase was only induced by polyols containing the D-xylo configuration that is, sorbitol and xylitol. They found also that SDH oxidised D-xylo polyols seven times faster than does D-ribo polyols. The substrates oxidised include L-iditol, sorbitol, xylitol and

ribitol and the corresponding 2-ketoses, L-sorbose, D-fructose, D-xylulose and D-ribulose are reduced. The purified enzyme was found to have a specific activity 20 fold higher than that of the crude extracts.

Elsasser et al. (71), studied SDH activity in relation to the growth phase of Acetobacter melanogenus in shaking culture and found that the cells harvested after 11 h "culture" corresponding more or less to the first half of the log phase showed maximum dehydrogenating activity. Quantities up to one half of the sorbitol supplied were dehydrogenated during the log phase, and the remainder was dehydrogenated in the stationary phase.

Schnarr et al. (72), reported that A. suboxydans cells were immobilised in polyacrylamide gels, whereas treatment with glutaraldehyde did not result in intercellular cross-linking, and that, cells from both procedures retained the capacity to oxidise alditols, and showed that the quantitative oxidation of some aldose diethyl dithioacetals was possible with both free cells and glutaraldehyde-modified cells.

1.4 The aims of this research

The work presented here was performed with the view of fulfilling the following objectives:

a) A quantitative study of the nutritional requirements for growth of A. suboxydans.

b) Optimisation of sorbitol dehydrogenase production from A. suboxydans NCIB 9108 under laboratory fermenter conditions.

c) Purification of sorbitol dehydrogenase and the evaluation of substrate and inhibitor kinetic constants.

2. MATERIALS

The chemicals used in this study are listed below in the relevant sections, together with abbreviated names of the suppliers. The full names and addresses of the suppliers are given at the end of this section.

Generally, the chemicals listed are of reagent grade.

Substrates

D(-) Fructose	Sigma
β-Nicotinamide adenine dinucleotide reduced	Sigma
Sodium pyruvate	B.D.H.
D(+) Xylose	Sigma

Enzymes and proteins

Bovine serum albumin	Sigma
Cytochrome c	Sigma
Egg albumin	Sigma
Lactate dehydrogenase	Boehringer
Lysozyme	Sigma

Chromatography components

5' AMP-Sepharose 4 B	Pharmacia
DEAE-cellulose (DE 32)	Whatman
Sephadex G-25 (fine)	Pharmacia

Miscellaneous

Acrylamide	B.D.H.
Agar No.3	Oxoid
p-Aminobenzoic acid	Sigma
Ammonium persulphate	Fisons
Ammonium sulphate	Sigma
Biotin	Sigma
Bromothymol blue	B.D.H.

p-Chloromercuribenzoate	Sigma
Calcium pantothenate	Sigma
Coomassie brilliant blue R_{250}	B.D.H.
Cupric sulphate pentahydrate	B.D.H.
Dipotassium hydrogen phosphate	May and Baker
Disodium hydrogen phosphate	May and Baker
Folin-Ciocalteau phenol reagent	B.D.H.
Glacial acetic acid	B.D.H.
D(+) Glucose	B.D.H.
Glycerol	B.D.H.
Hexamethyldisilazane (HMDS)	Sigma
Hydrochloric acid	B.D.H.
Inositol	Sigma
o-Iodosobenzoate	Sigma
Magnesium sulphate	Fisons
Manganese chloride	May and Baker
Manganese sulphate	May and Baker
D-Mannitol	May and Baker
Methanol (redistilled)	I.C.I.
N,N' methylene-bis acrylamide	B.D.H.
2-Mercaptoethanol	Sigma
Nicotinic acid	Sigma
Nicotinamide	B.D.H.
Peptone (bacteriological)	Oxoid
Potassium chromate	B.D.H.
Potassium dihydrogen phsophate	May and Baker
Potassium hydroxide	B.D.H.
Pyridine	B.D.H.

Salicylic acid	B.D.H.
Silcolapse 5000	Ambersile
Silver nitrate	B.D.H.
Sodium carbonate	B.D.H.
Sodium chloride	B.D.H.
Sodium dihydrogen phosphate	May and Baker
Sodium hydroxide	B.D.H.
Sodium potassium tartrate	May and Baker
Sodium lauryl sulphate (SDS)	B.D.H.
Sodium D(+) tartrate	May and Baker
D-Sorbitol	Sigma
D(-) Sorbose	May and Baker
N N N' N'-Tetramethylene diamine	B.D.H.
Thiamine hydrochloride	Sigma
Trisma base	Sigma
Trimethylchlorosilane (TMCS)	Sigma
Urea	B.D.H.
Xylitol	Sigma
Yeast extract	Oxoid

Ambersil Ltd., Basingstoke, Hants. England.

BDH Chemicals Ltd., Poole, England.

The Boehringer Corporation Ltd., London, England.

Fisons Scientific Apparatus Ltd., Loughborough, England.

May and Baker Ltd., Dagenham, Essex, England.

Oxoid Ltd., London, England.

Pharmacia, Uppsala 1, Sweden.

Sigma London Chemical Co. Ltd., Poole, England.

Whatman Biochemicals Ltd., Kent, England.

3. GENERAL METHODS

3.1 Bacteriological media

Bacteriological media were prepared in glass distilled water, the pH being adjusted to 5.5 prior to autoclaving at 15 psi for 20 min or 1 h depending on the volume of medium. Solid media were prepared by incorporating agar No.3 to a concentration of 15 $g.l^{-1}$.

3.1.1 Simple media

Unless otherwise stated, a chemically defined medium was used, containing ($g.l^{-1}$) 1 g $(NH_4)_2SO_4$, 0.4 g $MgSO_4.7H_2O$, 10 g sorbitol and 10 ml mineral salts solution in 25 mM potassium phosphate buffer pH 5.0.

The mineral salts solution contained ($g.l^{-1}$):

1 g $MnSO_4.4H_2O$

1 g $FeSO_4.7H_2O$

1 g NaCl

3.1.2 Complex media

A general complex medium containing ($g.l^{-1}$) 5 g yeast extract and 10 g sorbitol in 25 mM potassium phosphate buffer pH 5.0 was used, its variations are indicated in the appropriate sections.

3.2. Cultures

A. suboxydans, strains NCIB 621, 3734 and 9108 were maintained in 20 ml of liquid growth medium (3.1.2), by subculturing weekly at 30°. Purity of strains was checked by regular subculture onto solid medium and microscopic examinations of gram-stained slides.

3.3 Growth measurement

3.3.1 Apparatus

3.3.1.1 Apparatus (i)

The apparatus illustrated in Fig. 3.3.1.1.1 consisted of a 5 l conical flask, containing 2 l of medium. Aeration was by means of an air line supplied from a pressure tank (pressure 5 psi; flow rate 1.5 $l.l^{-1}$ min^{-1}), air was filtered as follows:-

a) A course filter for removal of the large air suspended particles.

b) Oil filter for removal of fine oil droplets produced by the compressor.

c) Fine filter (Whatman filter tube, grade 12 - 80) for removal of air-borne organisms.

3.3.1.2 Apparatus (ii)

The apparatus used was the New Brunswick controlled environmental orbital shaker (New Brunswick Scientific Co. Inc. New Brunswick, N.J.) using a 500 ml bacteriological flask containing 250 ml of medium (3.1.2).

3.3.1.3 Apparatus (iii)

The apparatus illustrated in Fig. 3.3.1.3.1 consisted of a magnetically stirred pot vessel (working capacity 0.5 l) containing 400 ml of medium. Aeration subject to filtration as previously described was at 5 psi with a flow rate of 1.5 $l.l^{-1}.min^{-1}$ and was then dispersed by passing into the culture through a sintered glass bulb, the resultant small bubbles were then sparged by four stainless steel blades coupled to the 45 mm magnetic stirrer as in Fig.3.3.1.3.2.

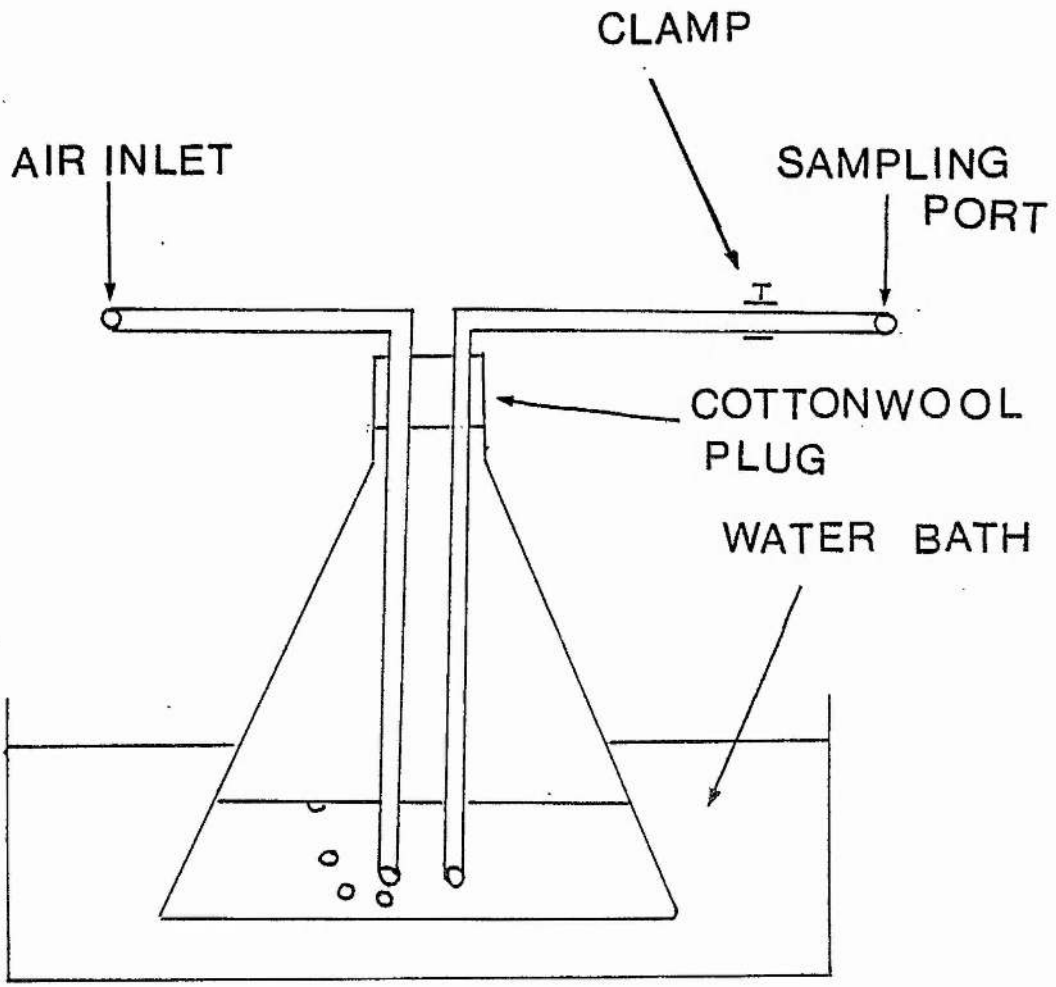


Fig. 3.3.1.1.1. Illustration of 5 l flask culture vessel.

Fig. 3.3.1.3.1 Magnetically stirred culture pot vessel

A. Thermometer
B. Air inlet
C. Sampling port
D. Baffles

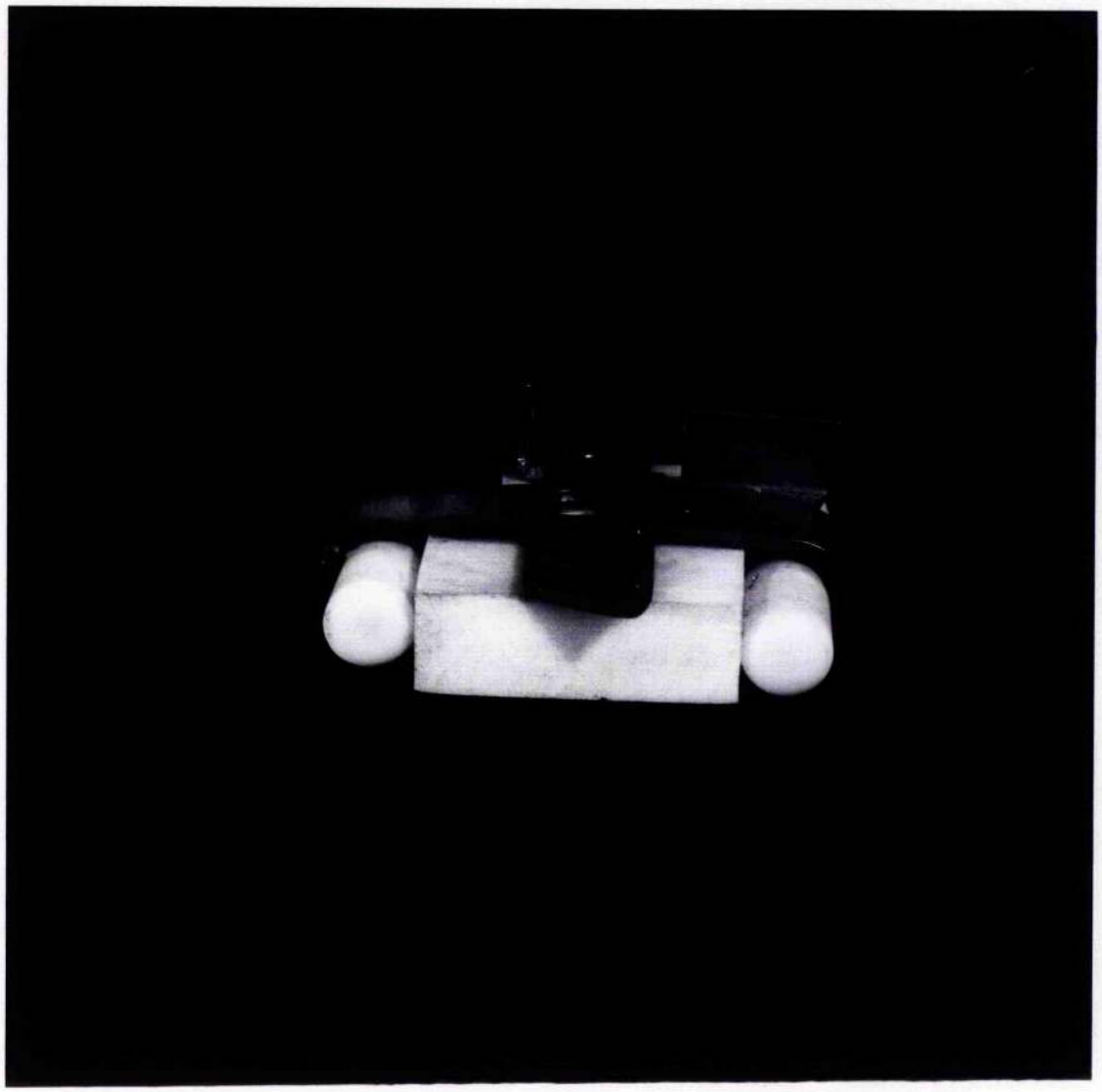


Fig. 3.3.1.3.2. Magnetic stirring system. The centre square is of P.T.F.E. to the sides of which are attached 2 x 45 mm P.T.F.E. magnetic followers. Four vertically mounted stainless steel agitator blades are attached to the P.T.F.E. block.

Temperature control was effected by means of an external thermostatted water bath.

3.3.1.4 Apparatus (iv) The New Brunswick MF 114 Fermenter-

This 14 l fermenter system was used with 12 l of medium containing 0.1% antifoam (Silcolapse 5000 Ambersil Ltd., Basingstoke, Hants.) the complete system was autoclaved for 1 h at 15 psi. Aeration was again subject to filtration as previously described at 5 psi with a flow rate of 1.5 $l.l^{-1}$. min^{-1} and agitation of 300 rpm.

3.3.1.5 Apparatus (v)

The 5 l fermenter (New Brunswick Scientific Co. Inc.) was used with 3 l medium containing 0.1% antifoam (Silcolapse 5000), the complete system was autoclaved as in 3.3.1.4.

Aeration and agitation were the same as in 3.3.1.4.

3.3.1.6 Apparatus (vi)

This Vortex-stirred batch apparatus Fig. 3.3.1.6.1 was built in the departmental work-shop according to the specification of Dr. W. H. Holms, Department of Biochemistry, University of Glasgow. A 1 l round bottom flask containing 400 ml of medium was used, stirring was effected by a 45 mm magnetic follower at 2,000 rpm, and temperature control was by means of thermostatted water bath.

3.3.2 Turbidity measurements

Culture turbidity measurements were taken at 610 nm using a Unicam SP600 spectrophotometer (Unicam Instruments, Cambridge). Samples were diluted with sterile media to give absorbance in the range 0.1 - 0.4., sterile media was used as control.

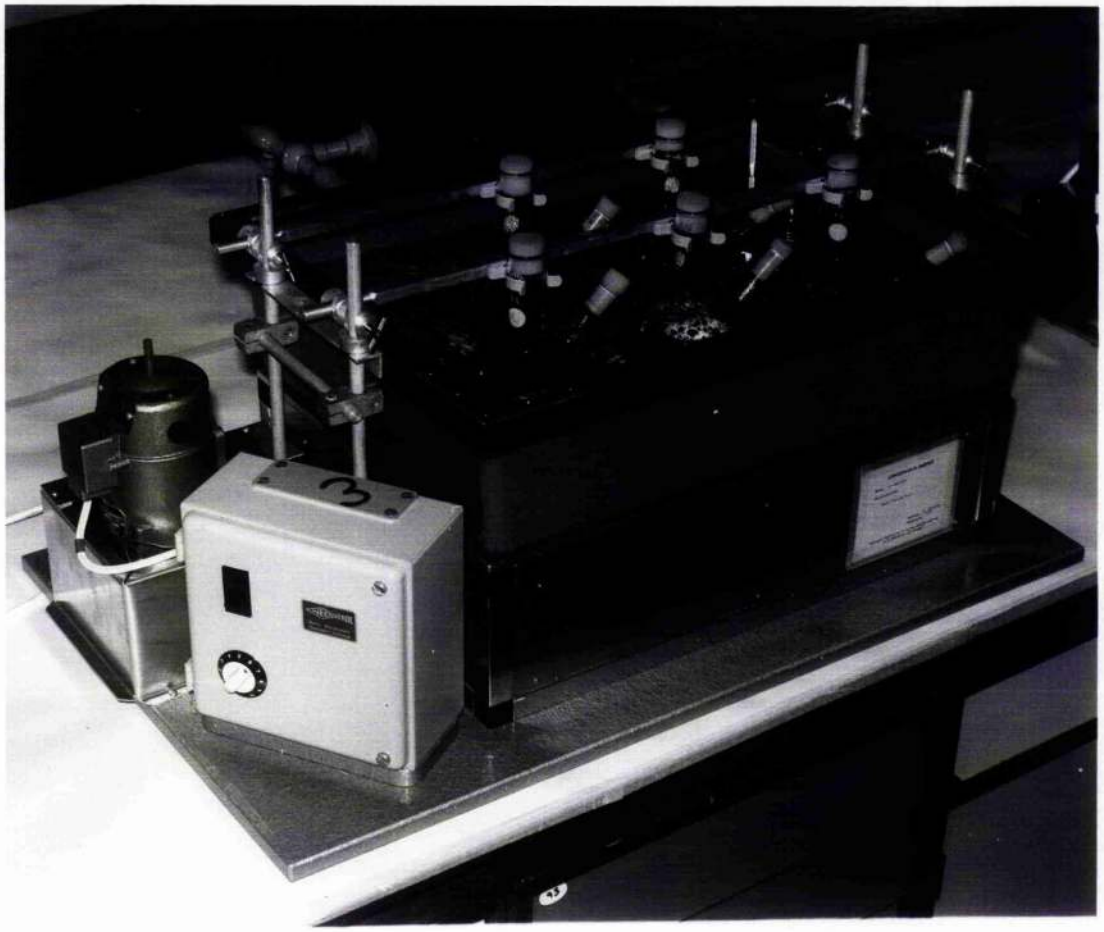


Fig. 3.3.1.6.1 Vortex-stirred batch culture apparatus.

3.3.3 Bacterial growth kinetics

During the exponential phase of growth, the number of cells at time t(X_t) (or the concentration at time t) is related to the initial number of cells X_o by the following relationship

$$X_t = X_o \, e^{\mu t}$$

where e is the base of natural logarithms and μ is the specific growth rate. Algebraic manipulation of this equation can yield the form

$$\mu = \frac{\ln X - \ln X_o}{t}$$

a plot of lnX versus time will therefore, during exponential growth, yield a straight line of gradient μ.

3.3.4 Turbidity/dry weight calibration graph

Three accurately measured 1 ml samples of a thick but pipettable homogeneous cell suspension which had been washed twice with 20 mM Tris-HCl buffer, pH 7.6 and once with distilled water were placed into three pre-weighed crucibles and reduced to dryness at 110° overnight. The crucibles were allowed to cool in a dessicator and reweighed. A series of dilutions (within the absorbance range 0.1 - 0.4) of the cell suspension were prepared and the absorbance measured for each dilution as in 3.3.2.
Figure 3.3.4.1. is a graph of absorbance versus dry weight bacteria (μg).

3.4 Harvesting of large scale cultures

Cultures were harvested on an air turbine driven Sharples continuous flow centrifuge (Sharples Centrifuge Ltd., Peunwalt, Camberley, Surrey). The rate of flow through the centrifuge

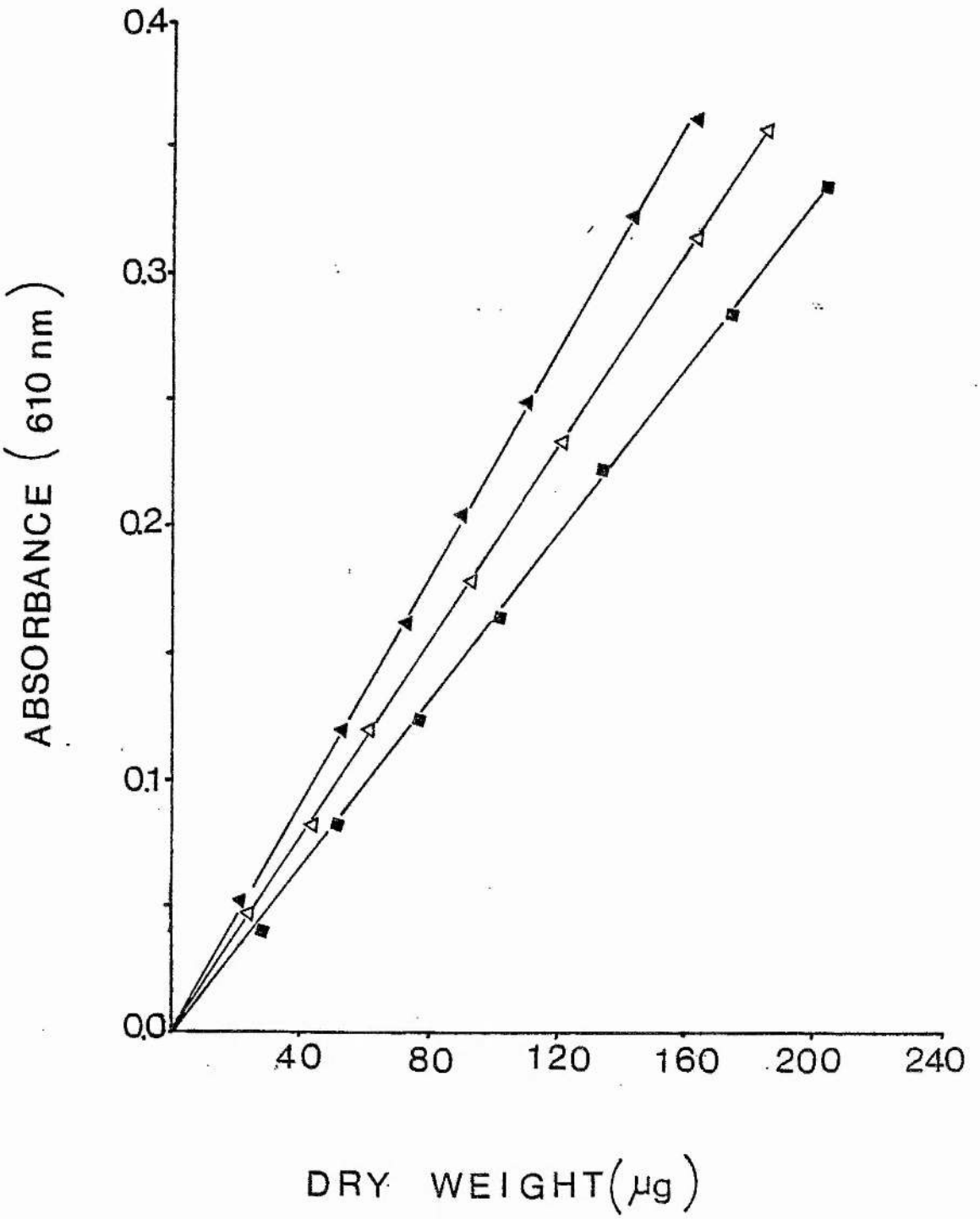


Fig. 3.3.4.1 Absorbance-dry weight calibration graph for A. suboxydans NCIB 3734 (▲), NCIB 621 (▷) and NCIB 9108 (■).

was adjusted to give the fastest flow rate compatible with clear effluent. After centrifugation, the solid cell paste was weighed and then stored at -15° until use.

3.5 Estimation of metabolic activity of whole washed cells

The Gilson Differential Respirometer was used to measure rates of metabolic activity of the whole washed cells. Thick, but pipettable, homogeneous washed cell suspensions (10 - 20 $mg.ml^{-1}$) were prepared. Rates of oxygen consumption were measured in a system of the following composition (final concentrations) in a total volume of 3.5 ml

14.3 mM phosphate buffer, pH 7.6

5.7 mM NAD^+

5.7 mM substrate.

Typically each system contained 10 mg (dry cell equivalent) of bacterial cells. The central well contained strip (3.5 x 10 mm) of Whatman No. 40 filter paper saturated with 5% potassium hydroxide. The flasks were equilibrated at 30°, the contents of the side arms were tipped into the main compartments and the rate of oxygen uptake was measured.

3.6 Cell disruption

3.6.1 Hughes press disruption

A 30% homogeneous washed cell suspension was prepared in 20 mM ice-cold Tris-HCl buffer pH 7.6 and was frozen drop by drop in liquid nitrogen. Frozen cells were loaded into the press which had been cooled to -35° in solid-CO_2/methanol mixture and then disrupted on a hydraulic press at 5,000 psi. The thawed broken cell preparation was

centrifuged (25,000 x g) at 4° for 30 min to remove cell debris and the clear supernatant was retained.

3.6.2 Ultrasonic disruption

A 30% homogeneous washed cell suspension was prepared in 20 mM ice-cold Tris-HCl buffer pH 7.6 and 20 ml aliquots were subjected to sonic treatment using a 60 w MSE ultrasonic disintegrator (MSE Ltd., Crawley, Sussex, England) with 3/8" probe, the tube containing the suspension was placed in an ice-bath prior to disruption and held under these conditions throughout the sonic treatment. The instrument was tuned for 1 min with a cooling period of 1 min between successive exposures. The clear supernatant was separated as in 3.6.1 and retained.

3.7 Methods of enzyme assay

Enzyme assays were carried out by the following methods using a Unicam SP800 Spectrophotometer AR45 recorder (Unicam Instruments Ltd., Cambridge). Unless otherwise stated, all enzyme unit definitions used in this thesis are those recommended by the IUPAC-IUB commission (1).

3.7.1 Sorbitol dehydrogenase (SDH) [EC1.1.1.14] assay

SDH was assayed spectrophotometrically by the decrease in absorbance at 340 nm when reduced nicotinamide-adenine dinucleotide (NADH) is oxidised in the presence of fructose.

In general, the reaction mixture was of the following composition (final concentrations) in a total volume of 3 ml

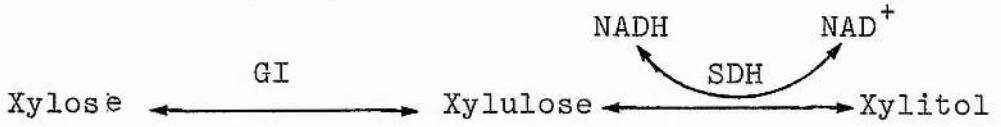
10.67 mM Tris-HCl buffer pH 7.6

93.3 mM D(-) Fructose

0.25 mM NADH

3.7.2 Glucose isomerase (GI) [EC 5.3.1.5] assay

GI was assayed spectrophotometrically on the basis of the following coupled reaction



The rate of decrease in absorbance at 340 nm was recorded when NADH is oxidised in the presence of xylulose. In general the reaction mixture was 3 ml total volume containing (final concentrations) 10.67 mM Tris-HCl buffer pH 7.6, 333.3 mM D(+) xylose and 0.25 mM NADH, with a GI/SDH ratio generally of 8:10.

3.7.3 Lactate dehydrogenase (LDH) assay

LDH was assayed spectrophotometrically by the decrease in absorbance at 340 nm when NADH is oxidised in the presence of sodium pyruvate. The reaction mixture of 3 ml total volume containing (final concentrations) 53.3 mM sodium phosphate buffer pH 7.0, 333.3 mM sodium pyruvate and 0.25 mM NADH.

3.8 Methods of protein determination

Protein determinations were carried out by one of the following methods, employing a Unicam SP600 spectrophotometer for data recordings.

3.8.1 Folin-Lowry Estimation

This method was developed by Lowry et al. (73), (involving the reaction of copper in alkaline solution and the reduction of Folin-Ciocalteau reagent).

Reagents

Solution A : 2% (w/v) sodium carbonate in 0.1 M NaOH

Solution B : 1 g sodium tartrate dissolved in glass distilled water.

0.5 g cupric sulphate pentahydrate
10 ml 1 M NaOH were added and the volume made up to 100 ml with glass distilled water

Solution C : 50 ml A + 1 ml B

Solution D : Folin-Ciocalteau phenol reagent diluted to 1 M with glass distilled water.

A calibration graph (Fig. 3.8.1.1.) was prepared for a range of 1-100 $\mu g.ml^{-1}$ of bovine serum albumin in glass distilled water.

Assay

An aliquot, 0.5 ml, of protein solution (10 - 100 μg) was added to 5 ml solution C, mixed well and left for 10 min followed by the addition of 0.5 ml solution D. The mixture was left for 30 min, and the absorbance measured at 500 and 750 nm against a control in which distilled water replaced the protein solution.

3.8.2 Biuret Estimation

Llayne (74), first described the Biuret method which depends upon a reaction between an alkaline - copper solution and peptides or proteins with a minimum requirement for two peptide bonds, to produce a purple colour with a maximum absorption at 540 nm.

Assay

A 1 ml aliquot of protein solution (containing less than 10 $mg.ml^{-1}$) was mixed with 4 ml Biuret reagent and left at room temperature (20 - 25°) for 30 min, the absorbance of the resulting solution was then read at 540 nm against a control in which distilled water replaced the protein solution.

A calibration graph (Fig. 3.8.2.1) was prepared for a

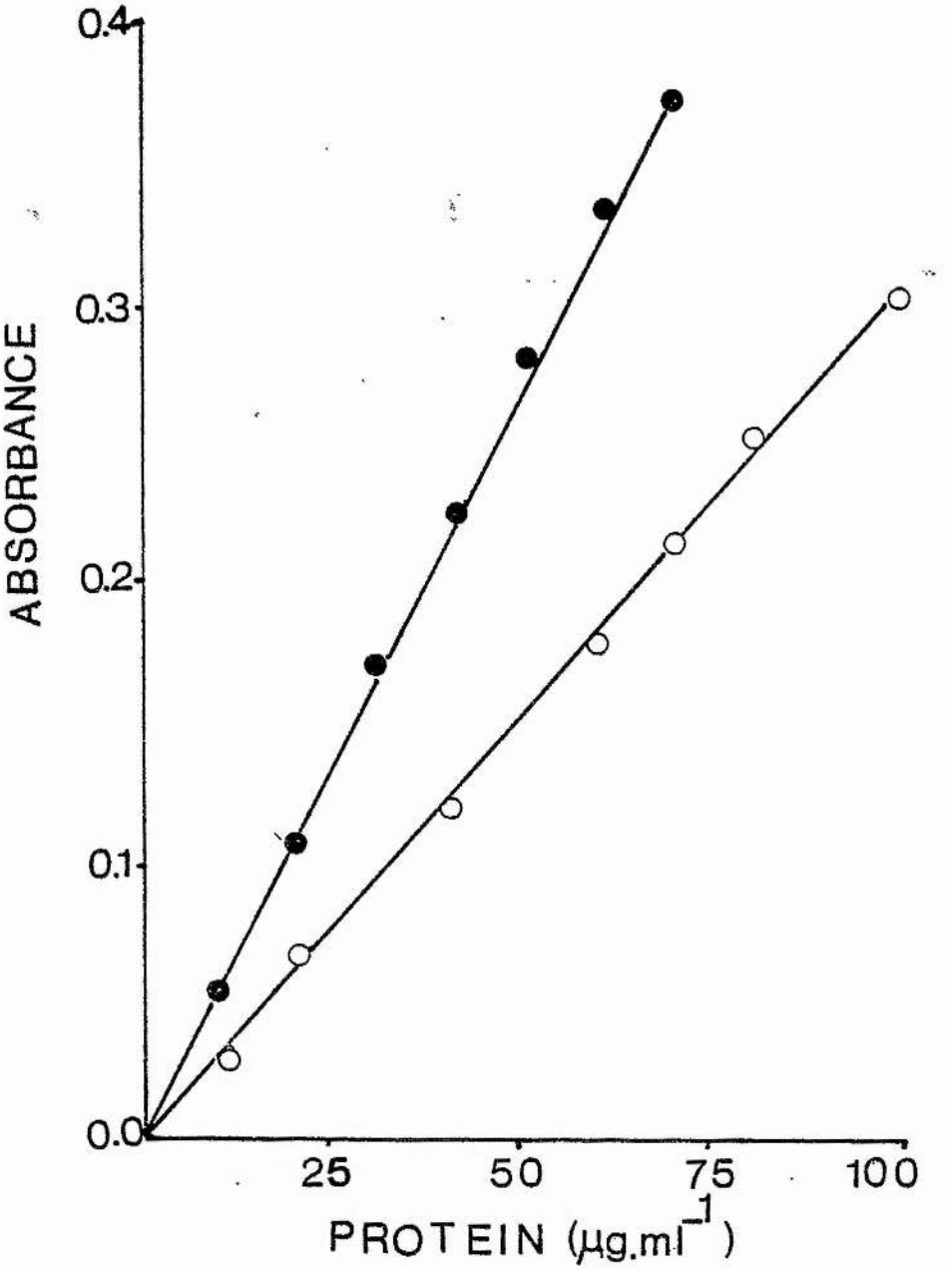


Fig. 3.8.1.1 Calibration graph for the estimation of protein by the method of Lowry et al. at 500 nm (○) and 750 nm (●).

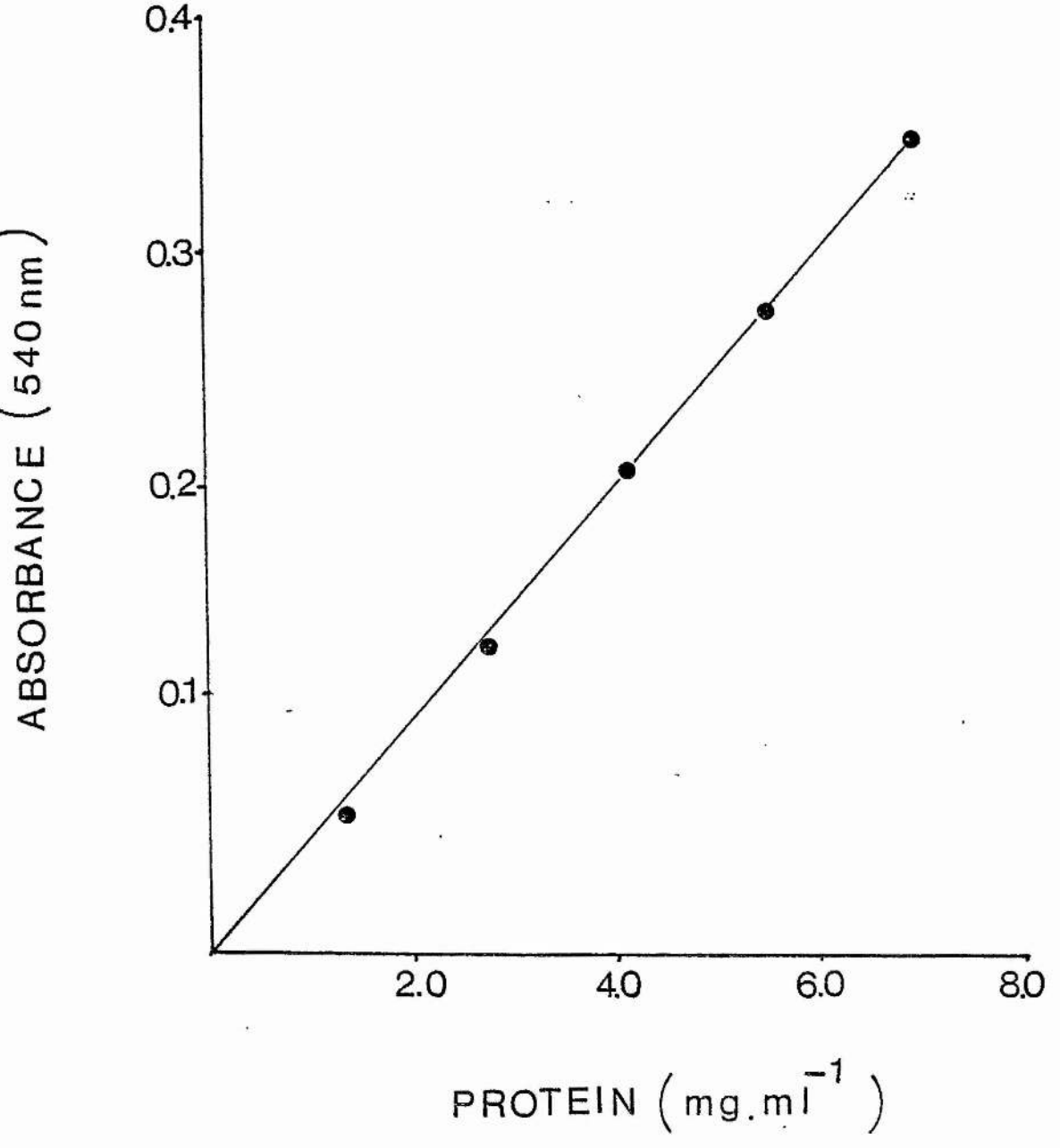


Fig. 3.9.2.1 Calibration graph for the estimation of protein by the Biuret method.

range of 1 - 8 $mg.ml^{-1}$ of bovine serum albumin.

3.9 Gas-liquid Chromatographic (G.L.C.) Analysis of carbohydrates

3.9.1 Apparatus

G.L.C. analysis were performed on a PYE Model 104 gas chromatograph fitted with a flame ionisation detector and using nitrogen as the carrier gas. Samples were injected onto the top of the column through a gas tight septum using a 5 µl calibrated microlitre syringe. The nitrogen flow rate was calibrated using a bubble flow meter. (The individual peaks were recorded on a Phillips P800 chart recorder).

3.9.2 Preparation of material and analysis

Culture samples (10 ml) were centrifuged (600 x g, 15 min) and 0.25 ml aliquots of clear supernatant dried overnight at 35^{o} under vacuum. Solutions of separate and mixed standards (Sorbitol, sorbose and fructose) at concentrations of 25 $mg.ml^{-1}$ of sugar were prepared and 40 µl aliquots were dried as above.

Dried aliquots of culture supernatants and standards were converted into trimethylsilyl (TMS) derivatives by the method of Holligan (75), by adding 0.5 ml of TMS silylating reagent. The reaction was allowed to proceed at room temperature for 30 min and then the mixture was centrifuged to remove the white precipitate (a mixture of ammonium and pyridine chloride).

Samples 2 µl, were injected onto the top of the column and the materials analysed under the following conditions:

Column	5 ft. glass
Column packing	3% SE_{30} on 100 - 200 mesh celite
Column oven temperature	175^{o}
Detector Oven temperature	200^{o}
Injection port heater temperature	230^{o}
Attenuation	1×10^{4}
N_2 carrier flow	45 $ml.min^{-1}$
H_2 flow to detector	45 $ml.min^{-1}$
Air flow to detector	600 $ml.min^{-1}$
Chart speed	10 $mm.min^{-1}$

3.9.3 Determination of peak area and identification

Individual peak areas of traces obtained on the chart recorder from the G.L.C. were calculated by multiplying the peak height (mm) by the width of the same peak at half height (mm). The area of each peak was then used to calculate the sugar concentration from the calibrated standard graphs.

The retention times of the standards were used for the identification of contents of the unknown samples.

Graph calibrations (Fig. 3.9.3.1 and Fig. 3.9.3.2) were prepared for sorbitol and sorbose (range 2-12 $\mu g.ml^{-1}$) respectively as a function of peak area (cm^2) versus sugar ($\mu g.\mu l^{-1}$).

3.10 Chloride Estimation

Reagents

0.1 M Silver nitrate

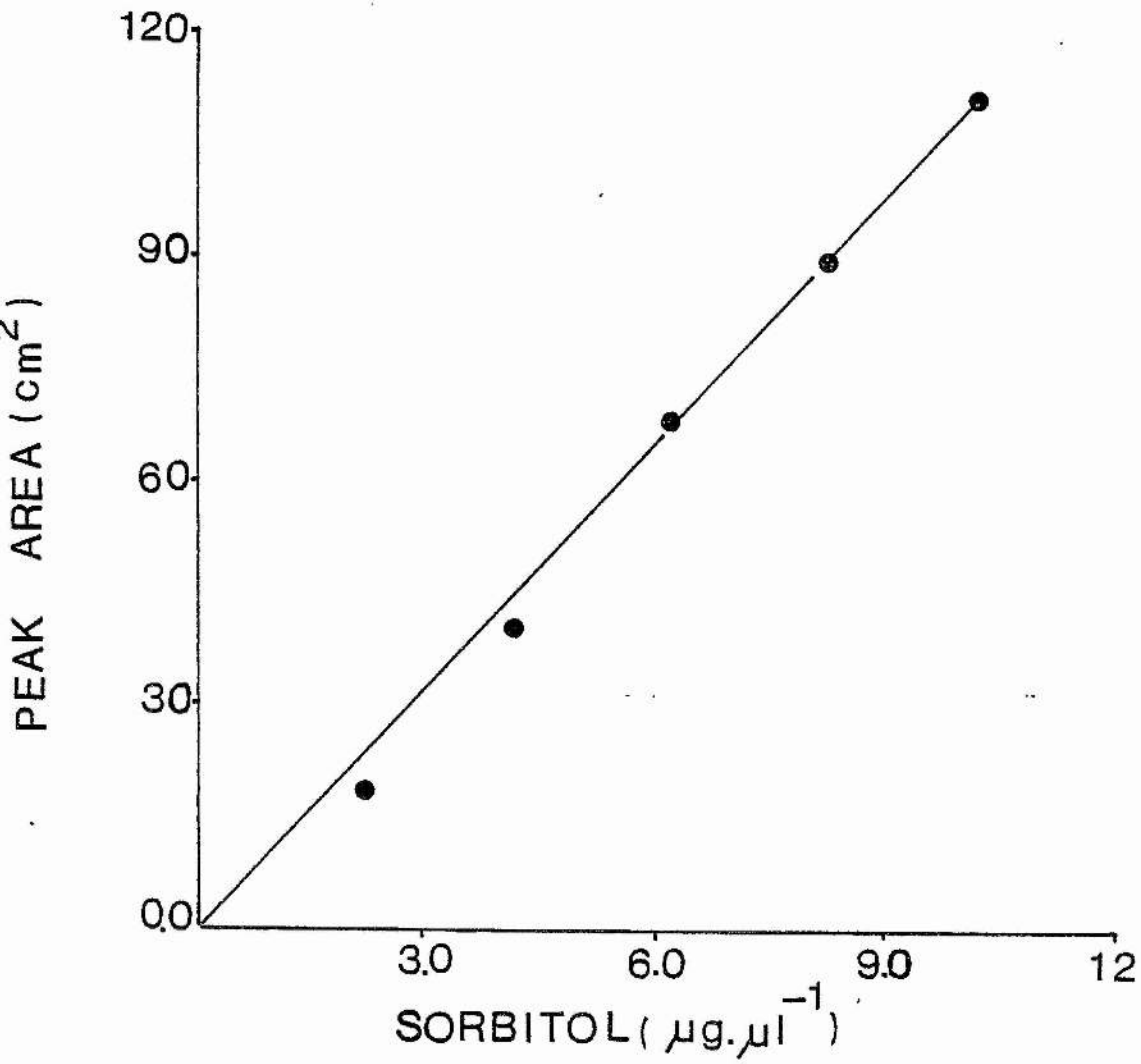
5.0% Potassium chromate

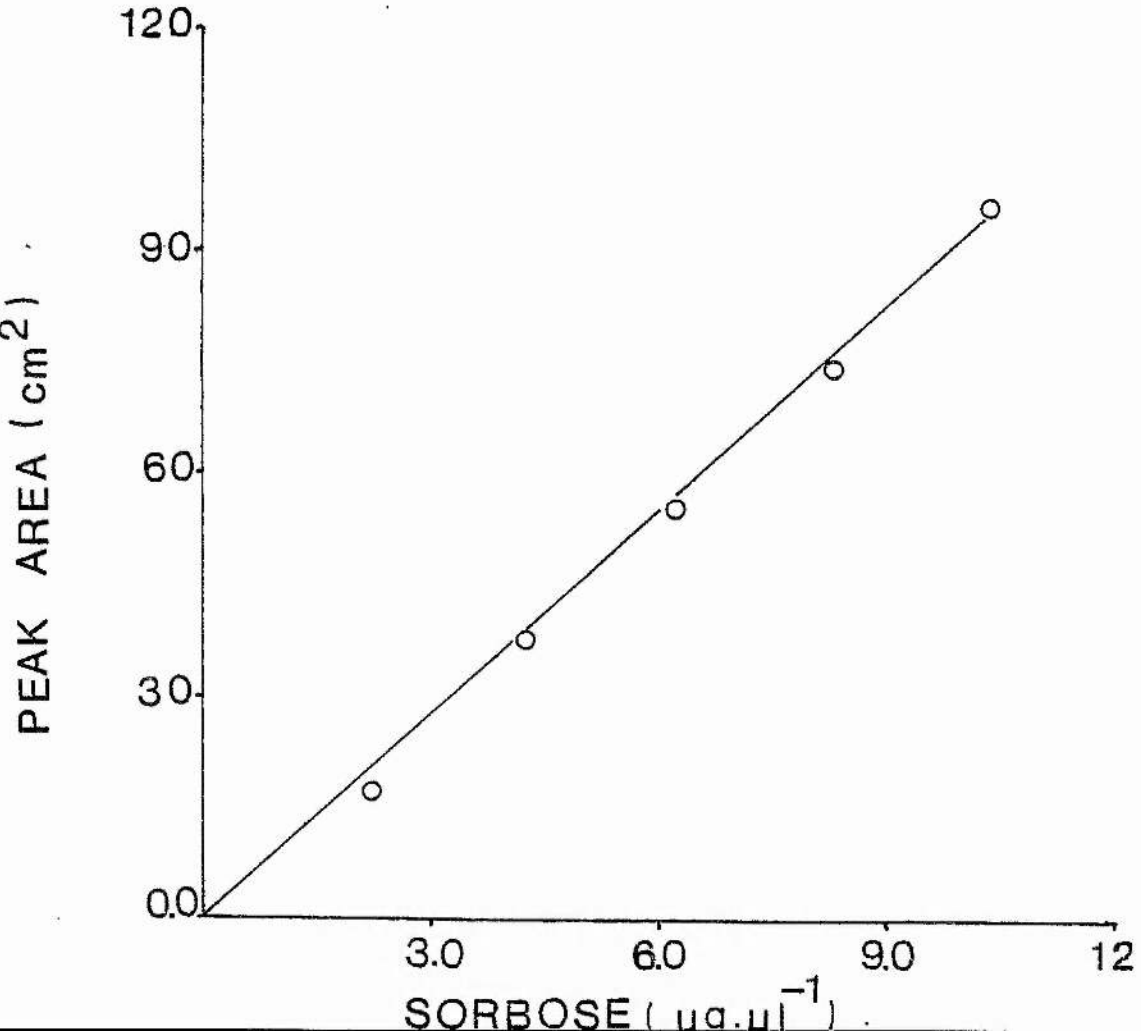
Assay

To 1 ml sample in a 50 ml Erlenmyer flask (containing

Fig. 3.9.3.1 Calibration graph for the estimation of sorbitol by the G.L.C.

Fig. 3.9.3.2 Calibration graph for the estimation of sorbose by the G.L.C.


120
90
60
30
0.0
PEAK AREA (cm^2)
3.0
6.0
9.0
12
SORBITOL ($\mu g.\mu l^{-1}$)


120
90
60
30
0.0
PEAK AREA (cm^2)
3.0
6.0
9.0
12
SORBOSE ($\mu g.\mu l^{-1}$)

less than 0.6 M Cl ion), two drops of 5% potassium chromate were mixed by gentle swirling, then titration was carried out from a 10 ml graduated burette containing 0.1 M silver nitrate with continuous mixing, the end point was taken as the appearance of a red colour.

A calibration graph was established using sodium chloride as a standard (Fig. 3.10.1) .

3.11 Enzyme purification

3.11.1 Preparation of cell-free extract

A 30% homogeneous washed cell suspension was prepared in 20 mM Tris-HCl buffer pH 7.6 and 20 ml aliquots were subjected to ultrasonication for 12 min as described in 3.6.2. The supernatant was separated by centrifugation (25,000 x g) for 30 min at 4^{o}.

3.11.2 Nucleic acid precipitation

The supernatant from 3.11.1 was treated with 0.5 M ice-cold manganese chloride (ratio 1 : 10) to give a final concentration of 0.05 M with respect to the salt. After gentle mixing the pH was adjusted to 6.0 and allowed to stand for 1 h at 4^{o}, after which the supernatant was separated by centrifugation as before.

3.11.3 Ammonium sulphate precipitation

Solid ammonium sulphate crystals were added to the supernatant obtained from 3.11.2 to give a final saturation of 30%. After gentle mixing, the solution was allowed to stand at 4^{o} for 1 h.

The precipitate was then removed by centrifugation (25,000 x g) at 4^{o} for 30 min and the supernatant was adjusted to 61% saturation, allowed to stand for 1 h at

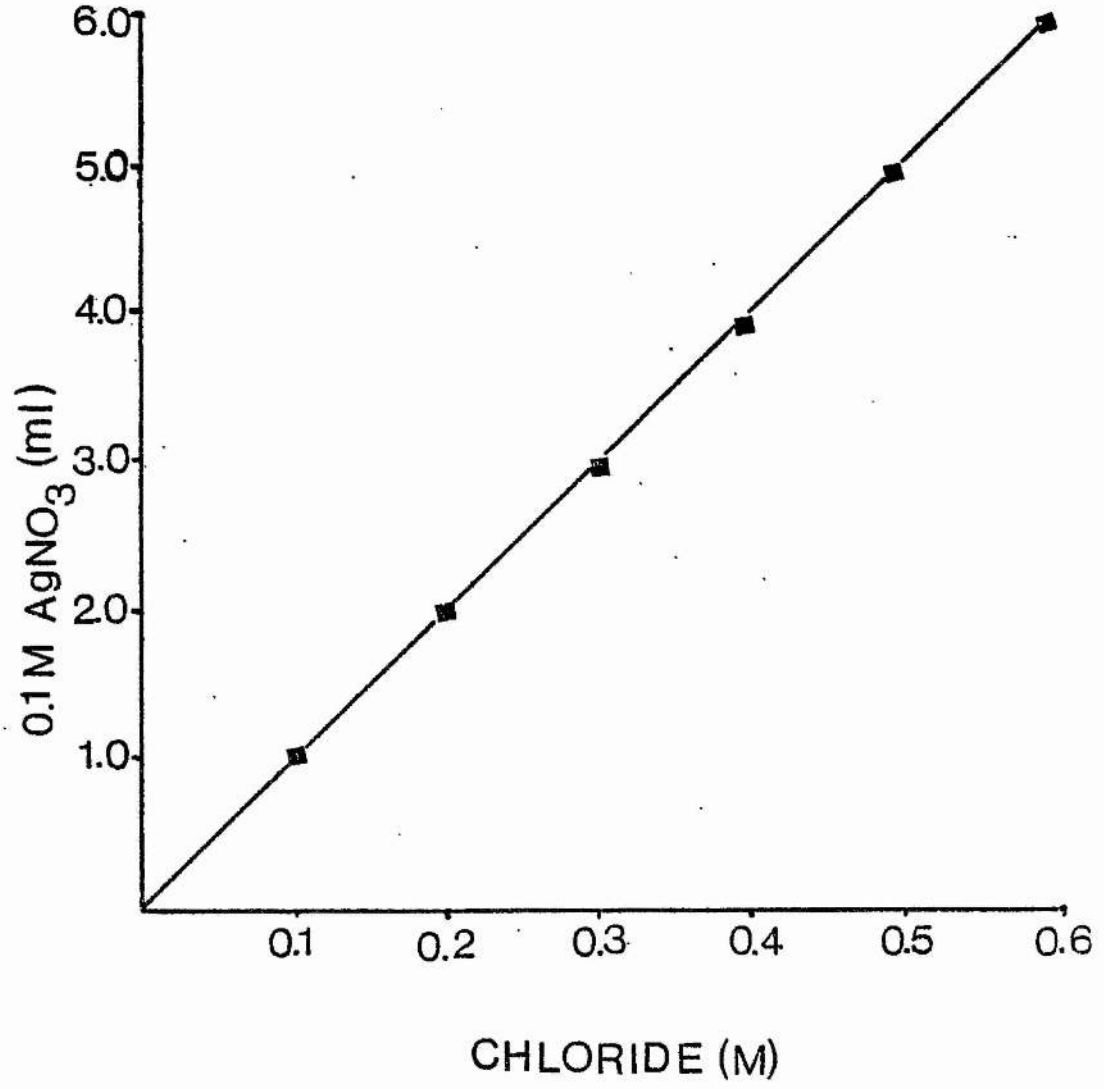


Fig. 3.10.1 Calibration graph for chloride estimation

4° and centrifuged as before, the precipitate being stored at -15° until use.

3.11.4 Ion-Exchange chromatography on DEAE-Cellulose

Preparation of DEAE-Cellulose (DE32)

30 g of DEAE-Cellulose (DE32) were swollen in 1 l distilled water for three days at room temperature and washed several times by sedimentation in distilled water to remove the finings. The DEAE-Cellulose was then precycled as recommended by the manufacturers, by washing alternatively with 0.5 M HCl, distilled water and 0.5 M NaOH, distilled water until the pH value of the washing was below 8.0 then filtered and stirred into 450 ml of the acid component of the Tris-HCl buffer and the pH was adjusted to 4.5, then degassed under vacuum and titrated with the basic component of the buffer, until the pH was 9.0 followed by two changes with 450 ml 20 mM Tris-HCl buffer pH 9.0.

The precycled DEAE-Cellulose was made into a slurry with the same buffer and packed into a 2.5 x 40 cm Pharmacia column (Pharmacia Fine Chemicals, Sweden) to a final bed size of 2.5 cm diameter x 20 cm (height). The column was then equilibrated with 20 mM Tris-HCl buffer pH 9.0 (stepwise elution) and with 0.1 M NaCl in 20 mM Tris-HCl buffer pH 9.0 for gradient elution.

3.11.4.1 Chromatography by stepwise elution

A 5 ml aliquot of crude cell-free extract from 3.11.1 containing 25 - 30 mg protein was applied to the DEAE-Cellulose column. The column was then washed with 200 ml Tris-HCl buffer pH 9.0 to remove any unbound protein. SDH was eluted stepwise with increasing NaCl concentration

as follows:-

250 ml 0.1 M NaCl

125 ml { 0.2 M NaCl
0.3 M NaCl
0.4 M NaCl

250 ml 0.5 M NaCl

The column effluent was monitored by an L.K.B. U.V. recorder and the fractions were collected by an L.K.B. fraction collector.

3.11.4.2 Chromatography by gradient elution

A 5 ml aliquot of enzyme solution from 3.11.3 which had been dialysed in 2 l 20 mM Tris-HCl buffer pH 9.0 overnight at 4^{o} containing 50 - 60 $mg.ml^{-1}$ protein was applied to the column which was then washed with 200 ml 0.1 M NaCl in 20 mM Tris-HCl buffer pH 9.0 to elute any unbound protein. SDH was eluted by a linear gradient of NaCl (0.2 - 0.4 M), total volume 1 l, each gradient chamber contains 500 ml. The column effluent was monitored and the fractions were collected as in 3.11.4.1.

3.11.5 Gel filtration on Sephadex

Preparation of the Sephadex G-25 column

30 g of Sephadex G-25 (Fine) were suspended in 400 ml 20 mM Tris-HCl buffer pH 7.6 and allowed to stand at room temperature for a few hours to swell. The finings were removed by successive decantations and the swollen gel was made into a slurry with the same buffer and packed into a 2.5 x 40 cm Pharmacia column to a final bed size of 2.5 cm (diameter) x 35 cm (height). The Sephadex column was washed with 2 l of the same buffer.

Application of sample

A 5 ml aliquot of enzyme solution from 3.11.3 was applied to the Sephadex column and eluted with 20 mM Tris-HCl buffer pH 7.6. The column effluent was monitored as before and 6 ml fractions were collected as described previously.

3.11.6 Bioaffinity Chromatography on 5' AMP-Sepharose 4 B

2 g lyophilised 5' AMP-Sepharose 4 B (Pharmacia Fine Chemicals) were suspended in 8 ml 0.1 M sodium phosphate buffer pH 7.0 and allowed to swell at room temperature for 3 h. The 5' AMP-Sepharose 4B gel was washed in a sintered glass funnel with 300 ml of the same buffer and the gel was made into slurry with the same buffer and was packed into 0.9 x 10 cm water-jacketed column to a final bed size of 0.9 cm (diameter) x 8.5 cm (height). The column was then equilibrated with 0.1 M sodium phosphate buffer, pH 7.0, and 1 ml aliquot of protein solution from 3.11.3 dissolved in 0.1 M sodium phosphate buffer pH 7.0 was applied to the column. The column was eluted with 40 ml of the same equilibrating buffer to elute unbound protein followed by a wash with 10 ml 0.2 M NaCl in 0.1 M sodium phosphate buffer pH 7.0 to remove any non-specifically bound protein. SDH was eluted with 1 mM NADH in the equilibrating buffer, the effluent was collected in 1.3 ml fractions at a flow rate of 0.2 $ml.min^{-1}$ as described previously.

Fig. 3.11.6.1 is schematic representation of the Sepharose bound 5' AMP analogue.

3.12 SDH polyacrylamide gel electrophoresis

Purity check and molecular weight estimation were performed by SDS polyacrylamide gel electrophoresis according to Weber et al. (76).

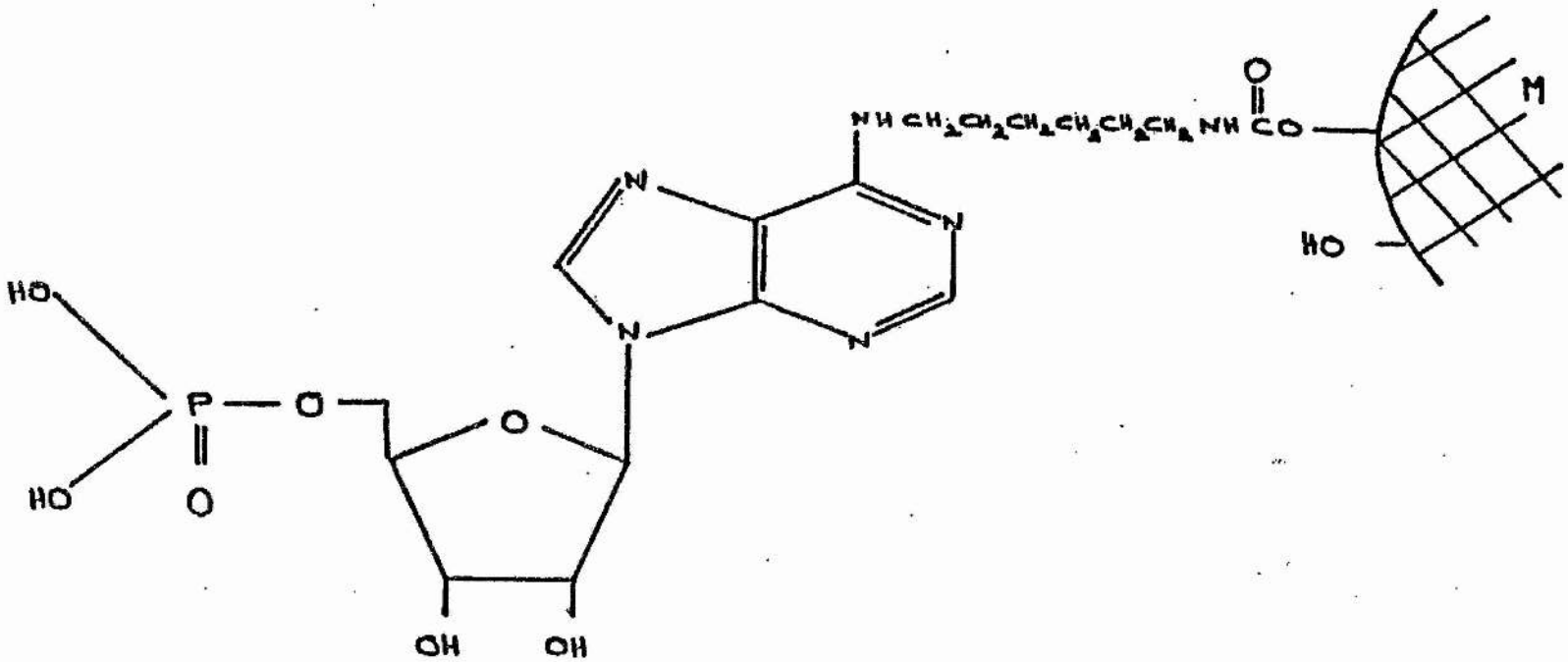


Fig. 3.11.6.1 Schematic representation of the Sepharose bound 5' AMP analogue.

Procedure

The following steps were used:-

a) 0.6 x 10 cm smooth end glass tubes were soaked in chromic acid solution overnight, rinsed with distilled water, and oven dried. Each tube was marked at 7 cm indicating the required gel length, the ends were closed with parafilm and placed in a vertical position in a corrugated plastic rack.

b) 2.5 ml acrylamide stock solution was added to 10 ml gel buffer, mixed and 7.5 ml distilled water added, and well mixed followed by 20 mg ammonium persulphate and 30 µl TEMED, mixed and loaded into tubes up to the mark. Small volumes of distilled water were layered on top of the gels to prevent meniscus formation and ensure a flat gel surface.

c) Stock protein standard solution (5 $mg.ml^{-1}$) were diluted 1 : 10 (0.5 + 4.5 ml) in the chamber buffer and 45 µl 2-mercaptoethanol added to each and then incubated at 37^{o} for 2 h to ensure complete denaturation and reduction of any disulphide bonds, after which time the tubes were removed and cooled to room temperature. The unknown samples were treated in the same way.

d) Into each of two small test tubes, 5µl of tracking dye solution was added then mixed with 1 drop of glycerol. To the first tube 10 µl of each standard was added and to the second tube 10 µl of the unknown sample. These were loaded to the appropriate gel after removal of the water layers. The tubes were then placed in the electrophoresis apparatus, the lower tray was filled with chamber buffer

and the upper tray placed in position; then over both sample and standard tubes chamber buffer was layered using a pasteur pipette and the upper tray was filled with the same buffer. The power supply was connected with the anode in the lower tray and electrophoresis performed using 2 mA per gel until the marker dye migrated to the bottom of the gels.

e) Gels were removed from the glass tubes, the dye position and gel length were measured and proteins were stained using Coomassie brilliant blue R_{250}, after staining, gels were scanned on Vitatron TLD 100 Densitometer under the following conditions:-

Bulb	: Phosphotungstin
Slit	: 2.5 x 0.5
Mode Switch	: $\log^{-}$
Level Switch	: $\mathcal{L}$
Chart speed	: 3 $cm.min^{-1}$
Machine switch	: 3

f) Mobilities were calculated using the following relationship:-

$$\text{Mobility} = \frac{\text{distance moved by protein}}{\text{distance migrated by tracking dye}} \times \frac{\text{length of gel before staining}}{\text{length of gel after staining}}$$

Fig. 3.12.1 is a standard graph obtained by running bovine albumin (MW 68,000), egg albumin (MW 43,000), myoglobin (MW 17,000) and cytochrome c (MW 11,700).

Reagents

Acrylamide stock solution

38.7 g acrylamide and 2.66 g N,N'-methylene-bis

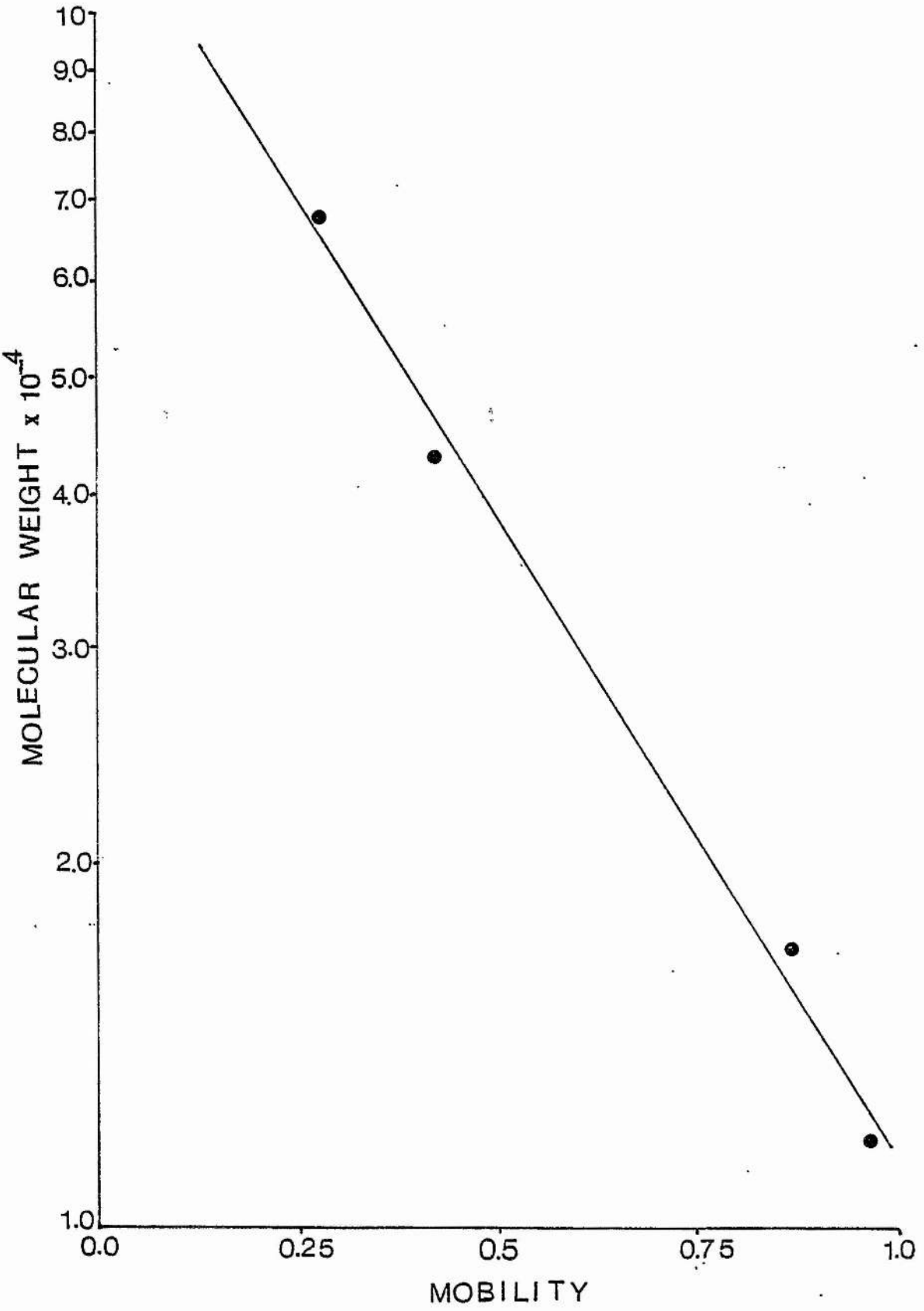


Fig. 3.12.1 Electrophoretic mobilities of the standard proteins on 5% SDS-acrylamide gel.

acrylamide were dissolved in a total volume of 100 ml with glass distilled water and stored at 4^{o}.

Gel buffer

Gel buffer was prepared by dissolving 0.5 g SDS, 0.38 ml TEMED and 120 g urea in 30 ml 0.1 M disodium hydrogen phosphate and 20 ml 0.1 M sodium dihydrogen phosphate. The solution was then made up to 250 ml with glass distilled water and was stored at 4^{o}.

Chamber buffer

Chamber buffer was made of 3 g SDS dissolved in 60 ml 0.3 M disodium hydrogen phosphate and 40 ml 0.3 M sodium dihydrogen phosphate, then the volume was made up to 3000 ml with glass distilled water (final pH 7.0 - 7.2).

Staining solution

Staining solution was made up of 1.25 g coomassie brilliant blue R_{250}, 454 ml 50% methanol and 46 ml glacial acetic acid.

Destaining solution

75 ml glacial acetic acid was added to 250 ml methanol and the volume was made up to 1000 ml with glass distilled water.

Tracking dye

0.05% bromophenol blue in 0.01 M phosphate buffer pH 7.0.

4. RESULTS

4.1 Preparation of inocula

Inoculum of A. suboxydans was prepared from pure culture of the organism on solid sorbitol/yeast extract/agar. All inocula were scaled up (0.25 l and larger) through sorbitol/salts medium (3.1.1), its variations or sorbitol/yeast extract (3.1.2) and its variations where appropriate.

4.2 Effect of batch culture design on culture growth rate

A 10% (v/v) inoculum of A. suboxydans NCIB 621 was introduced into the appropriate apparatus containing sorbitol/yeast extract medium (3.1.2) (culture conditions as described in 3.3.1.1 - 3.3.1.6; temperature 30°). Growth was monitored over a period of eight hours and the specific growth rates (μ) were calculated (see Table 4.2.1).

Table 4.2.1 Effect of culture design on culture growth rate.

Apparatus used	Specific growth rate ($\mu = h^{-1}$)
The New Brunswick controlled environmental orbital shaker (3.3.1.2)	0.39
A magnetically stirred pot vessel (3.3.1.3)	0.414
The New Brunswick MF 114 fermenter (3.3.1.4)	0.41
The New Brunswick 5 l fermenter (3.3.1.5)	0.55
The vortex-stirred batch apparatus (3.3.1.6)	0.633

Evaporation problems rendered the results obtained for the growth in a 5 l conical flask (3.3.1.1) unsatisfactory.

4.3 Measurement of substrate oxidation rates by whole washed cell preparations

A 10% (v/v) inoculum of A. suboxydans NCIB 621 was introduced into 3 l carbohydrate/yeast extract medium (3.1.2) in a 5 l New Brunswick fermenter (culture conditions as described in 3.3.1.5; temperature 30°). Growth was allowed until the stationary phase was reached. The cells were harvested as described in 3.4 and washed three times in 25 mM phosphate buffer pH 7.6. The rates of substrate oxidation were measured as described in 3.5 using sorbitol, xylitol, xylose and glucose.

Graphs of oxygen uptake (µl) versus time (min) were plotted for sorbitol grown cells (Fig. 4.3.1.), glucose grown cells (Fig. 4.3.2 and xylose grown cells (Fig. 4.3.3.).

Table 4.3.1.4 is a summary of the results obtained.

Table 4.3.1.4 Substrate oxidation by whole washed cells of A. suboxydans

Substrate	Oxidation rates (µl O_2.mg dry cell^{-1}.h^{-1})		
	Sorbitol grown cells	Glucose grown cells	xylose grown cells
Sorbitol	9.6	7.45	8
Xylitol	3.6	5.79	8
Xylose	4.2	3.3	8
Glucose	6.0	6.2	5.7

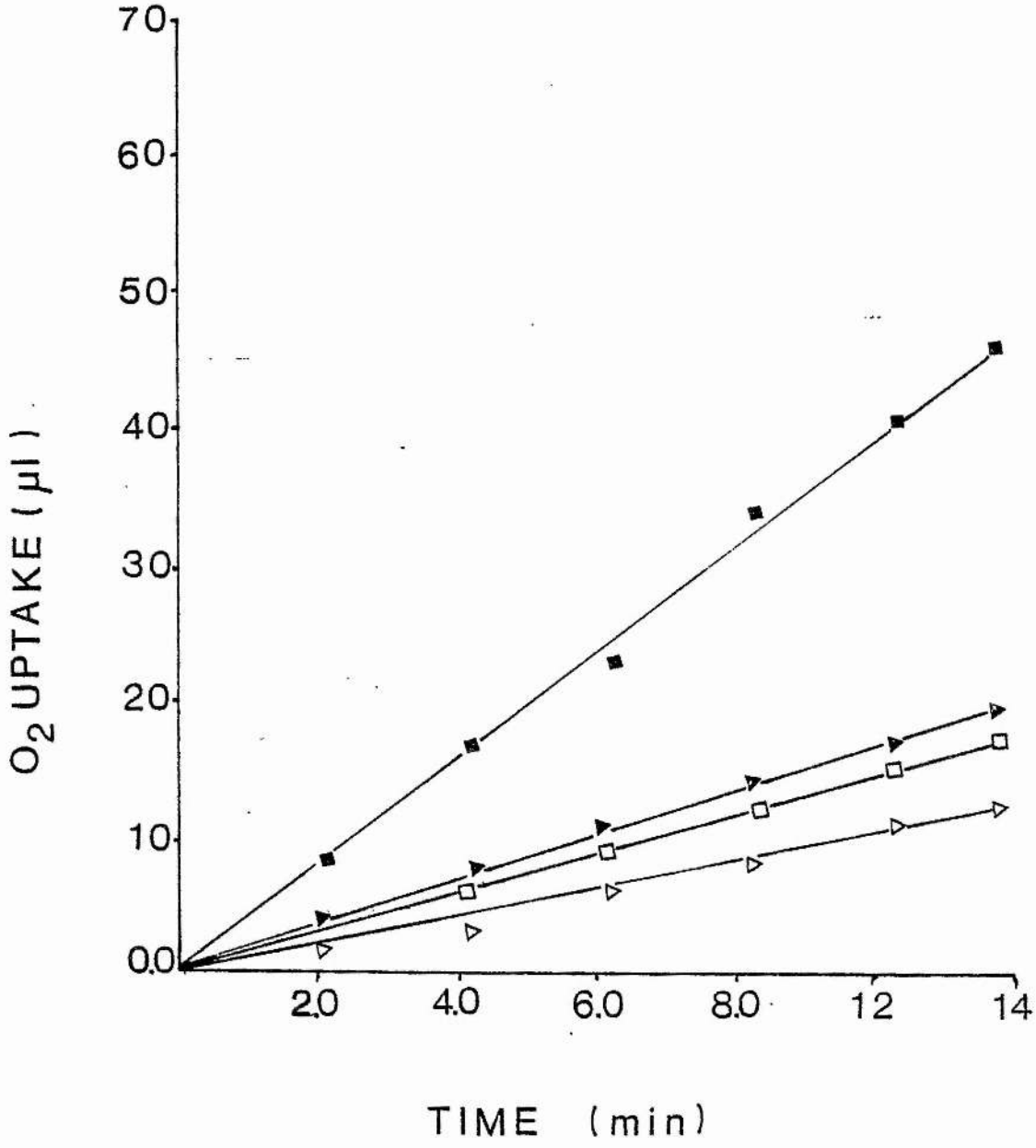
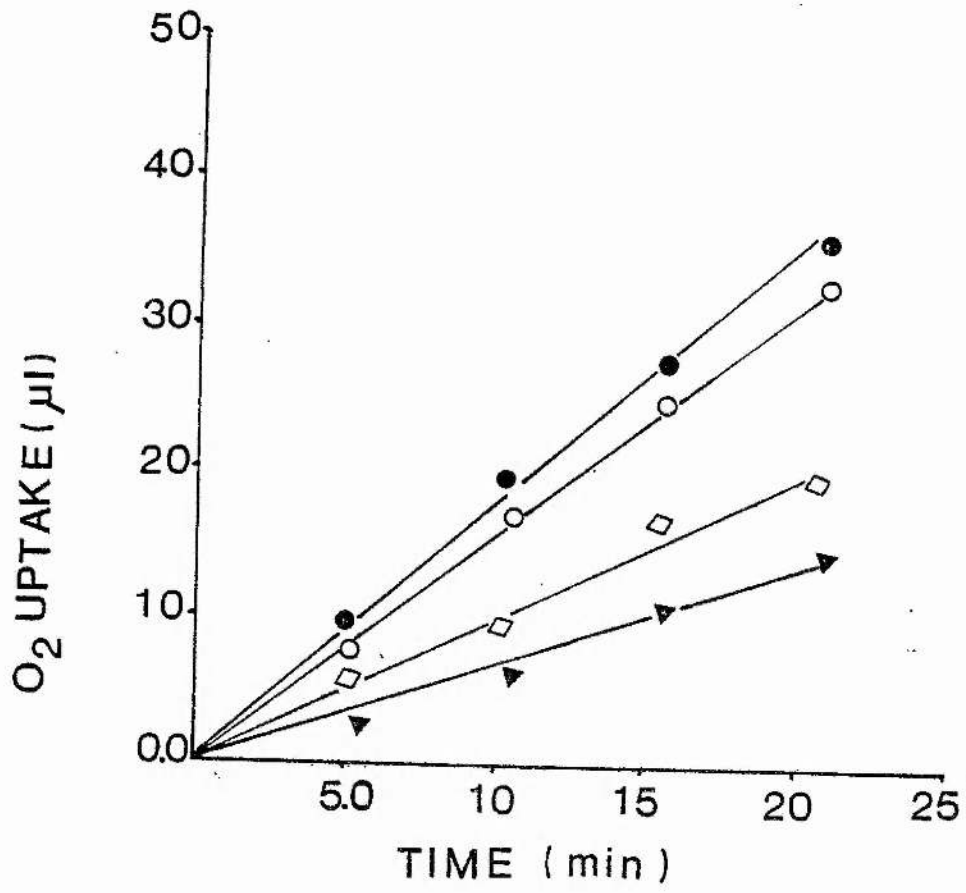
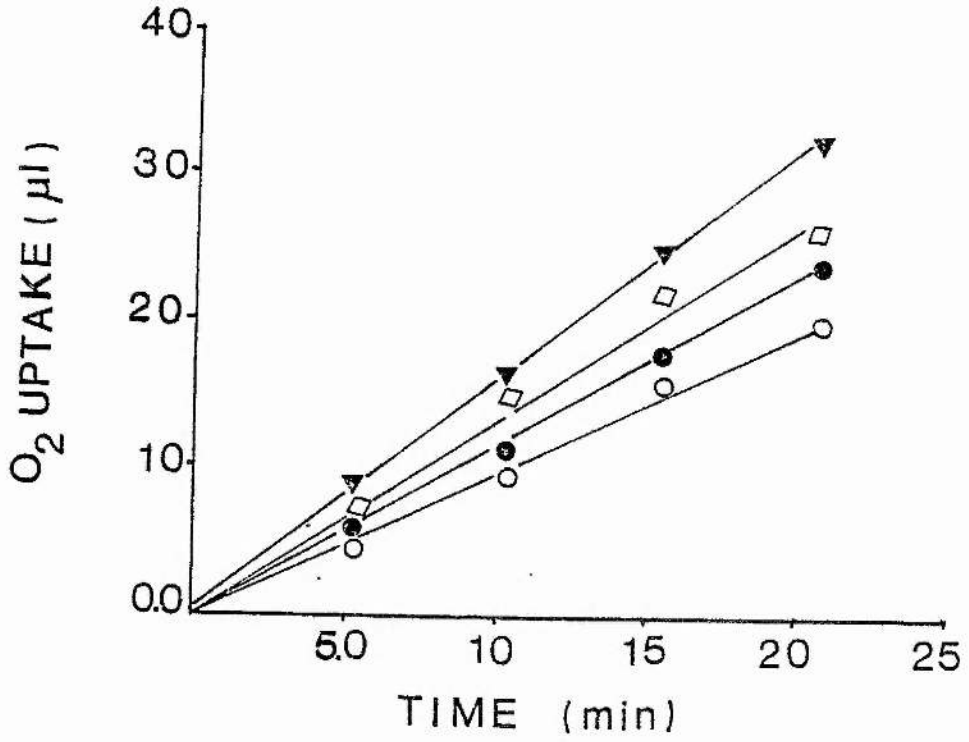


Fig. 4.3.1.1 Oxidation of sorbitol (■), xylitol (▶), glucose (□) and xylose (▷) by sorbitol grown cells of A. suboxydans NCIB 621 (10 mg dry cell equivalent).

Fig. 4.3.1.2 Oxidation of sorbitol (●), xylitol (▶), glucose (○) and xylose (◇) by glucose grown cells of A. suboxydans NCIB 621 (10 mg dry cell equivalent).

Fig. 4.3.1.3 Oxidation of sorbitol (●), xylitol (▶), glucose (○) and xylose (◇) by xylose grown cells of A. suboxydans NCIB 621 (10 mg dry cell equivalent).


50
40
30
20
10
0.0
O_2 UPTAKE (µl)
5.0
10
15
20
25
TIME (min)


40
30
20
10
0.0
O_2 UPTAKE (µl)
5.0
10
15
20
25
TIME (min)

4.4 A. suboxydans strain selection

A. suboxydans strains NCIB 3734, NCIB 9108 as well as a new copy of NCIB 621 were grown in sorbitol/yeast extract medium (3.1.2), harvested and washed as described in 4.3. The rate of substrate oxidation was measured as in 3.4 using sorbitol. Graphs of oxygen uptake (μl) versus time (min) were plotted for A. suboxydans NCIB 9108 (Fig. 4.4.1), NCIB 621 and NCIB 3734 (4.4.2).

Table 4.4.3 is a summary of the results obtained.

Table 4.4.3 Sorbitol oxidation rate by three strains of A. suboxydans

A. suboxydans	Oxidation rate (μl O_2.mg dry cell^{-1}.h^{-1})
NCIB 9108	43.8
NCIB 621	21.6
NCIB 3734	8.4

From straight forward observations of the rate of sorbitol oxidation of the three strains, it became apparent that strain NCIB 9108 has a higher rate of sorbitol oxidation and therefore was decided to be more suitable for subsequent experiments.

4.5 Basic nutritional requirements of A. suboxydans NCIB 9108

4.5.1 Effect of vitamins on the growth of A. suboxydans

A 10% (v/v) inoculum of A. suboxydans NCIB 9108 was introduced into 3 l sorbitol/salts medium (3.1.1) and sorbitol/salts medium containing one of the following vitamins:-

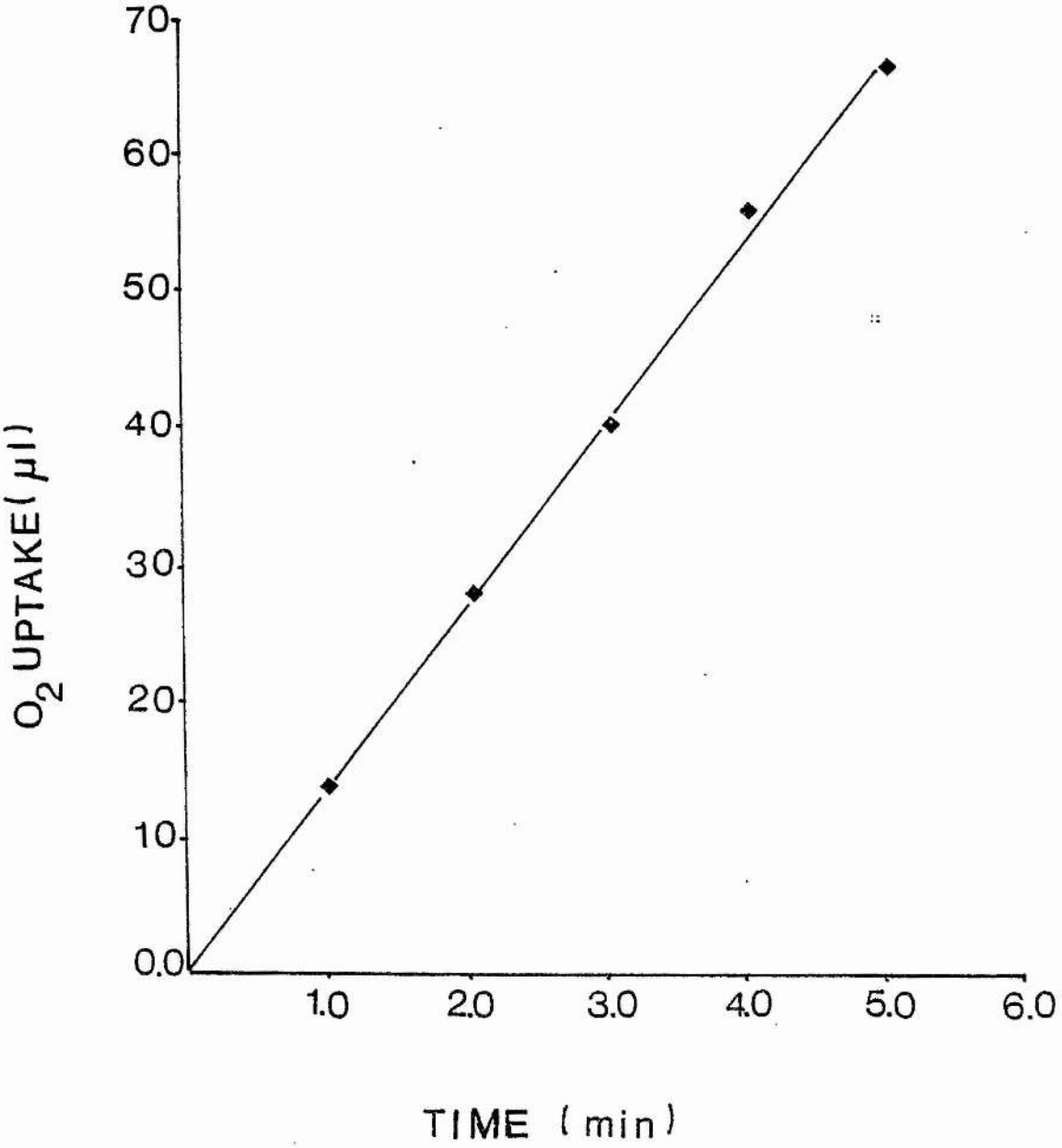


Fig. 4.4.1 Sorbitol oxidation by sorbitol grown cells of A. suboxydans NCIB 9108 (10 mg dry cell equivalent).

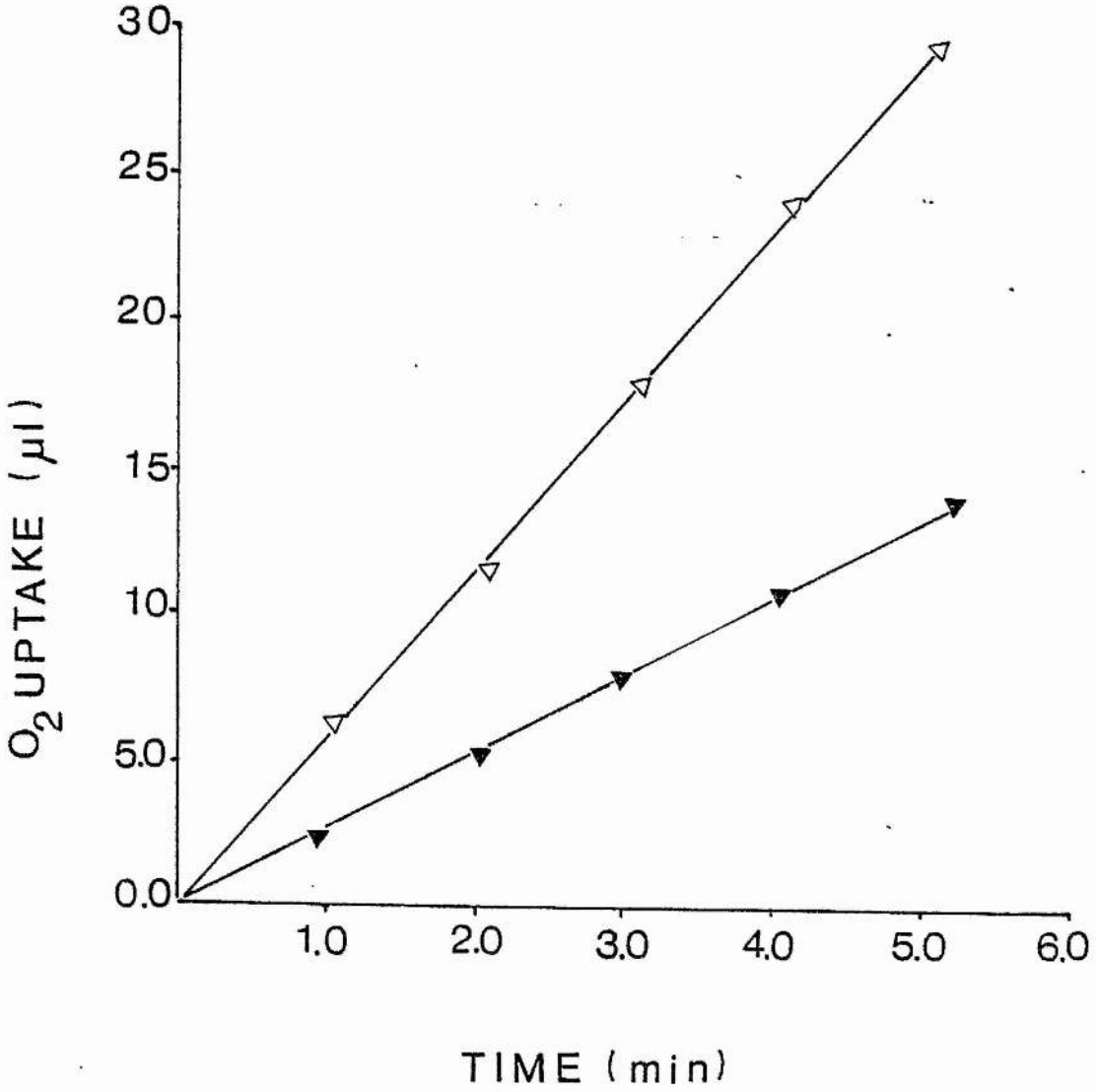


Fig. 4.4.2 Sorbitol oxidation by sorbitol grown cells of A. suboxydans NCIB 621 (▷) and NCIB 3734 (▶) (10 mg dry cell equivalent).

biotin, thiamine-HCl, inositol, nicotinic acid, pantothenic acid, pyridoxine-HCl or p-aminobenzoic acid in a 5 l New Brunswick fermenter (culture conditions as in 4.3).

Growth was monitored over a period of 12 to 34 h and the specific growth rates were calculated see Table 4.5.1.1.

Table 4.5.1.1 Effect of vitamins on the specific growth rate (μ) of A. suboxydans and the vitamin concentration used.

Growth medium	Vitamin Concentration ($mg.l^{-1}$)	Specific growth rate ($\mu = h^{-1}$)
Sorbitol/salts medium	0	0.043
Sorbitol/biotin/salts medium	0.02	0.07
Sorbitol/thiamine-HCl/salts medium	5.0	0.085
Sorbitol/inositol/salts medium	20.0	0.1
Sorbitol/nicotinic acid/salts medium	0.4	0.104
Sorbitol/pantothenic acid/ salts medium	0.5	0.124
Sorbitol/pyridoxine-HCl/ salts medium	1.0	0.138
Sorbitol/p-aminobenzoic acid/ salts medium	0.4	0.153

Fig. 4.5.1.2 is a growth curve of A. suboxydans on sorbitol/ salts medium (3.1.1).

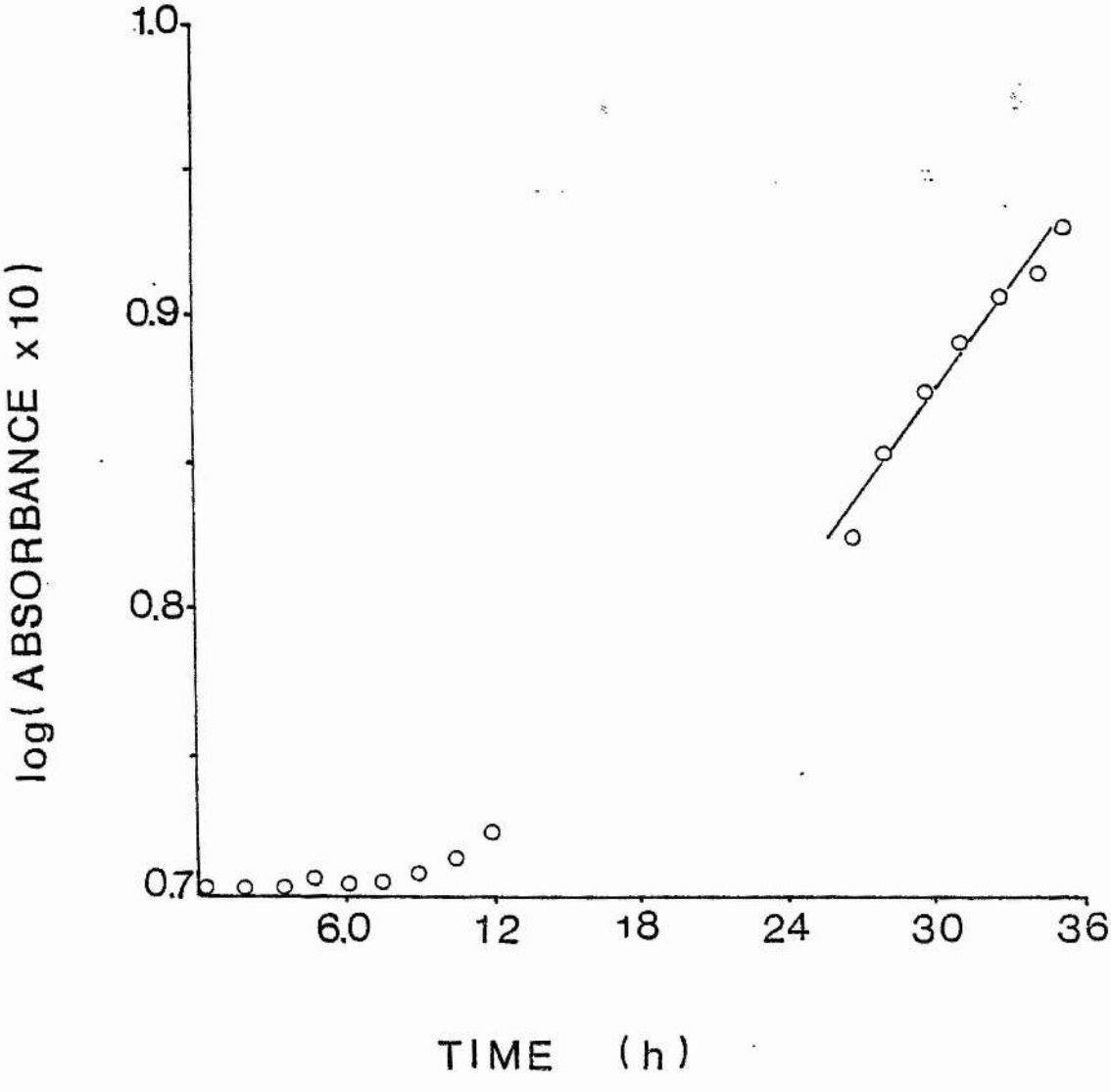


Fig. 4.5.1.2 Growth of A. suboxydans NCIB 9108 on sorbitol/salts medium (3.1.1).

4.5.2 Effect of yeast extract on the growth of *A. suboxydans*

4.5.2.1 Effect of yeast extract on the specific growth rate

A 10% inoculum of *A. suboxydans* NCIB 9108 was introduced into 3 l sorbitol/salts medium (3.1.1) containing 0.01%, 0.03% or 0.05% yeast extract in a 5 l New Brunswick fermenter (culture conditions as in 4.5.1). Growth was monitored over a period of 12 h and the specific growth rates were calculated see Table 4.5.2.1.1.

Table 4.5.2.1.1 Effect of yeast extract on the specific growth rate (μ)

Growth media	Specific growth rate ($\mu = h^{-1}$)
Sorbitol/salts medium	0.043
Sorbitol/0.01% yeast extract/ salts medium	0.115
Sorbitol/0.03% yeast extract/ salts medium	0.16
Sorbitol/0.05% yeast extract salts medium	0.207

4.5.2.2 Effect of yeast extract on the specific growth rate in the presence of 0.03% peptone

A. suboxydans was grown as in 4.5.2.1 using sorbitol/salts medium (3.1.1) containing 0.03% peptone with 0.01, 0.03 or 0.05% yeast extract.

Growth was monitored and the specific growth rates were calculated (see Table 4.5.2.2.1).

Table 4.5.2.2.1
Effect of yeast extract on the specific growth rate in the presence of 0.03% peptone

Growth media	Specific growth rate ($\mu = h^{-1}$)
Sorbitol/peptone/salts medium	0.102
Sorbitol/peptone/0.01% yeast extract/salts medium	0.19
Sorbitol/peptone/0.03% yeast extract/salts medium	0.25
Sorbitol/peptone/0.05% yeast extract/salts medium	0.29

4.5.3 Effect of carbon source on the specific growth rate

From the growth curve Fig. 4.5.2, it was obvious that the lag phase was too long. This was shortened by sub-culturing the organism through the medium to be used, then introducing a 25% (v/v) inoculum to 50ml of the medium which was incubated overnight in the New Brunswick controlled environmental orbital shaker (3.3.1.2). In this way it was ensured that the culture would be in the log phase when inoculated into 400 ml carbohydrate/salts medium (3.1.1), the appropriate carbon source being incorporated using a vortex-stirred batch apparatus (culture conditions as described in 3.3.1.6 : temperature 30°).

Growth was monitored over a period of 12 h and the specific growth rates were calculated (see Table 4.5.3.1).

Table 4.5.3.1

Effect of carbon source on the specific growth rate

Carbon source	Specific growth rate ($\mu = h^{-1}$)
Fructose	0.05
Glucose	0.067
Xylitol	0.075
Mannitol	0.08
Sorbitol	0.094

4.6 Investigation of the oxidation of sorbitol to products during the growth of A. suboxydans

4.6.1 Identification of product

Three standard solutions of sorbitol, sorbose and fructose were prepared and analysed by G.L.C. as in 3.9.2 individually and as a mixture.

Fig. 4.6.1.1 is a tracing of the individual peaks obtained on the chart recorder. From the retention time of the three standards, the product of sorbitol oxidation during the growth of A. suboxydans NCIB 9108 was found to be sorbose.

4.6.2 Production of sorbose from sorbitol

A 10% inoculum of A. suboxydans was introduced into 3l sorbitol/yeast extract medium (3.1.2) in a 5 l New Brunswick fermenter (culture conditions as in 3.3.1.5; temperature 30°). Growth was monitored for 12 h and samples being treated as in 3.9.2 for the quantitative analysis of sorbose produced from sorbitol during the growth of the organism.

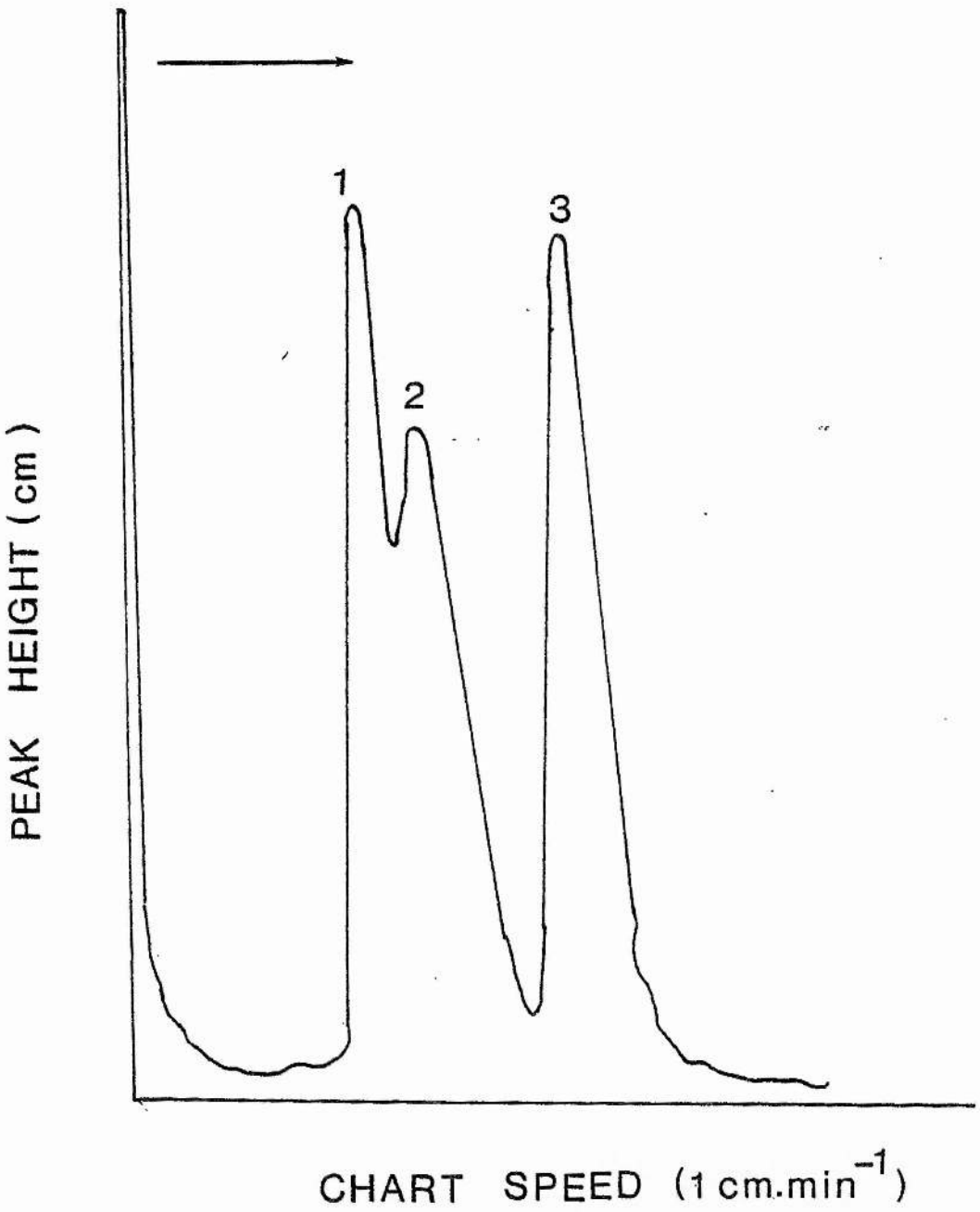


Fig. 4.6.1.1 G.L.C. tracing of a mixture of three carbohydrate standards as their TMS-ether derivatives on SE_{30} column, isothermal at 175°. S=solvent, 1 = fructose, 2 = sorbose and 3 = sorbitol.

Fig. 4.6.2.1 is a tracing of the G.L.C. peaks obtained on the chart recorder which shows a proportional increase of sorbose and a decrease of sorbitol with time, after eight hours sorbitol was completely converted into sorbose.

Fig. 4.6.2.2 illustrates the growth of A. suboxydans NCIB 9108 with concomitant substrate utilisation and product formation.

4.7 Enzyme release upon cell disruption

4.7.1 Effect of sonication time on enzyme activity and protein concentration

A 30% cell suspension was prepared in 20 mM Tris-HCl buffer pH 7.6 and 20 ml aliquots were subjected to sonication treatment as in 3.6.2. Aliquots, 1 ml, were removed at suitable intervals during ultrasonication. Each sample was centrifuged (25,000 x g) for 30 min at 4^{o} and the supernatants were assayed for SDH activity (3.7.1) and for protein concentration by the Biuret method (3.8.2).

Fig. 4.7.1.1 is a graph of specific activity of SDH and protein release versus time.

4.7.2 Comparison between enzyme release by ultrasonication and Hughes press

A 2 ml aliquot of 30% washed cell suspension was subjected for 12 min ultrasonication as in 3.6.2 and the clear supernatant was separated by centrifugation (25,000 x g) for 30 min at 4^{o} and assayed for SDH activity as in 3.7.1 and protein concentration by the Biuret method (3.8.2).

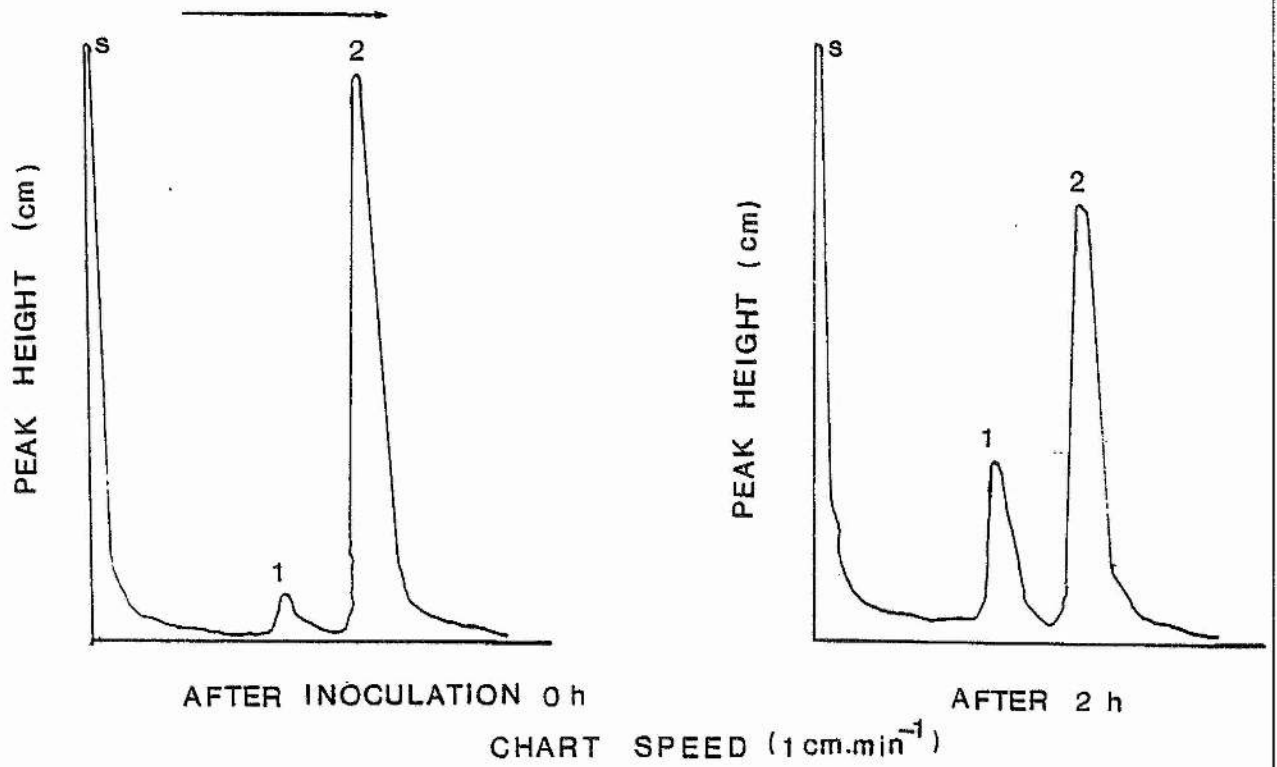
A similar volume of the same washed cells suspension

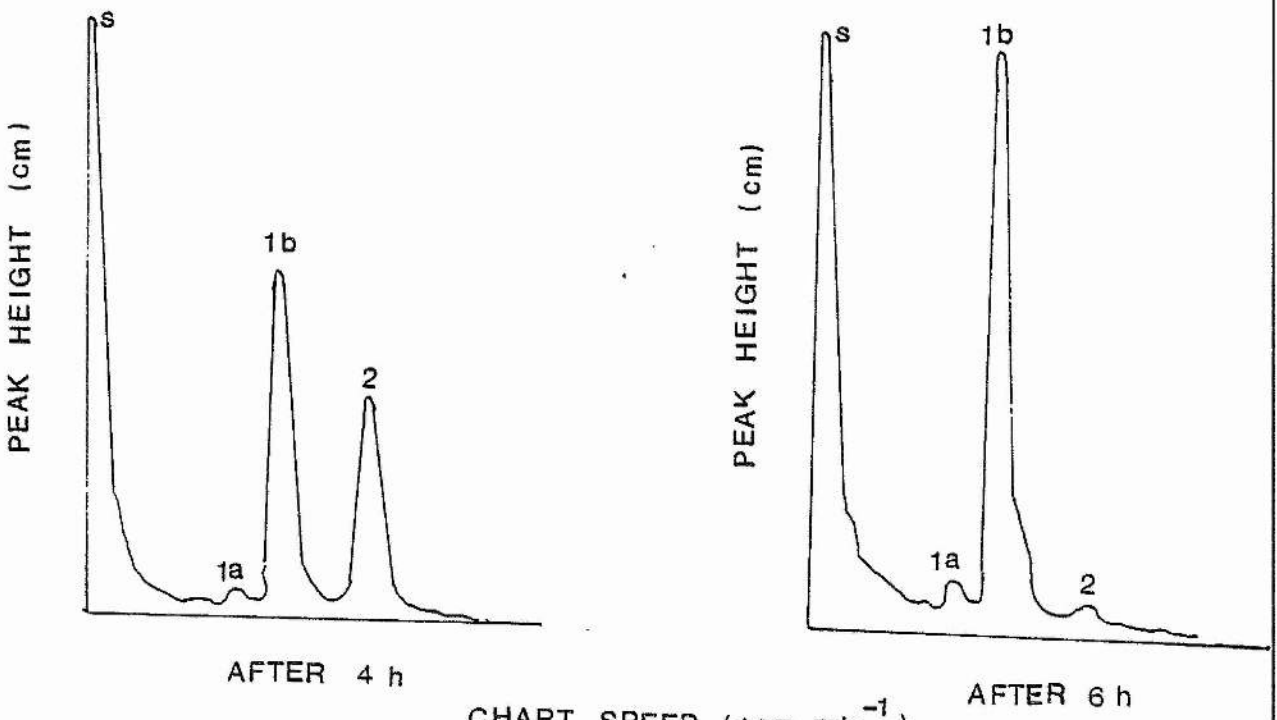
Fig. 4.6.2.1 G.L.C. traces indicating time course of sorbose production from sorbitol during the growth of A. suboxydans NCIB 9108 separated as their TMS-ether derivative on SE_{30} column, isothermal at 175°.

s = solvent

1, 1a and 1b = sorbose

2 = sorbitol


PEAK HEIGHT (cm)
s
1
2
AFTER INOCULATION 0 h
PEAK HEIGHT (cm)
s
1
2
AFTER 2 h
CHART SPEED (1 cm.min^{-1})


PEAK HEIGHT (cm)
s
1a
1b
2
AFTER 4 h
PEAK HEIGHT (cm)
s
1a
1b
2
AFTER 6 h
CHART SPEED (1cm. min^{-1})

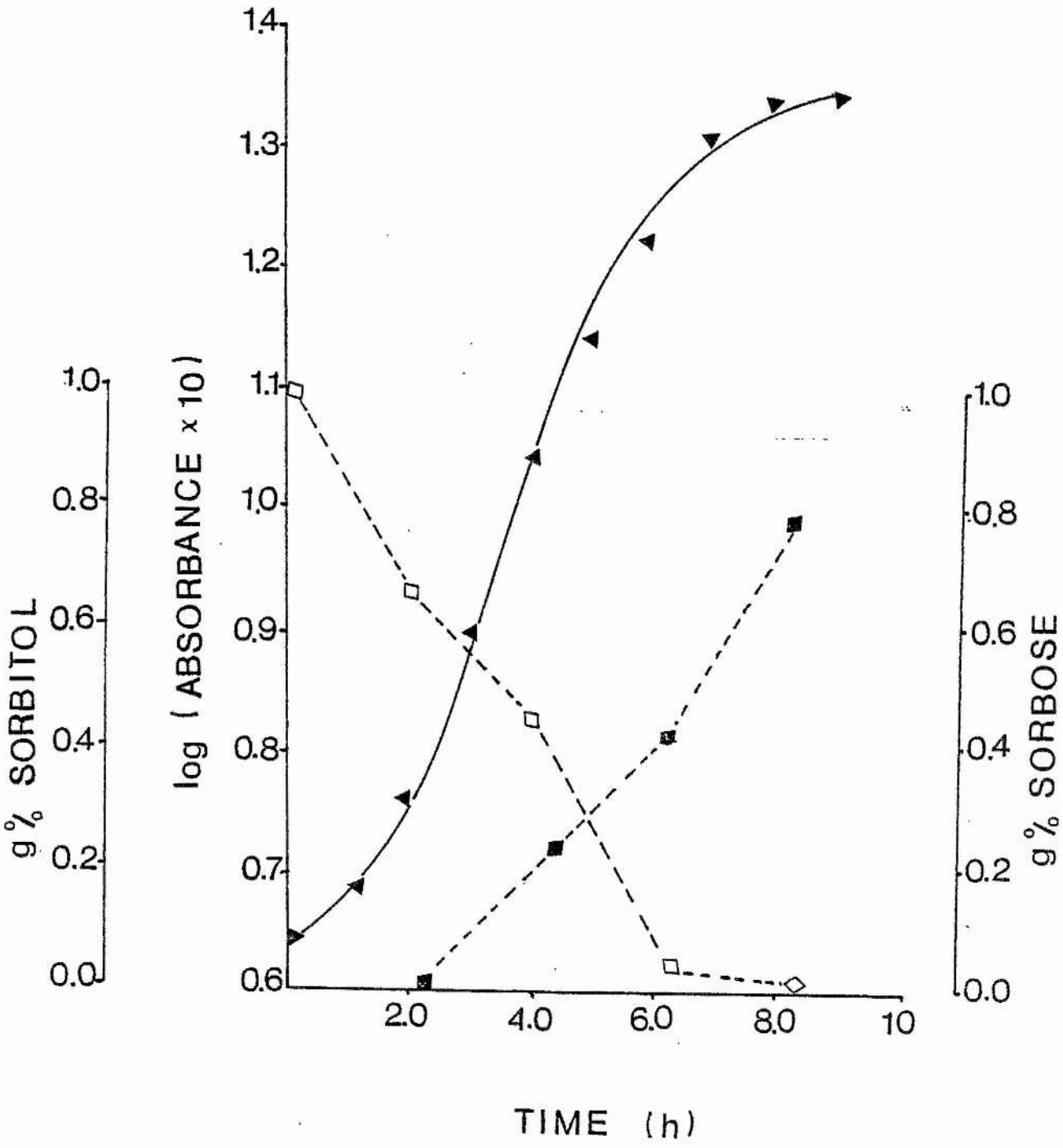


Fig. 4.6.2.2 Growth of A. suboxydans NCIB 9108 (▶) with concomitant substrate utilisation (◇) and product formation (◆).

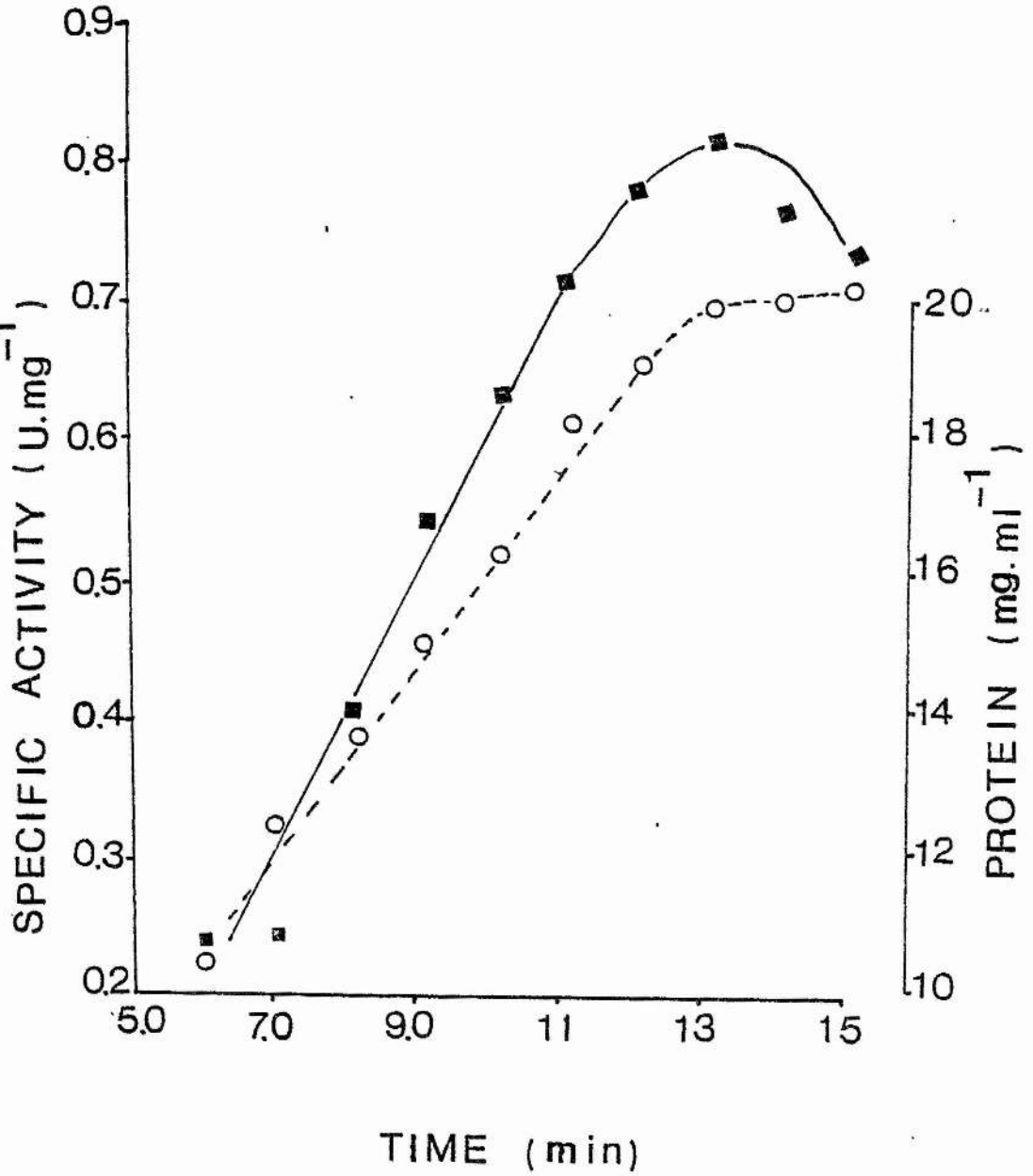


Fig. 4.7.1.1 Effect of sonication time on enzyme activity.

(■) Indicates SDH specific activity

(o) Indicates protein.

was subjected to Hughes press treatment as in 3.6.1 and the clear supernatant was separated by centrifugation and assayed for SDH activity and protein concentration as above (see Table 4.7.2.1).

Table 4.7.2.1

Enzyme release by ultrasonication and Hughes press cell disruption

Method used	Total SDH activity (U)	Total protein (mg)	Specific activity of SDH($U.mg^{-1}$)
Ultrasonication	3.4	7	0.4857
Hughes press	6.77	12.6	0.537

4.8 Production of sorbitol dehydrogenase (SDH) by A. suboxydans in the presence of selected carbon sources.

A 10% (v/v) inoculum of A. suboxydans was introduced into 3 l carbohydrate/yeast extract medium (3.1.2) containing 0.5% peptone and the appropriate carbohydrate being incorporated in a 5 l New Brunswick fermenter (culture conditions as in 4.3). Growth was monitored over a period of 14 h and the samples (200 ml each) were harvested by centrifugation (2,000 x g, 20 min) and washed three times in 20 mM Tris-HCl buffer pH 7.6. The washed cells were made into a homogeneous suspension in the same buffer and then subjected to ultrasonication for 12 min as in 3.6.2. The crude extracts were separated by centrifugation (25,000 x g, 30 min), assayed for SDH activity as in 3.7.1 and total

protein by the Biuret method (3.8.2).

Graphs illustrating growth of A. suboxydans with concomitant production of SDH were plotted for sorbitol grown cells (Fig. 4.8.1), glucose grown cells (Fig. 4.8.2), fructose grown cells (Fig. 4.8.3), sorbose grown cells (Fig. 4.8.4), xylitol grown cells (Fig. 4.8.5) and mannitol grown cells (Fig. 4.8.6) see Table 4.8.1.

Table 4.8.1

SDH produced by A. suboxydans in the presence of selected carbon sources

Carbon sources	Specific activity ($U.mg^{-1}$)
Fructose	0.096
Sorbose	0.108
Glucose	0.2
Xylitol	0.321
Mannitol	0.378
Sorbitol	0.494

4.9 Media formulation to maximise SDH production.

4.9.1 Optimisation with respect to sorbitol as a Carbon source

A series of sorbitol concentrations were used with 1% yeast extract as a nitrogen source in 25 mM potassium phosphate buffer pH 5.0. A 10% (v/v) inoculum of A. suboxydans was introduced into 250 ml sorbitol/yeast extract medium containing the appropriate concentration of sorbitol in a vortex-stirred batch apparatus (culture

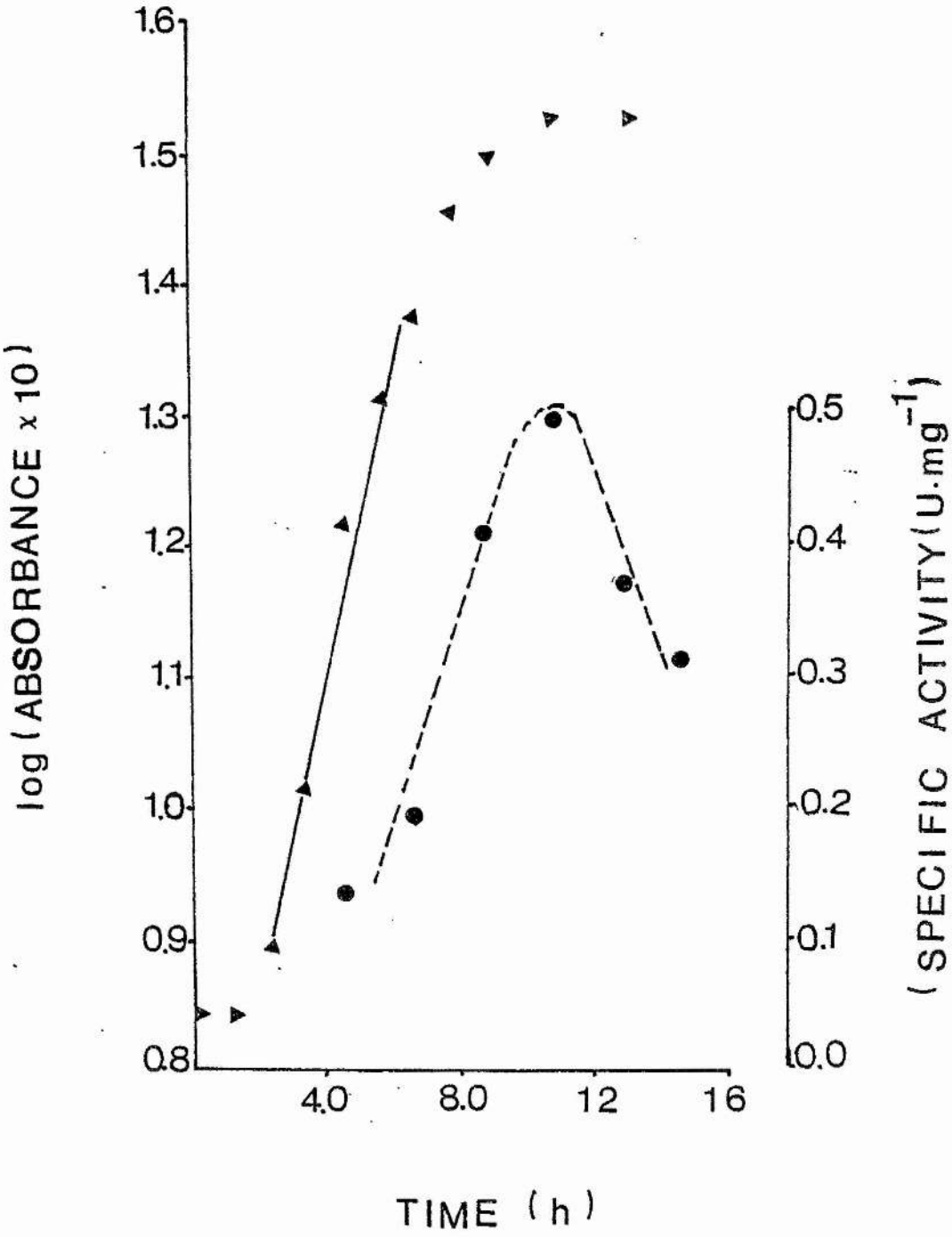


Fig. 4.8.1 Growth of A. suboxydans on sorbitol as a carbon source.

(▶) Indicates cell growth (log(absorbance x 10)).

(●) Indicates cell specific activity of SDH.

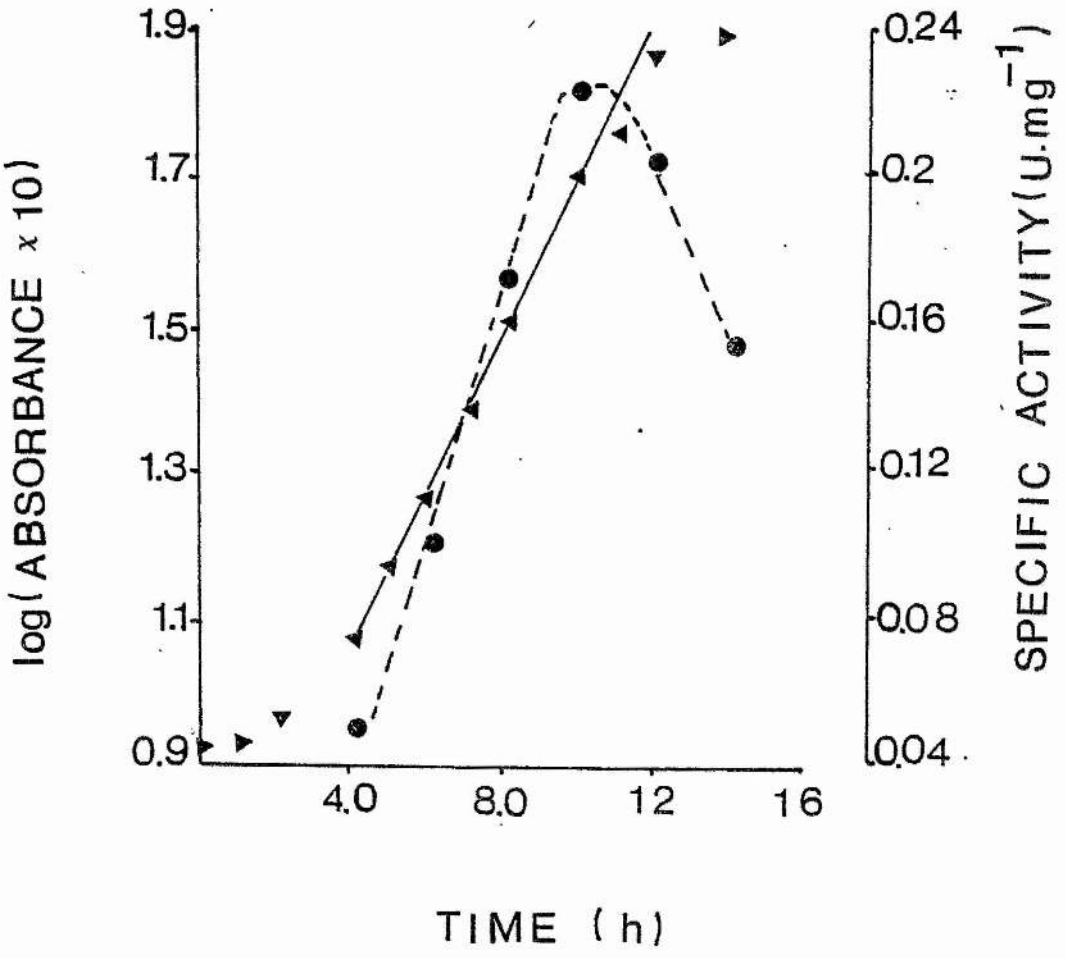


Fig. 4.8.2 Growth of A. suboxydans on glucose as a carbon source.

(▶) Indicates cell growth (log(absorbance x 10)).

(●) Indicates cell specific activity of SDH.

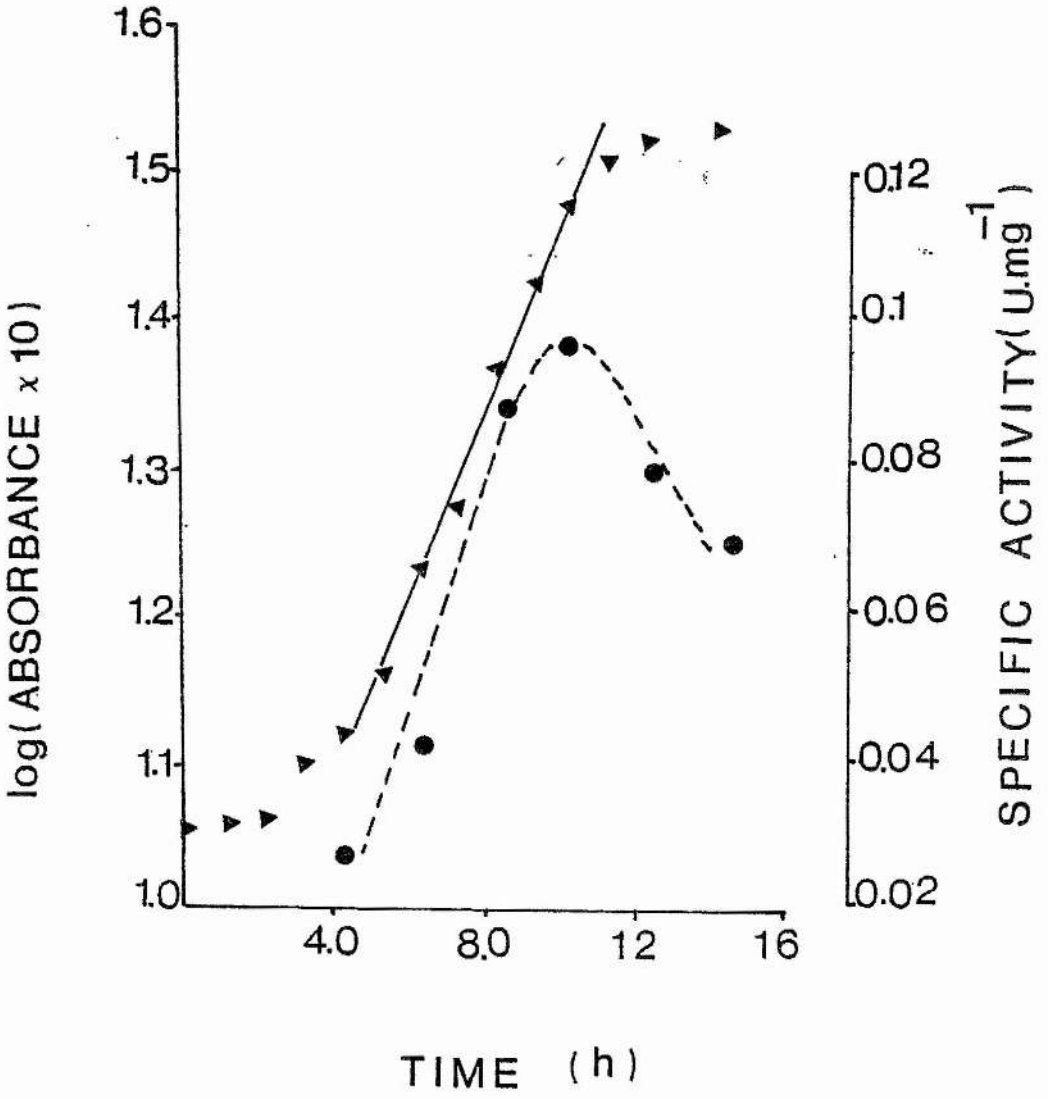


Fig. 4.8.3 Growth of A. suboxydans on fructose as a carbon source.

(▶) Indicates cell growth (log(absorbance x 10)).

(●) Indicates cell specific activity of SDH.

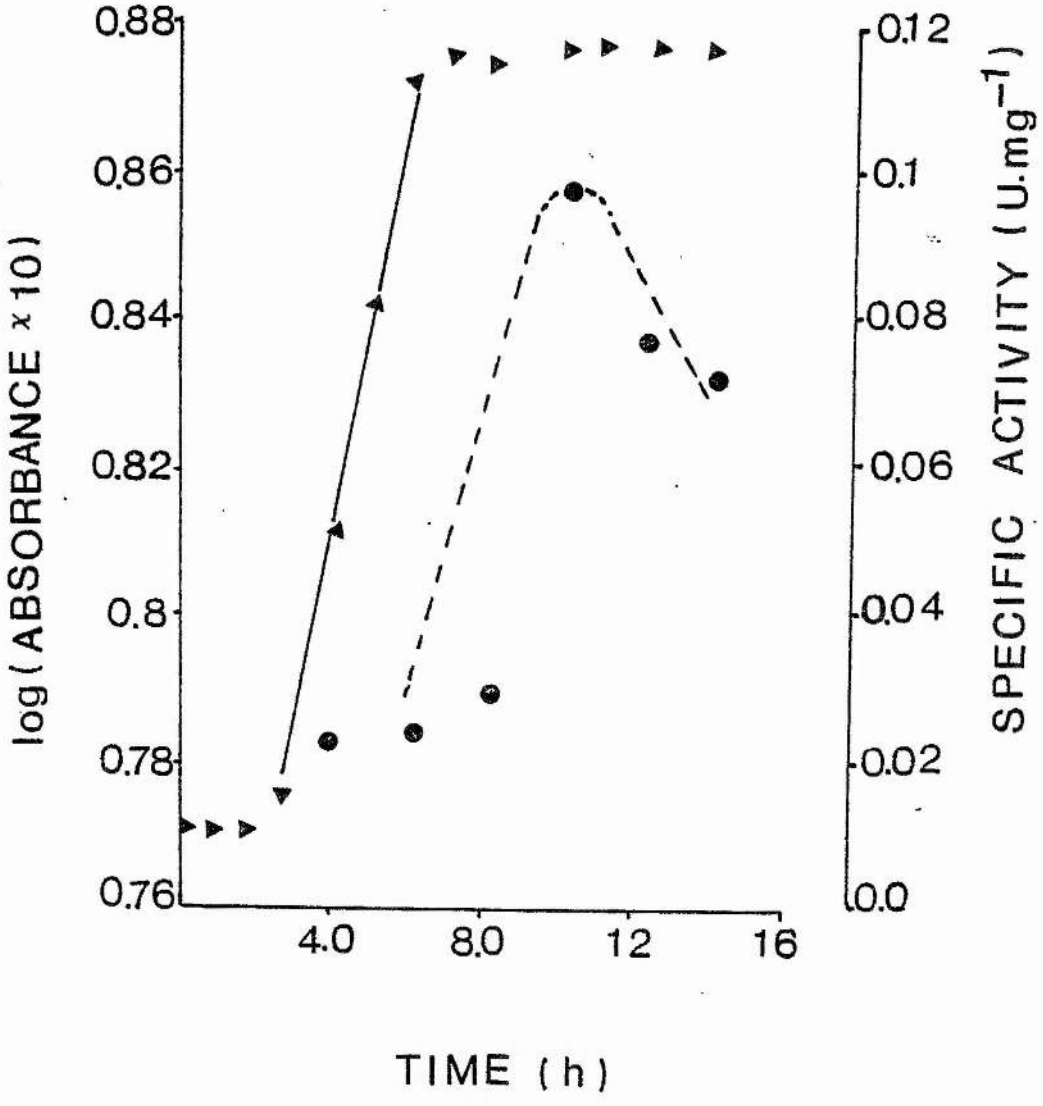


Fig. 4.8.4 Growth of A. suboxydans on sorbose as a carbon source.

(▶) Indicates cell growth (log(absorbance x 10)).

(●) Indicates cell specific activity of SDH.

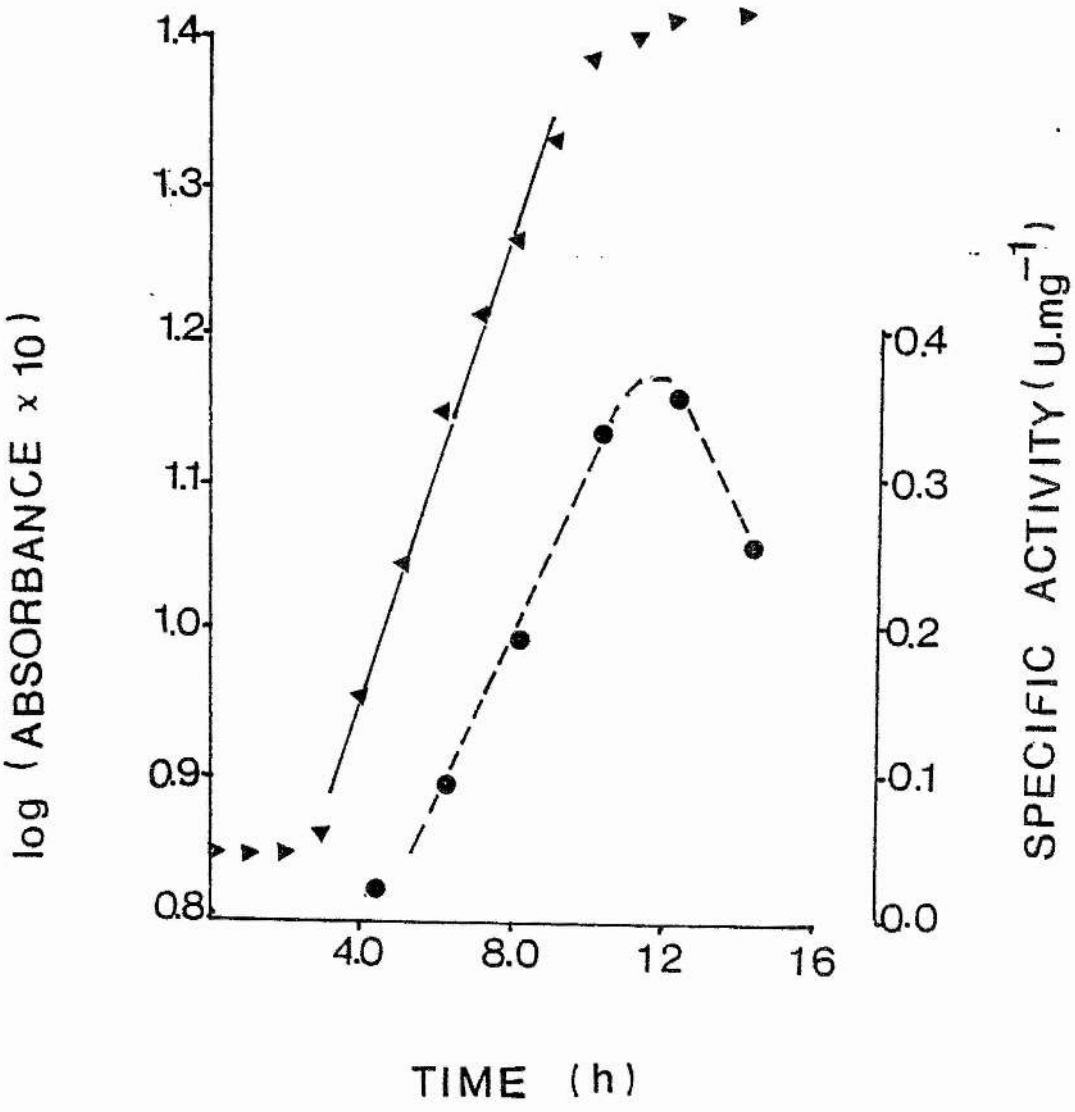


Fig. 4.8.5 Growth of A. suboxydans on xylitol as a carbon source.

(▶) Indicates cell growth (log(absorbance x 10)).

(●) Indicates cell specific activity of SDH.

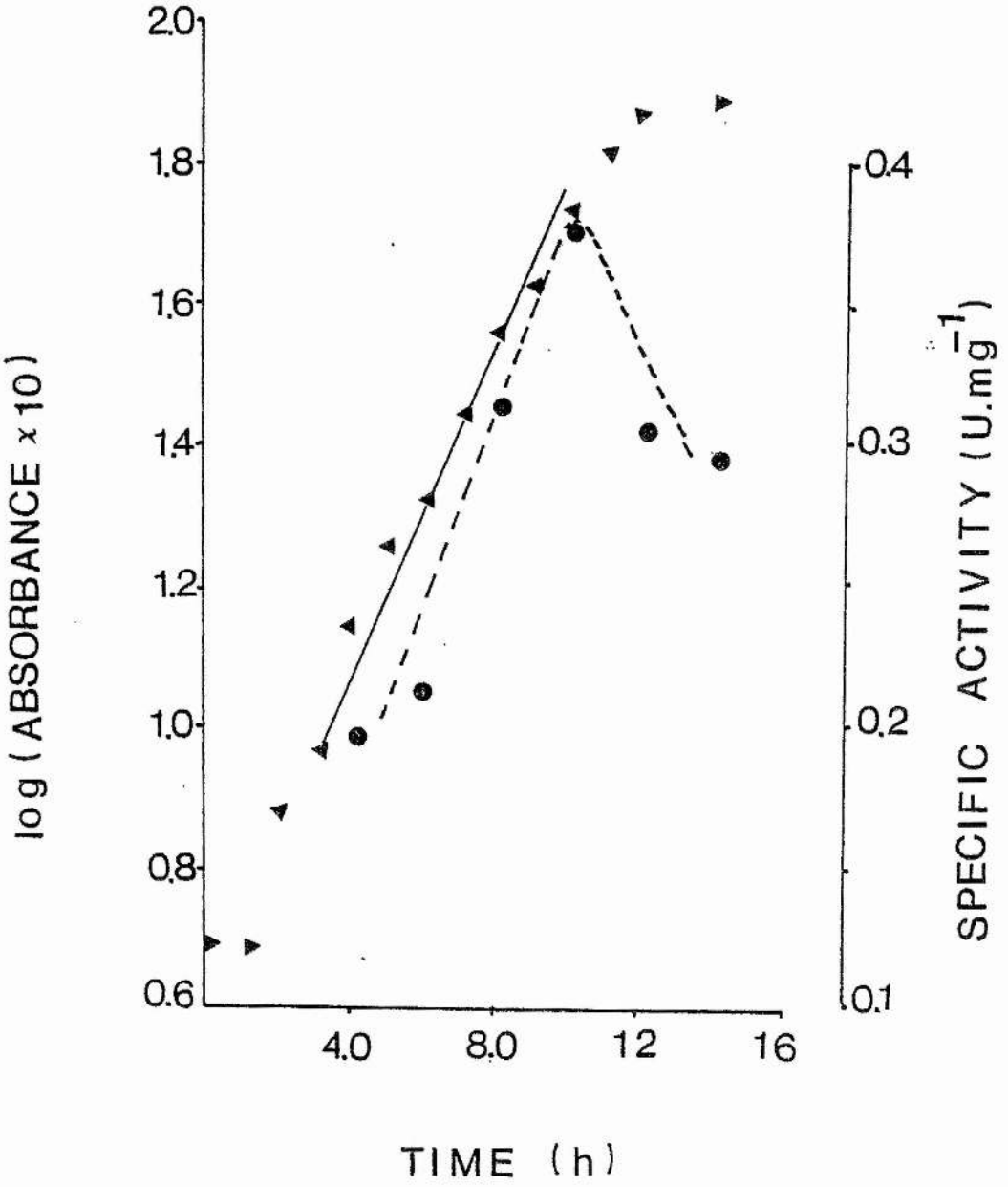


Fig. 4.8.6 Growth of A. suboxydans on mannitol as a carbon source.

(▶) Indicates cell growth (log (absorbance x 10)).

(●) Indicates cell specific activity of SDH.

conditions as described in 3.3.1.6; temperature 30°).

Growth was allowed to proceed for 10 h and the cells were harvested as in 4.8, washed three times in ice-cold 20 mM Tris-HCl buffer, pH 7.6, and made into a homogeneous suspension in a total volume of 2 ml in the same buffer. Each suspension was subjected to 12 min ultrasonication as in 3.6.2 and the crude extract was separated by centrifugation (25,000 x g, 30 min), measured and assayed for SDH activity as in 3.7.1 and total protein by the Biuret method (3.8.2). Fig. 4.9.1.1 is a graph of SDH specific activity versus sorbitol concentration.

4.9.2 Optimisation with respect to yeast extract

A series of yeast extract concentrations were used with 2% sorbitol as a carbon source in 25 mM potassium phosphate buffer pH 5.0. A 10% (v/v) inoculum of A. suboxydans was introduced into 250 ml sorbitol/yeast extract medium containing the appropriate concentration of yeast extract and was grown and treated as in 4.9.1. Fig. 4.9.2.1 is a graph of SDH specific activity versus yeast extract concentration.

4.9.3 Optimisation with respect to yeast extract in the presence of 0.5% peptone

A series of yeast extract concentrations were used with 2% sorbitol and 0.5% peptone in 25 mM potassium phosphate buffer pH 5.0. A 10% (v/v) inoculum of A. suboxydans was introduced into 250 ml sorbitol/yeast extract/peptone medium containing the appropriate concentration of yeast extract. Growth was allowed to proceed for 10 h and the harvested

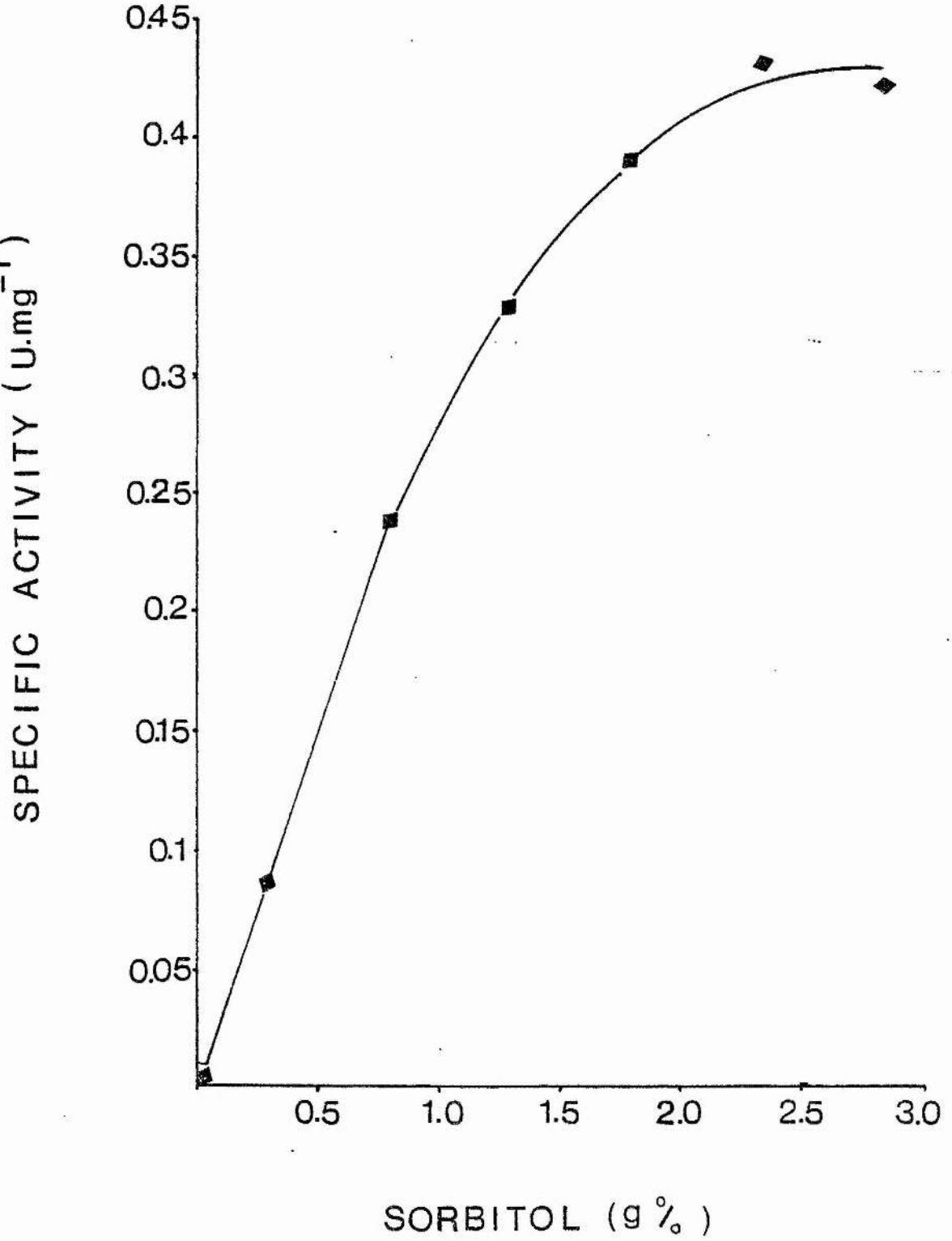


Fig. 4.9.1.1 Relationship between SDH specific activity and sorbitol concentration.

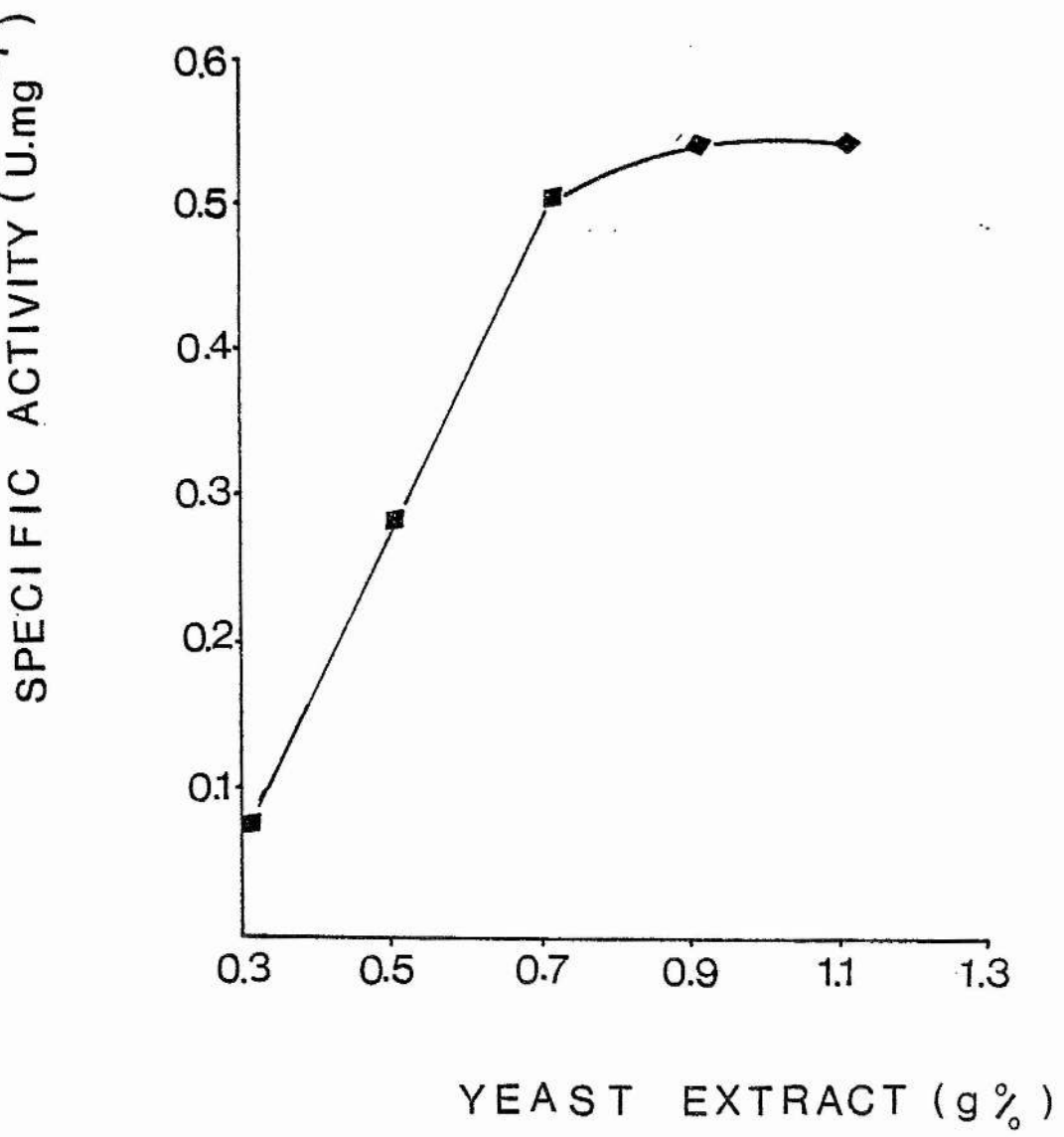


Fig. 4.9.2.1 Relationship between SDH specific activity and yeast extract concentration.

cells were treated as in 4.9.1.

Fig. 4.9.3.1 is a graph of SDH specific activity versus yeast concentration.

Table 4.9.3.2 shows the growth medium formulated for the production of SDH by A. suboxydans NCIB 9108.

Table 4.9.3.2 Growth medium for the production of SDH by A. suboxydans

Sorbitol	20 $g.l^{-1}$
Yeast extract	5 $g.l^{-1}$
Peptone	5 $g.l^{-1}$
in 25 mM potassium phosphate buffer pH 5.0.	

The sorbitol/peptone/yeast extract medium (4.9.3.2) was used for the production of SDH by A. suboxydans throughout the purification studies.

4.10 Studies on the purification of sorbitol dehydrogenase (SDH)

The crude enzyme extract was prepared as in 3.11.1. Nucleic acid precipitation and ammonium sulphate fractionation were performed as in 3.11.2 and 3.11.3 respectively.

4.10.1 Chromatography on DEAE-Cellulose (DE 32) by step-wise elution

The chromatography of the crude extract obtained from 3.11.1 and adjusted to pH 9.0 was carried out by step-wise elution as described in section 3.11.4.1. The flow rate was 2.5 $ml.min^{-1}$ and the effluent stream was analysed as previously described by an LKB-UV recorder.

A typical pattern of the chromatographic behaviour of SDH from A. suboxydans NCIB 9108 on DEAE-Cellulose (DE32)

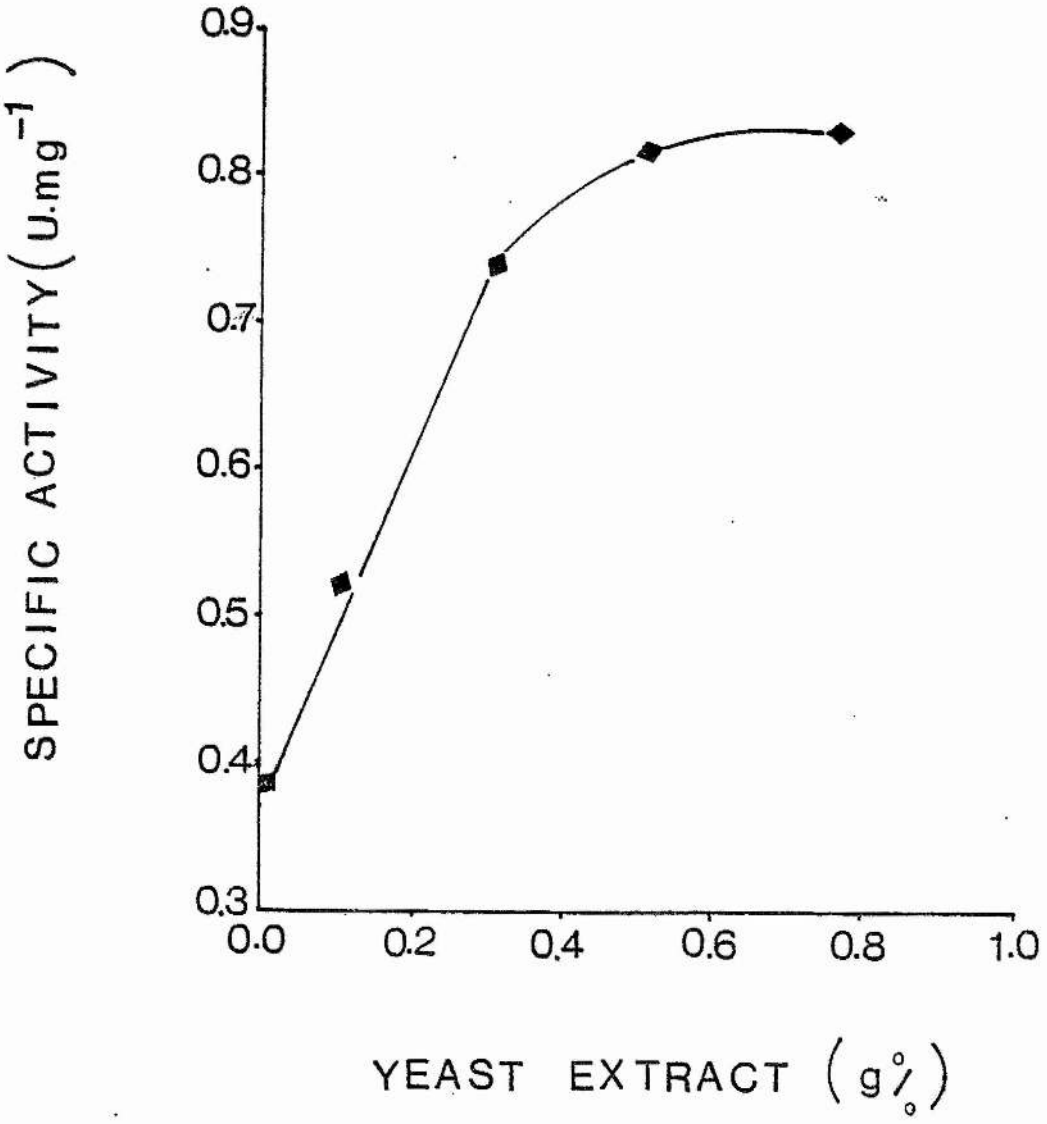


Fig. 4.9.3.1 Relationship between SDH specific activity and yeast extract in the presence of 0.5% peptone.

following this procedure is shown in Fig. 4.10.1.1. This elution profile was obtained by using 4 ml of crude extract containing 5.3 $mg.ml^{-1}$ protein. SDH activity was obtained in peak IV following elution with 0.2 M NaCl in 20 mM Tris-HCl buffer pH 9.0.

4.10.2 Chromatography on DEAE-Cellulose (DE32) by gradient elution

A 5 ml aliquot of enzyme solution from 3.11.3 containing 57.5 mg protein was desalted by dialysis as described in 3.11.4.2 and was applied to the top of a 2.5 cm (diameter) x 20 cm (height) column of DEAE-Cellulose that had been washed with 20 mM Tris-HCl buffer until the pH value of the washing was about 9.0 and finally with 0.1 M NaCl in the same buffer pH 9.0 until equilibration. The sample was washed in with 0.1 M NaCl in 20 mM Tris-HCl buffer pH 9.0 and the column was washed with 200 ml 0.1 M NaCl in the same buffer to remove the unbound material as described in 3.11.4.2. A linear gradient elution of sodium chloride (0.2 - 0.4 M) was set up in 20 mM Tris-HCl buffer pH 9.0. The flow rate was 2 $ml.min^{-1}$. SDH and GI were co-chromatographed as a single peak being completely eluted at a NaCl concentration of 0.2 M. The protein was recorded by continuous ultraviolet monitoring of the effluent stream with an LKB-UV recorder.

The tubes containing the bulk of the activity were pooled, assayed for SDH activity (3.7.1), for GI activity (3.7.2) and total protein by the Folin-Lowry method (3.8.1). A typical elution profile can be seen in Fig. 4.10.2.2. Table 4.10.2.2 is a summary of the data for this chromatographic step on DEAE-Cellulose after ammonium sulphate fractionation, a total of 17 fold purification was achieved.

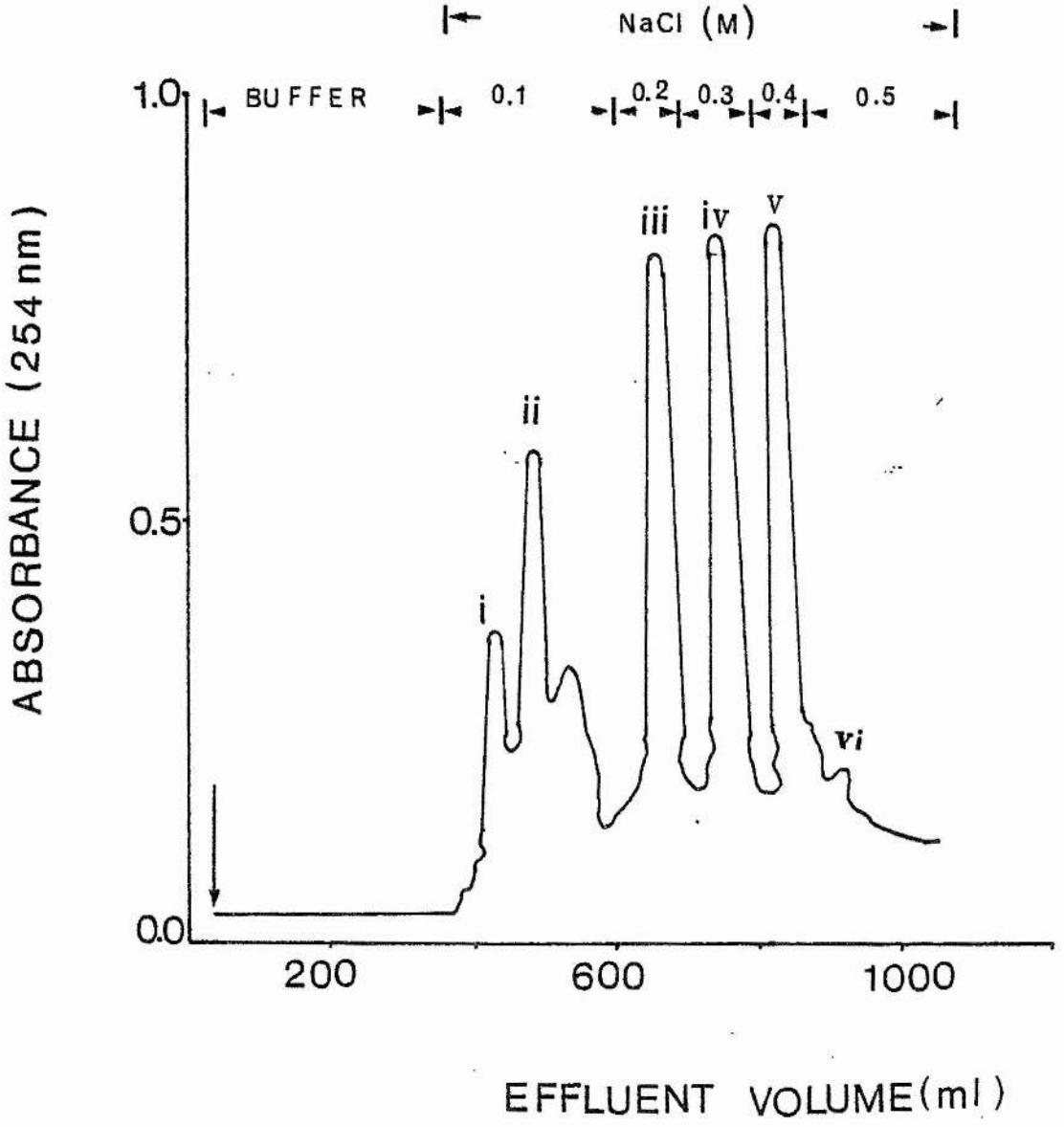


Fig. 4.10.1.1 Elution profile obtained for the chromatography of protein from 3.11.1 upon a DEAE-Cellulose (DE32) column (2.6cm diameter x 20cm height) by stepwise elution using a sample of 4 ml containing 5.3 $mg.ml^{-1}$ protein. The elution was carried out at a flow rate of 2.5 $ml.min^{-1}$ and as indicated in the chromatogram washing unbound protein from the column with 20mM Tris-HCl buffer pH 9.0 prior to stepwise elution with 0.1 to 0.5M NaCl (in steps of 0.1M) in the same buffer. The effluent was monitored as previously described and SDH activity was found in peak IV.

↓ Indicates point of sample application.

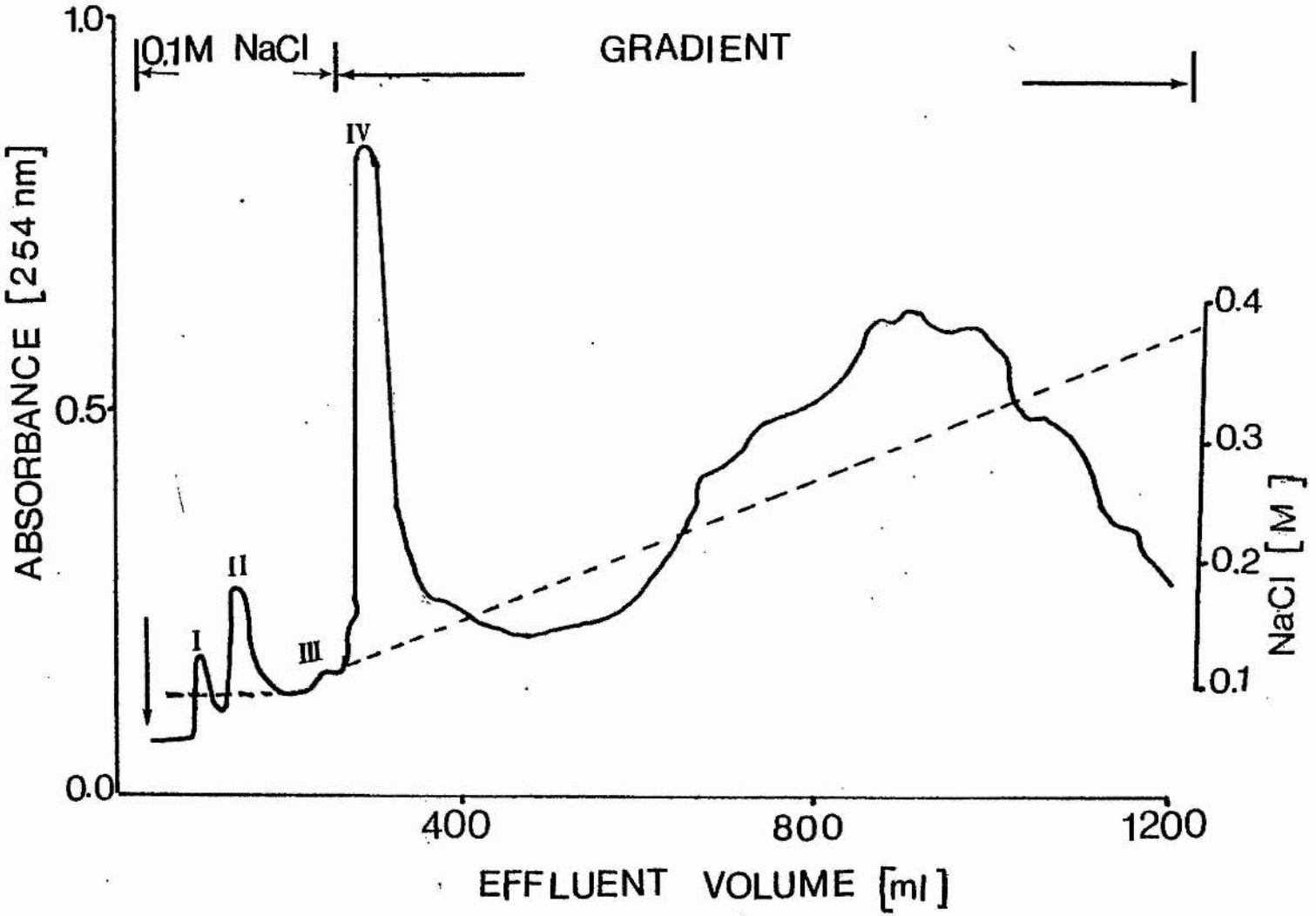

1.0
0.5
0.0
ABSORBANCE [254 nm]
0.1M NaCl
GRADIENT
I
II
III
IV
0.4
0.3
0.2
0.1
NaCl [M]
400
800
1200
EFFLUENT VOLUME [ml]

Fig. 4.10.2.2 Elution profile obtained for the chromatography of protein from 3.11.3.2 on a DEAE-Cellulose column (2.6 cm diameter x 20 cm height) by gradient elution using 5 ml sample of 11.5 $mg.ml^{-1}$ protein. The flow rate was 2 $ml.min^{-1}$ and as indicated in the chromatogram washing unbound protein with 0.1 M NaCl in the same buffer. A gradient elution was provided by sodium chloride in 20 mM Tris-HCl buffer pH 9.0 (0.2 - 0.4 M NaCl). Effluent was monitored as described in 4.10.1.1.

SDH and GI activities were found in peak IV.

↓ Indicates point of sample application.

Table 4.10.2.2 Purification of SDH and GI from A. suboxydans NCIB 9108 on DEAE-Cellulose chromatography after ammonium sulphate fractionation.

Step	Vol. (ml)	Protein		SDH					GI				
		$mg.ml^{-1}$	Total (mg)	Activity		Specific activity ($U.mg^{-1}$)	Recovery %	Purification factor (fold)	Activity		Specific activity ($U.mg^{-1}$)	Recovery %	Purification factor (fold)
				$U.ml^{-1}$	Total (U)				$U.ml^{-1}$	Total (U)			
Crude extract	50	14	700	12.72	636	0.91	100	1	10.7	535	0.764	100	1
0.05 M $MnCl_2$	54	6.2	335	8.5	459	1.37	72	1.5	7.1	383.4	1.14	71	1.49
30%$(NH_4)_2SO_4$	54	3.44	185.8	7.8	421	2.3	66	2.5	6	324	1.75	60	2.3
61%$(NH_4)_2SO_4$	4.9	20	98	78	382	3.9	60	4.3	52	254.8	2.6	47	3.4
*Dialysed sample	5	11.5	57.5 (1x1.6)	39.4	197 (1x1.6)	3.43	-	-	31.2	156 (1x1.6)	2.71	-	-
DEAE-Cellulose chromato-graphy	75	0.14	10.5 (1x1.6)	2.2	165 (1x1.6)	15.85	42	17	1.6	120 (1x1.6)	11.53	36	15

* Total volume obtained upon dialysis was 8 ml.

4.10.3 SDS polyacrylamide gel electrophoresis

The enzyme solution purified by DEAE-CEllulose (DE32) chromatography after ammonium sulphate fractionation (4.10.2) was concentrated by treating it with dry Sephadex G-25 beads and allowed to swell for 10 min, the gel grains were then removed by filtration. This was repeated until the protein concentration was 0.45 $mg.ml^{-1}$.
The concentrated protein solution was subjected to SDS gel electrophoresis as described in section 3.12.
Fig. 4.10.3.1 is a tracing of the optical density scan obtained from the recorder chart. Five major peaks can be seen with a molecular weight range 17,000 - 100,000.

4.10.4 Desalting of ammonium sulphate fractionated protein by gel filtration on Sephadex G-25

A 2ml aliquot of an ammonium sulphate fractionated protein solution obtained from 3.11.3 was applied to a Sephadex G-25 column (2.5 cm diameter x 35 cm height). The elution was carried out with 20 mM Tris-HCl buffer pH 7.6 as described in 3.11.5. The elution profile obtained is shown in Fig. 4.10.4.1 with the majority of SDH and GI activities occurring in the first peak.

4.10.5 Enzyme inactivation studies on SDH/GI complex

4.10.5.1 Acid treatment

The desalted protein solution obtained from 4.10.4 was divided into five separate 10 ml aliquots. Each aliquot was placed in a 25 ml beaker in an ice-bath and 0.2 M HCl was added dropwise with continuous stirring until a predetermined pH was reached (3.0 - 4.5). The sample was

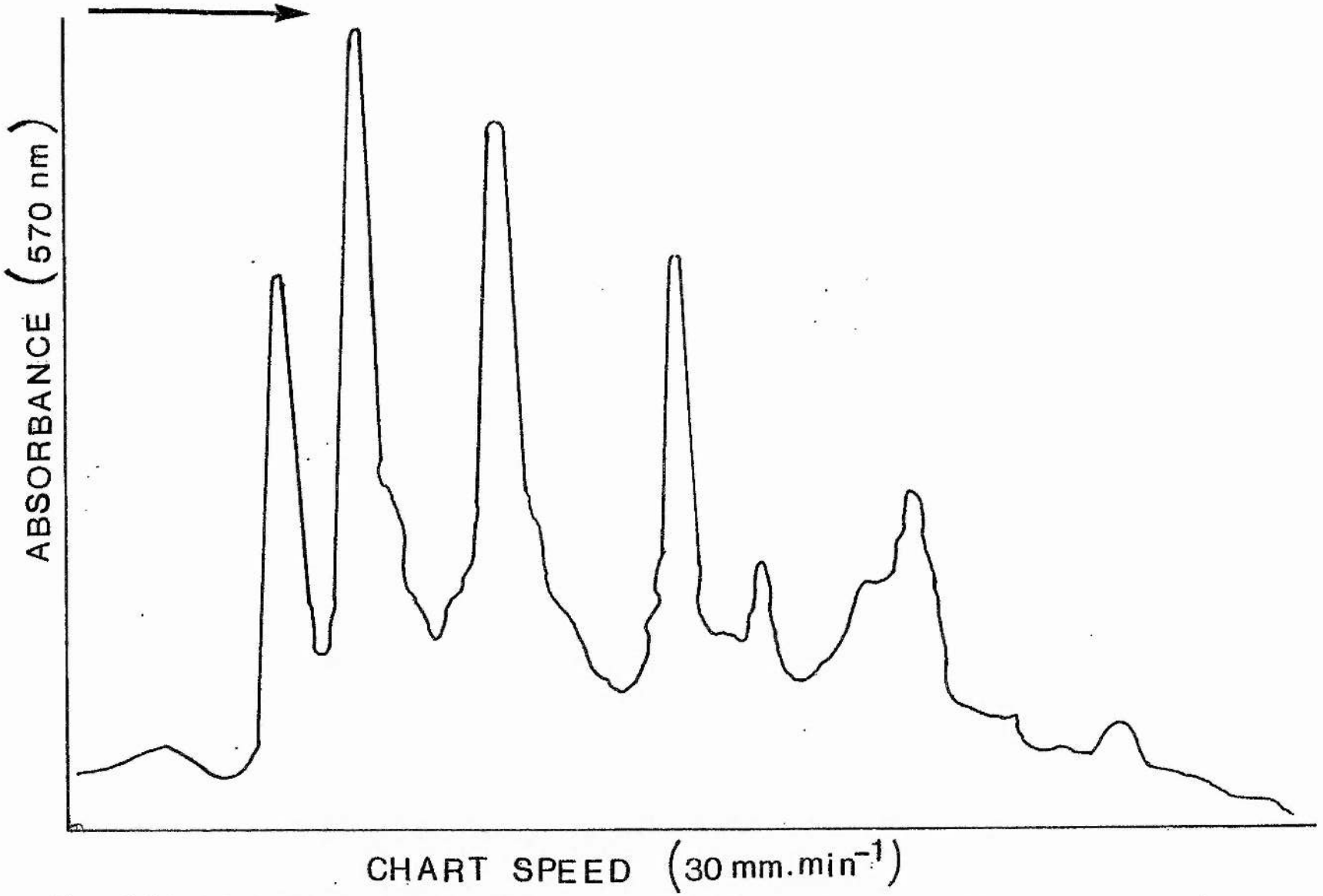


Fig. 4.10.3.1 Tracing of optical density scan obtained when protein sample purified by DEAE-Cellulose (DE32) chromatography was subjected to SDS polyacrylamide gel electrophoresis as described in 4.10.3.

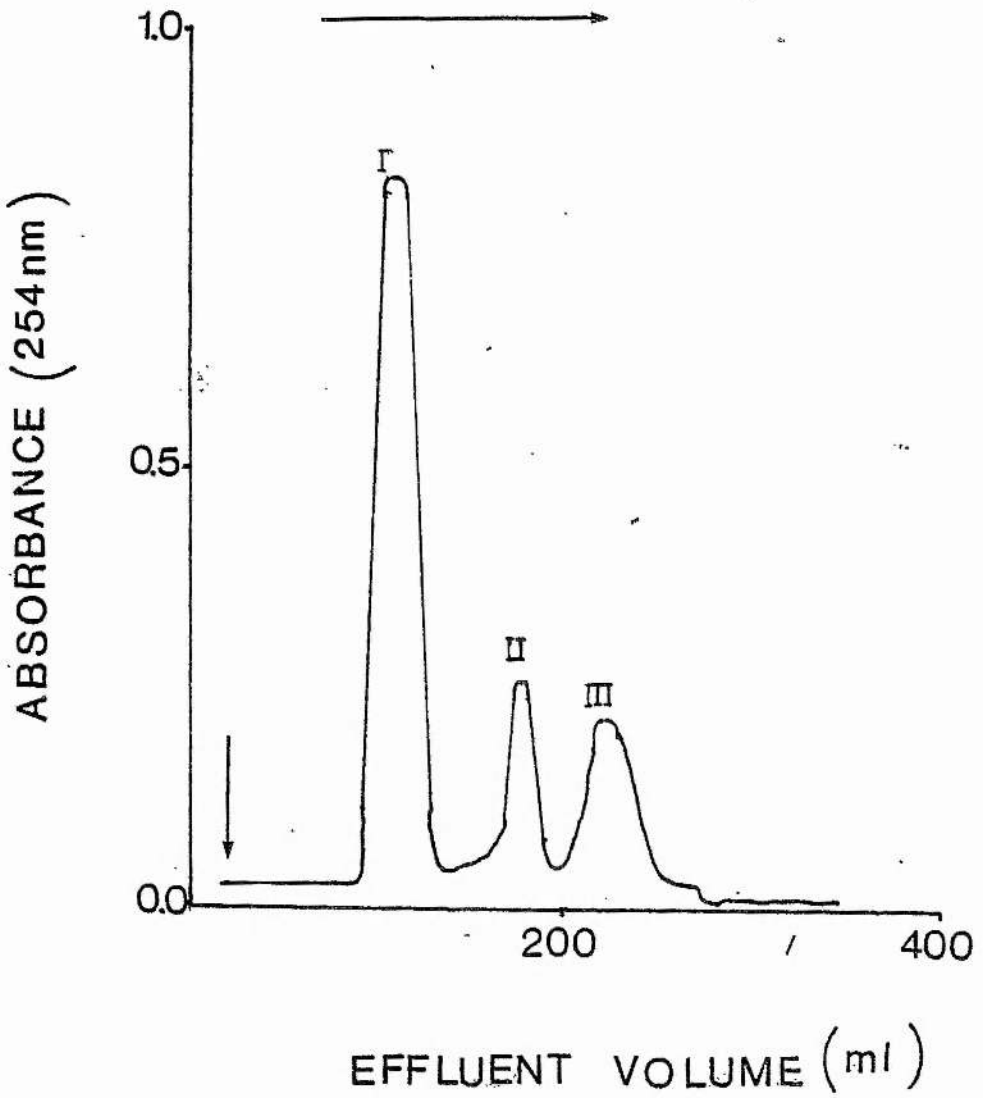


Fig. 4.10.4.1 Elution profile of the chromatography of the redissolved precipitate obtained after ammonium sulphate fractionation of the crude extract on a Sephadex G-25 (Fine) column (2.6 cm diameter x 35 cm height).
An aliquot of protein solution (2 ml, 22.2 mg.ml^{-1}) was applied to the column and elution was carried out at a flow rate of 1.5 ml.min^{-1} with 20 mM Tris-HCl buffer pH 7.6 and the effluent monitored for protein at 254 nm and assayed for SDH activity as in 3.7.1 and for GI activity as in 3.7.2.

Both SDH and GI were recovered in peak I.

↓ Indicates point of sample application.

allowed to stand for 30 min and assayed for SDH activity (3.7.1) and for GI activity as in (3.7.2). A plot of per cent original activity versus pH for each enzyme is presented in Fig. 4.10.5.1.1.

4.10.5.2 Heat treatment

A series of temperatures were decided upon. Aliquots, 0.5 ml, of enzyme solution obtained from 4.10.4 were placed in 100 x 12 mm test tubes and each was incubated for 30 min in a water bath equilibrated to the given temperature, placed in an ice-bath to cool and assayed for SDH activity (3.7.1) and for GI activity as in 3.7.2. A plot of per cent original activity versus temperature for each enzyme is presented in Fig. 4.10.5.2.1.

4.10.6 Bioaffinity chromatography of lactate dehydrogenase (LDH)

LDH purification on 5' AMP-Sepharose 4B was carried out according to Mosbach et al. (77).

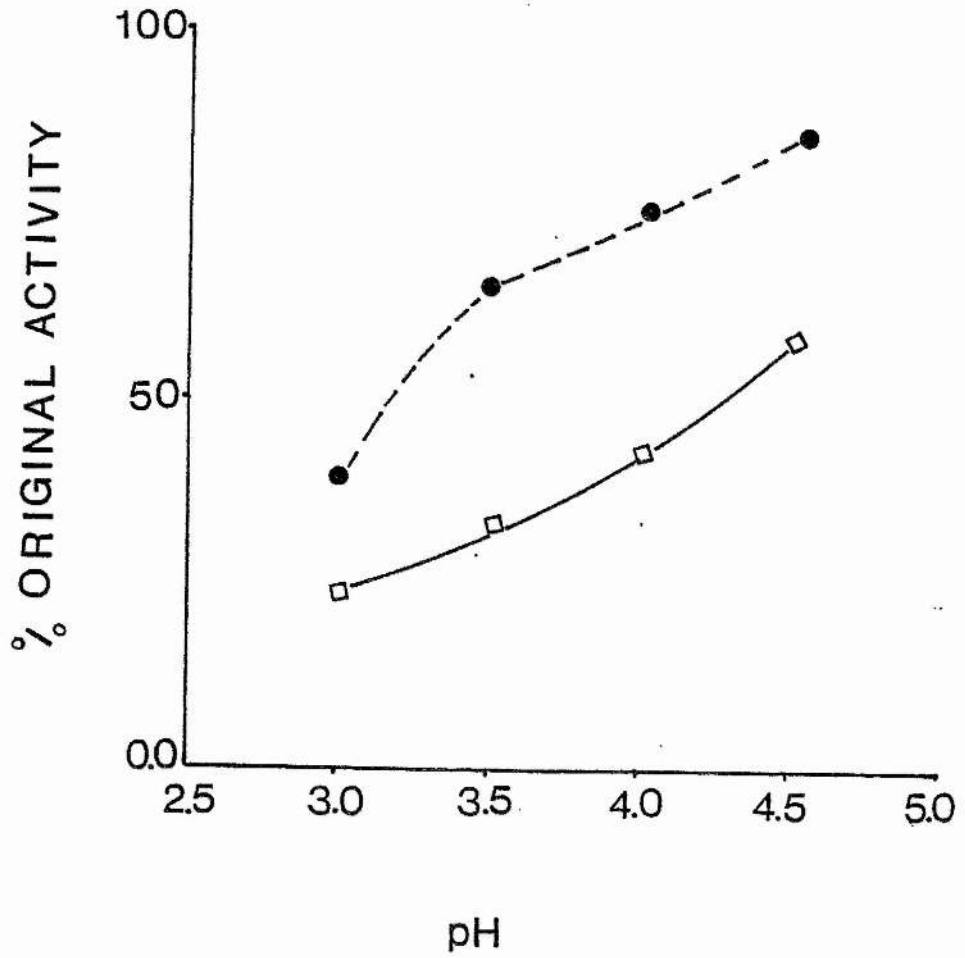
4.10.6.1 Determination of binding capacity

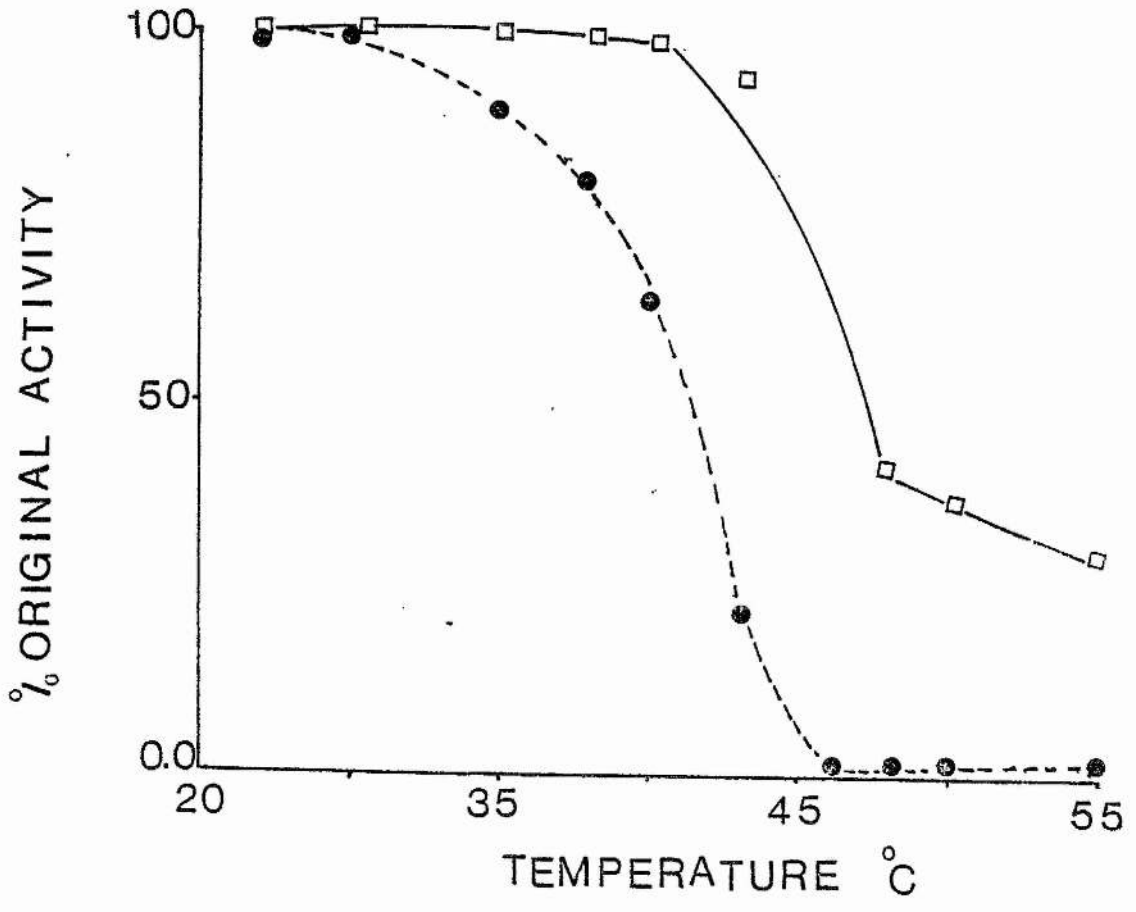
0.25 g lyophilised 5' AMP-Sepharose 4B was swollen in 0.1 M sodium phosphate buffer pH 7.0 and the gel was washed on sintered glass as in 3.11.6 giving a gel volume of 1 ml. The gel was suspended in the same buffer to a total volume of 2.5 ml, which was divided equally into five aliquots, placed in 16 x 120 mm test tubes in an ice-bath.

LDH solution containing 180 $U.ml^{-1}$ with a protein content of 2.5 $mg.ml^{-1}$ was prepared in ice-cold 0.1 M sodium phosphate buffer pH 7.0. Suitable aliquots of the

Fig. 4.10.5.1.1 Acid treatment of SDH (●) and GI (□).
(100% original activities were
determined at pH 7.6).

Fig. 4.10.5.2.1 Heat treatment of SDH (●) and GI (□).


100
50
0.0
% ORIGINAL ACTIVITY
2.5
3.0
3.5
4.0
4.5
5.0
pH


100
50
0.0
% ORIGINAL ACTIVITY
20
35
45
55
TEMPERATURE °C

enzyme solution were added to the test tubes containing gel and well mixed by rotation for five minutes, allowed to sediment and the supernatants were assayed for LDH activity as in 3.7.3 and for protein content by the Folin-Lowry method (3.8.1).

Fig. 4.10.6.1.1 shows the relationship between specific activity of the free enzyme and the percentage binding achieved.

4.10.6.2 Purification of LDH on 5' AMP-Speharose 4B

5' AMP-Sepharose 4B was swollen, washed and packed into the column as in 3.11.6. 0.5 ml enzyme sample was prepared in the same buffer which contains 180 $U.ml^{-1}$ with a protein concentration of 1.25 $mg.ml^{-1}$ and applied to the column. The column was then washed with 20 ml of the equilibrating buffer followed by 10 ml of 0.1 M phosphate buffer made 0.2 M with respect to NaCl to elute the non-specifically bound protein. LDH was then eluted with 1 mM NADH in 0.1 M phosphate buffer pH 7.0. The effluent was monitored and 2 ml fractions were collected using an LKB U.V. recorder and fraction collector. The fractions containing protein were assayed for LDH activity as in 3.7.3. The elution profile of this chromatography is presented in Fig. 4.10.6.2.1.

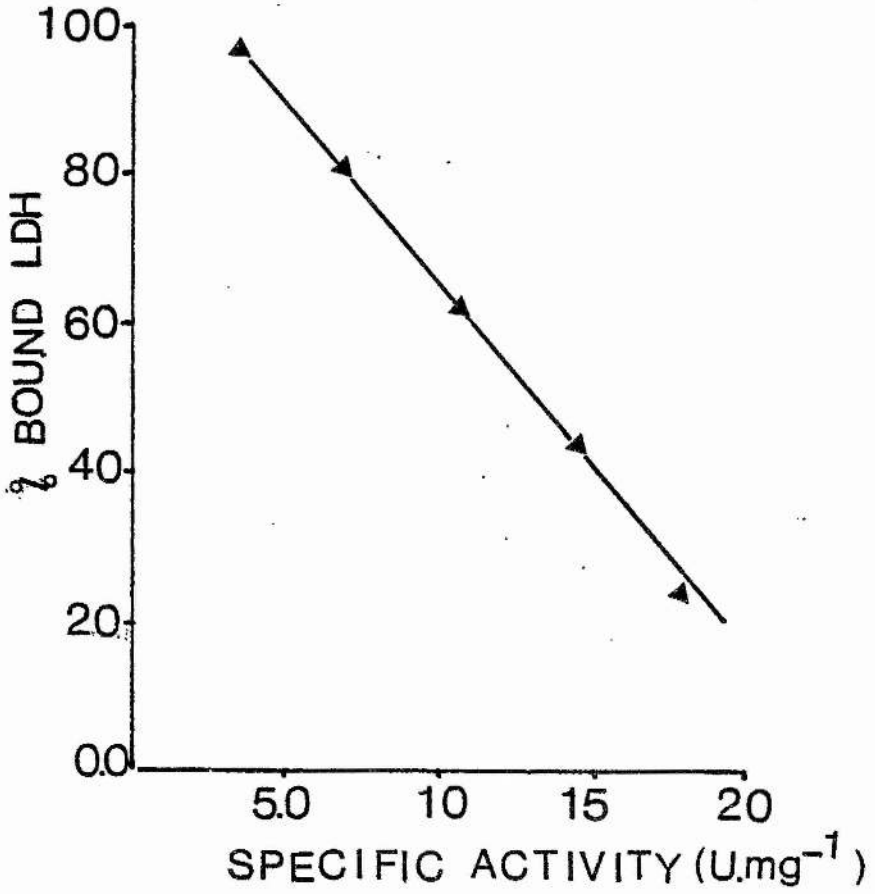
4.10.7 Bioaffinity chromatography of SDH on 5'AMP-Sepharose 4B

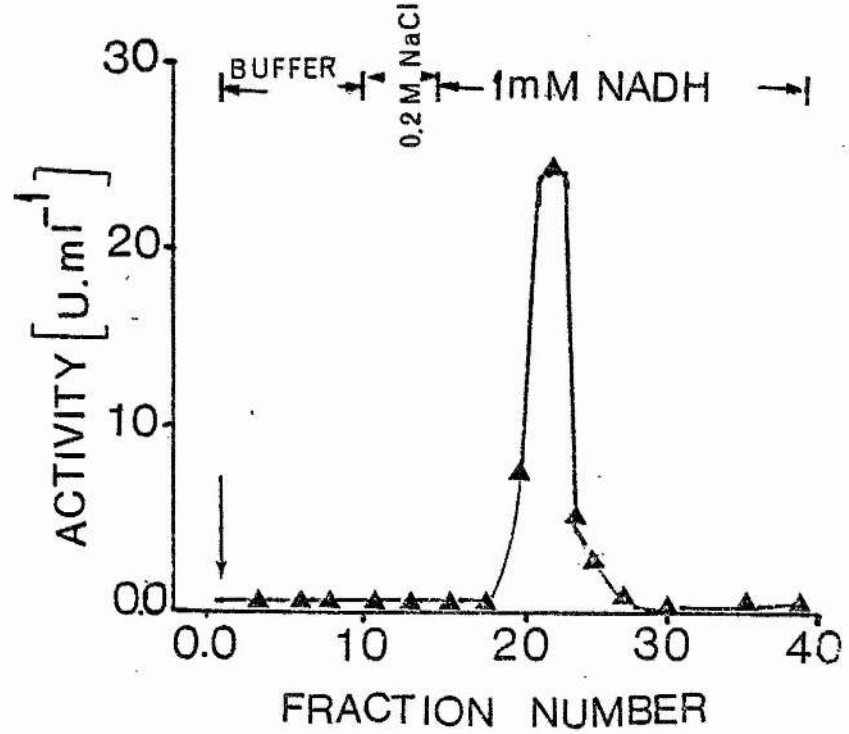
14 mg protein obtained from 3.11.3 were dissolved in 0.1 M ice-cold sodium phosphate buffer pH 7.0 and applied to the top of 0.9 cm (diameter) x 8.5 cm (height) column of 5' AMP-Sepharose 4B as described in 3.11.6. It was found

Fig. 4.10.6.1.1 Shows the relationship between specific activity of the free enzyme and the percentage binding achieved using aliquots of lactate dehydrogenase containing 3.6 to 18 $U.mg^{-1}$ with 0.5 ml gel of 5' AMP-Sepharose 4B. After mixing, the gels were assayed for LDH activity (3.7.3) and protein concentration by the Folin-Lowry method (3.8.1).

Fig. 4.10.6.2.1 Elution profile obtained for the bio-affinity chromatography of LDH on 5' AMP-Sepharose 4B using 0.5 ml sample containing 180 $U.mg^{-1}$ and protein concentration of 1.25 $mg.ml^{-1}$.
The flow rate was 0.2 $ml.min^{-1}$ and as indicated in the chromatogram washing the non-specifically bound protein was performed with 0.1 M sodium phosphate buffer pH 7.0 followed by elution with the same buffer made to 0.2 M with respect to sodium chloride.
LDH was specifically eluted with 1mM NADH in 0.1 M phosphate buffer pH 7.0.
Tubes were assayed periodically for LDH activity as described above.

↓ Indicates point of sample application.


100
80
60
40
20
0.0
% BOUND LDH
5.0
10
15
20
SPECIFIC ACTIVITY (U.mg^{-1})


30
20
10
0.0
ACTIVITY [U.ml^{-1}]
BUFFER
0,2 M NaCl
1mM NADH
0.0
10
20
30
40
FRACTION NUMBER

that it was not necessary to desalt the enzyme solution prior to application provided the enzyme solution was very clear, the slightest turbidity was removed by centrifugation (25,000 x g) at 4^{o} for 30 min.

Periodically tubes were assayed for SDH activity as in 3.7.1 and for GI activity as in 3.7.2.

A typical elution profile can be seen in Fig. 4.10.7.1.

Tubes containing the bulk of the activity were pooled, assayed for protein by the Folin-Lowry method 3.8.1 , for SDH activity as in 3.7.1 and for GI activity as in 3.7.2.

Table 4.10.7.2 is a summary of the data for this bio-affinity chromatography step on 5' AMP-Sepharose 4B after ammonium sulphate fractionation, a total of 80 fold purification was achieved.

The per cent recovery of SDH was 57% and GI was 51%.

The purified enzyme solution was subjected to SDS pol-acrylamide gel electrophoresis for purity check and molecular weight determination in the same way as described in section 3.10.3.

Fig. 4.10.7.3 is a tracing of the optical density scan obtained from the recorder chart.

Two peaks were obtained and the molecular weight calculated from their mobilities as in 3.12 were in the range between 55×10^3 and 77×10^3.

Fig. 4.10.7.1 Elution profile obtained for the bio-affinity chromatography of protein from 3.11.3 upon a 5' AMP-Sepharose 4B column (0.9 cm diameter x 8.5 cm height) using a 1 ml sample containing 14 $mg.ml^{-1}$ protein.

The flow rate was 0.2 $ml.min^{-1}$ and as indicated in the chromatogram washing unbound protein with 0.1 M sodium phosphate buffer pH 7.0 followed by a wash with 0.1 M phosphate buffer pH 7.0 made 0.2 M with respect to NaCl. Specific elution of SDH was performed with 1 mM NADH in the same buffer. Tubes were assayed periodically for SDH and GI activities using the method described in 3.7.1 and 3.7.2 respectively.

↓ Indicates point of sample application.

(▷) Indicates SDH activity

(▶) Indicates GI activity

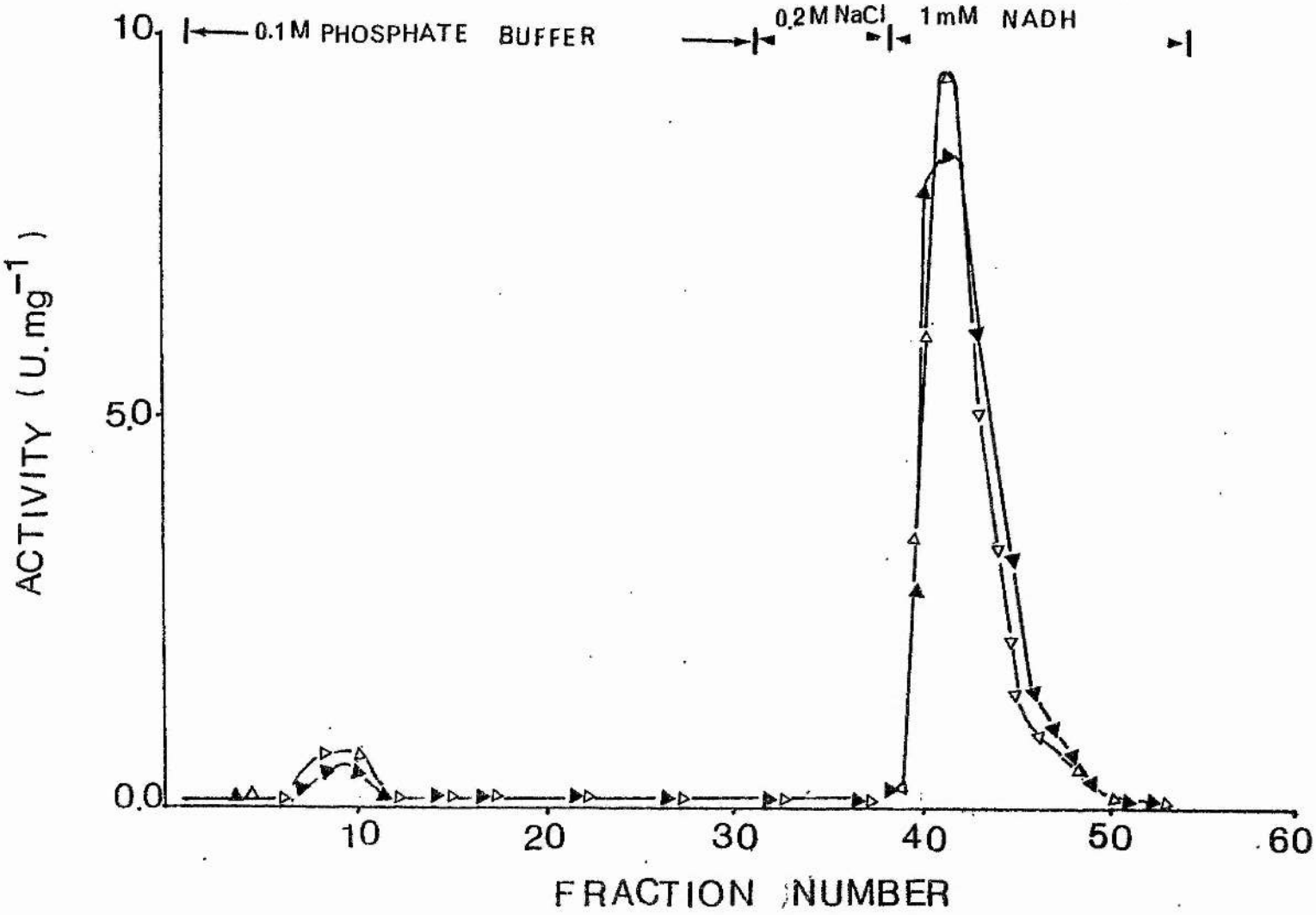

10
5.0
0.0
0.1M PHOSPHATE BUFFER
0.2M NaCl
1mM NADH
ACTIVITY (U.mg⁻¹)
10
20
30
40
50
60
FRACTION NUMBER

Table 4.10.7.2 Purification of SDH and GI complex from A. suboxydans NCIB 9108 on 5' AMP-Sepharose 4B bioaffinity chromatography after ammonium sulphate fractionation.

Step	Vol. (ml)	Protein		SDH					GI				
				Activity		Specific activity ($U.mg^{-1}$)	Recovery %	purification factor (fold)	Activity		Specific activity (U.mg)	Recovery %	purification factor (fold)
		$mg.ml^{-1}$	Total (mg)	$U.ml^{-1}$	Total (U)				$U.ml^{-1}$	Total (U)			
Crude extract	205	12.96	2656	11	2255	0.85	100	1	10.7	2193	0.83	100	1
0.05 M $MnCl_2$	212	5.6	1187	10.2	2162	1.8	96	2.1	9.1	1929	11.6	88	1.9
30%$(NH_4)_2SO_4$	212	3.2	678	9.1	1929	2.8	85	3.3	8	1696	2.5	77	3
61%$(NH_4)_2SO_4$	30	14	420	56.5	1695	4	75	4.7	50	1500	3.6	68	4.3
Applied sample to the 5' AMP-Sepharose column	1 (1x30)	14	14 (1x30)	56.5	56.5 (1x30)	4	75	4.7	45.2	45.2 (1x30)	3.6	68	4.3
Bioaffinity chromatography	7.8	0.08	0.624	5.5	42.9	68	57	80	4.8	37.44	60	51	72

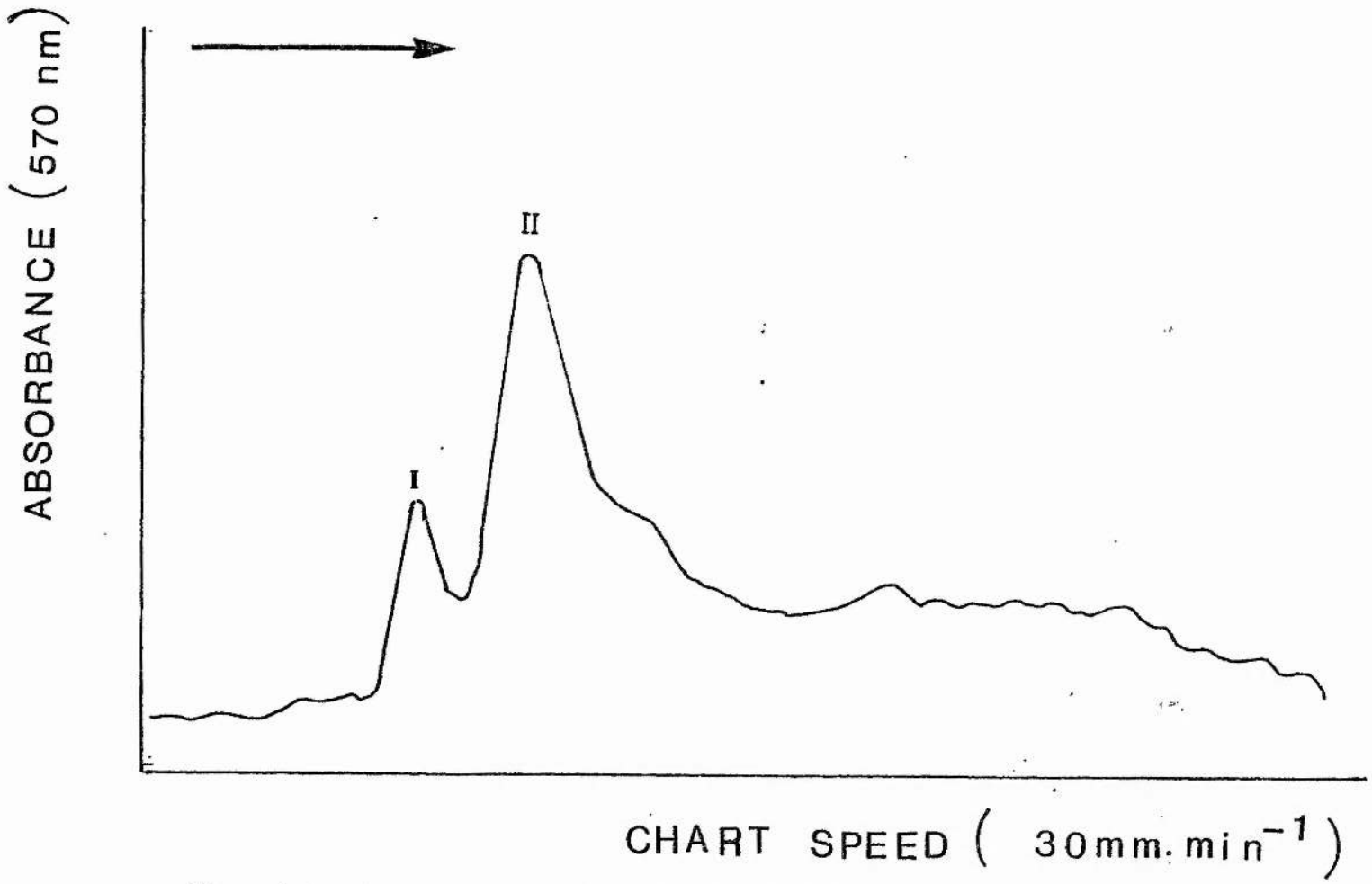


Fig. 4.10.7.3 Tracing of optical density scan obtained when protein sample purified by bioaffinity chromatography on 5' AMP-Sepharose column was subjected to SDS polyacrylamide gel electrophoresis as described in 4.10.3.

4.11 Kinetic studies on sorbitol dehydrogenase

4.11.1 pH-Activity profile

The pH profile of SDH was determined by incubating 93 mM fructose in 20 mM Tris-HCl buffer with 0.25 mM NADH at various pH values (4.0 - 9.5) using 0.25 U of the enzyme at 30°. SDH activity was assayed as described previously in section 3.7.1. The results were plotted as per cent maximum activity obtained against pH values (Fig. 4.11.1.1).

The optimum pH was found to lie between 6.5 and 7.0.

4.11.2 Temperature activity profile

0.25 units of SDH were added to the incubated assay mixture containing 93 mM fructose, 0.25 mM NADH in 20 mM Tris-HCl buffer pH 7.0 at various temperatures and the initial velocity measured as in section 3.7.1 at each temperature. Results were plotted as per cent maximum activity obtained against temperature (Fig. 4.11.2.1).

4.11.3 Determination of kinetic parameters

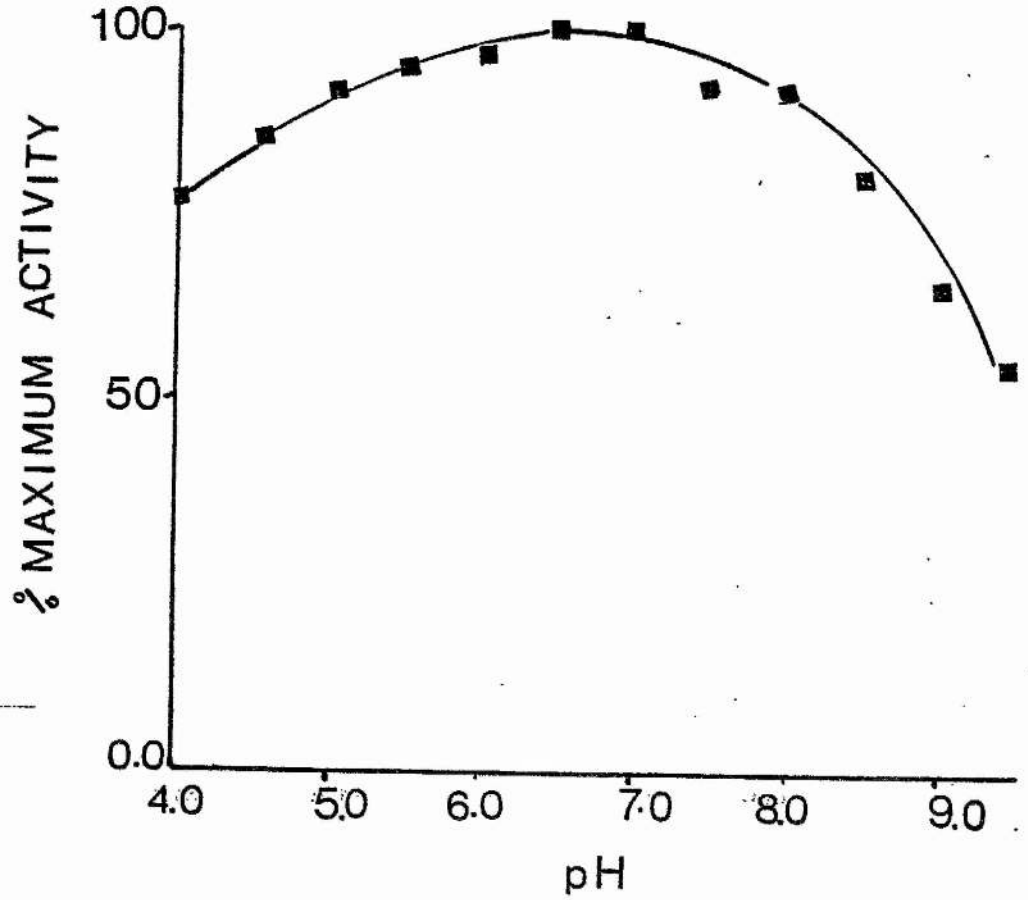
The kinetic parameters, Michaelis constant (K_m), maximum velocity (V_m) with respect to both fructose and NADH were determined by initial velocity studies. Data was analysed in two ways:

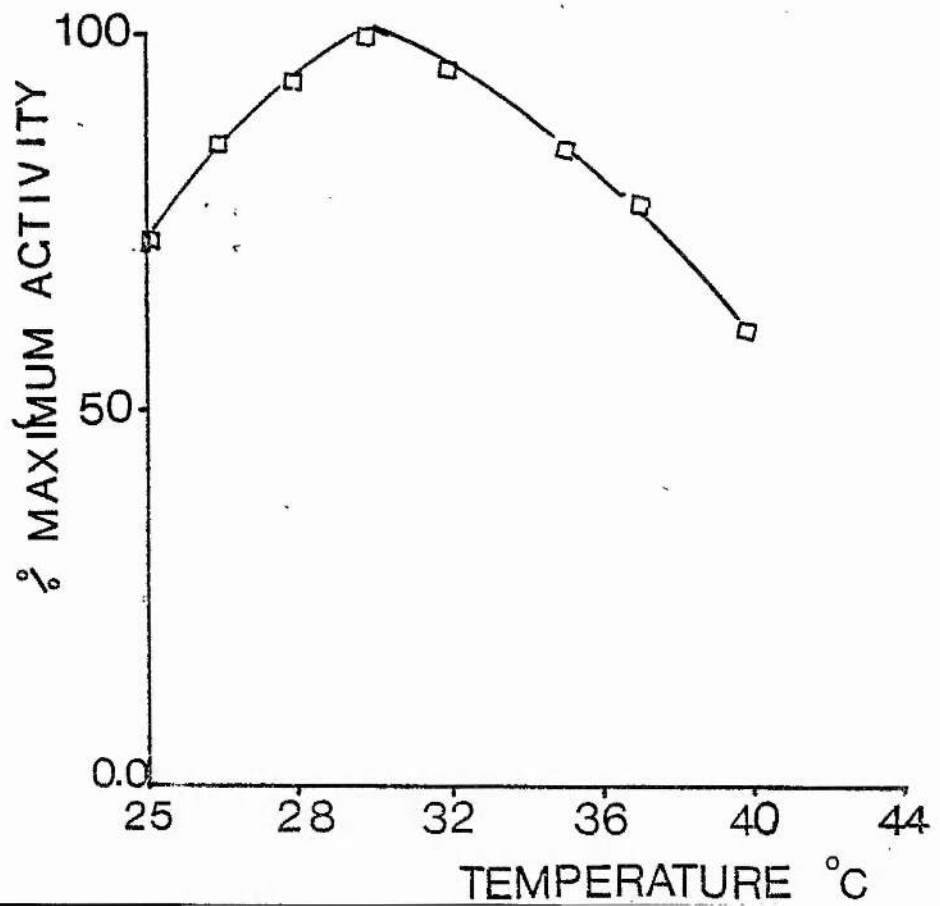
a) Graphically by the method of Lineweaver and Burk (78). The data was fitted by a least squares method to a standard FORTRAN IV bivariance programme (ESBIVAR: author, W. E. Stephens, Department of Geology, University of St. Andrews). The graphs were drawn by an off-line graph plotter (CIL 6011 digital plotter).

b) A computer method written in ALGOL-W (see appendix) which makes provisional estimates of K_m, V_m from initial

Fig. 4.11.1.1 pH-Activity profile obtained for purified SDH on 5' AMP-Sepharose 4B at 30°.

Fig. 4.11.2.1 Temperature-activity profile obtained for SDH purified on 5' AMP-Sepharose 4B column at pH 7.0.


100
50
0.0
% MAXIMUM ACTIVITY
4.0
5.0
6.0
7.0
8.0
9.0
pH


100
50
0.0
% MAXIMUM ACTIVITY
25
28
32
36
40
44
TEMPERATURE °C

velocity (v), substrate concentration, (s), data sets and then refits one of these estimates to the hyperbolic Michaelis-Menten function expressed linearly as Taylor expansion to give fine estimates. Assay solutions (3ml) of fructose (concentration range 8 - 900 mM) and NADH (concentration range 0.015 - 10 mM) in 20 mM Tris-HCl buffer pH 7.0 were incubated at 30^{o} with SDH (0.5 U) obtained from the 5' AMP-Sepharose 4B bioaffinity chromatography. The values of K_m and V_m for both fructose and NADH with their standard errors obtained by both methods are presented in Fig. 4.11.3.1.

Table 4.11.3.1 The values of K_m and V_m and their standard errors for both fructose and NADH obtained by direct computer analysis and Lineweaver-Burk plot fitted by linear regression (Bivariance program).

Substrate	$K_m \pm$ standard error (mM)		$V_m \pm$ standard error($min.\mu mole^{-1}$)	
	Lineweaver-Burk plot fitted by linear regression (Bivariance program)	Direct computer analysis (see appendix)	Lineweaver-Burk plot fitted by linear regression (Bivariance program)	Direct computer analysis (see appendix)
Fructose	166.66	176 ± 23	5.55	5.35 ± 0.31
NADH	0.027	0.028 ± 0.005	5.7	5.25 ± 0.19

Figs. 4.11.3.2 and 4.11.3.3 are traces for the double reciprocal plots obtained for SDH preparation with fructose and NADH respectively upon results computed by a FORTRAN IV bivariance program for correlation analysis and discussed in an appendix to this work.

4.11.4 Inhibition studies on sorbitol dehydrogenase

The determination of K_i values was carried out by the method of Dixon (79), from the equations:-

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \overset{k_2}{\longrightarrow} E + P \quad \ldots\ldots\ldots\ldots (1)$$

$$E + I \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} EI \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (2)$$

$$K_i = \frac{k_{-3}}{k_3} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (3)$$

$$\text{and if } v = \frac{V_m\ (s)}{K_m + (s)} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (4)$$

$$\text{then } v = \frac{V_m\ (s)}{K_m\ (1 + \frac{I}{K_i}\ ) + (s)} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (5)$$

Fig. 4.11.3.2 Double reciprocal plot (Lineweaver Burk) for SDH. Inverse initial velocity plotted against inverse fructose concentration in the presence of 0.25 mM NADH in 20 mM Tris-HCl buffer pH 7.0 at 30^{o} (fructose concentration range 8 mM to 900 mM).

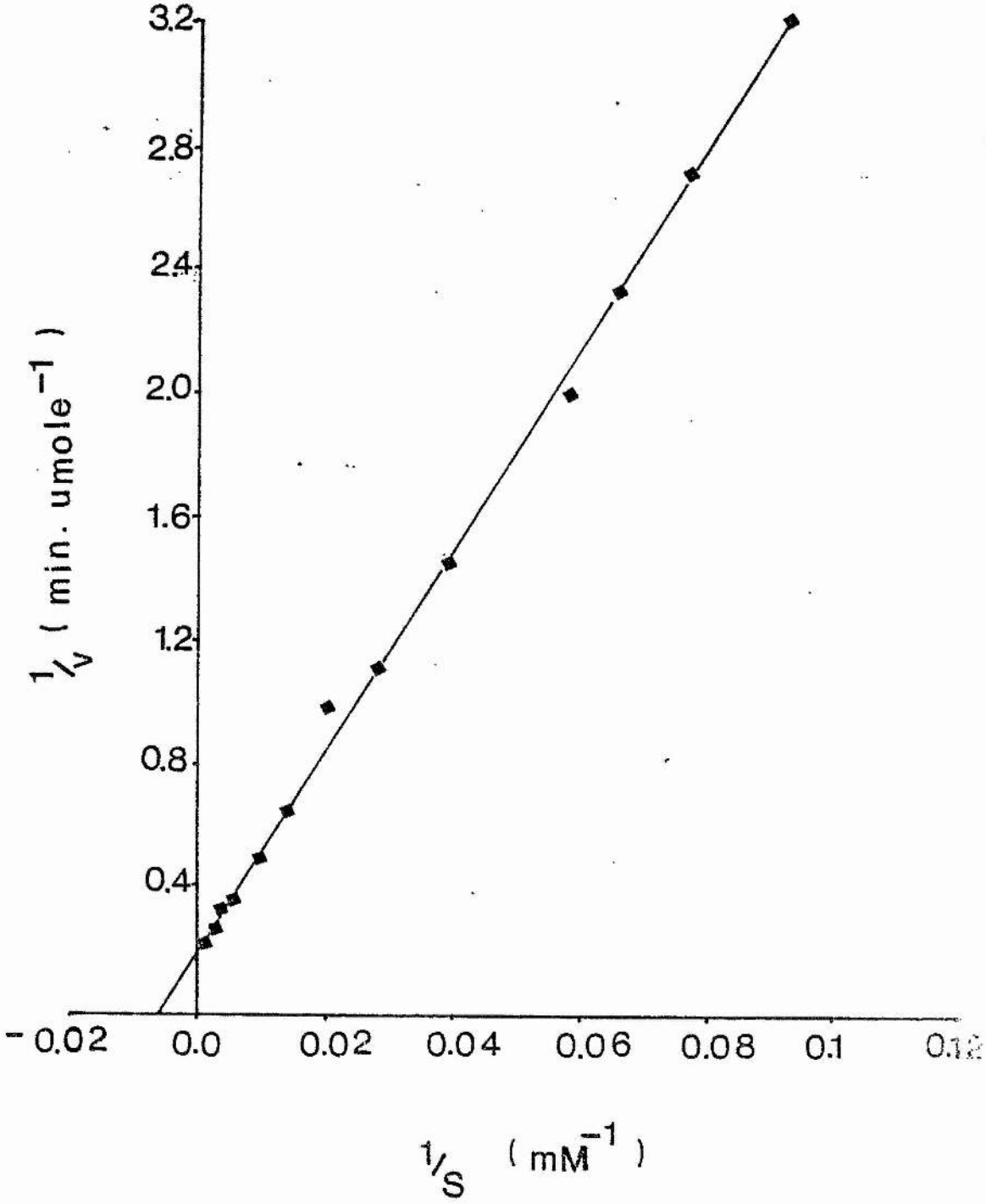
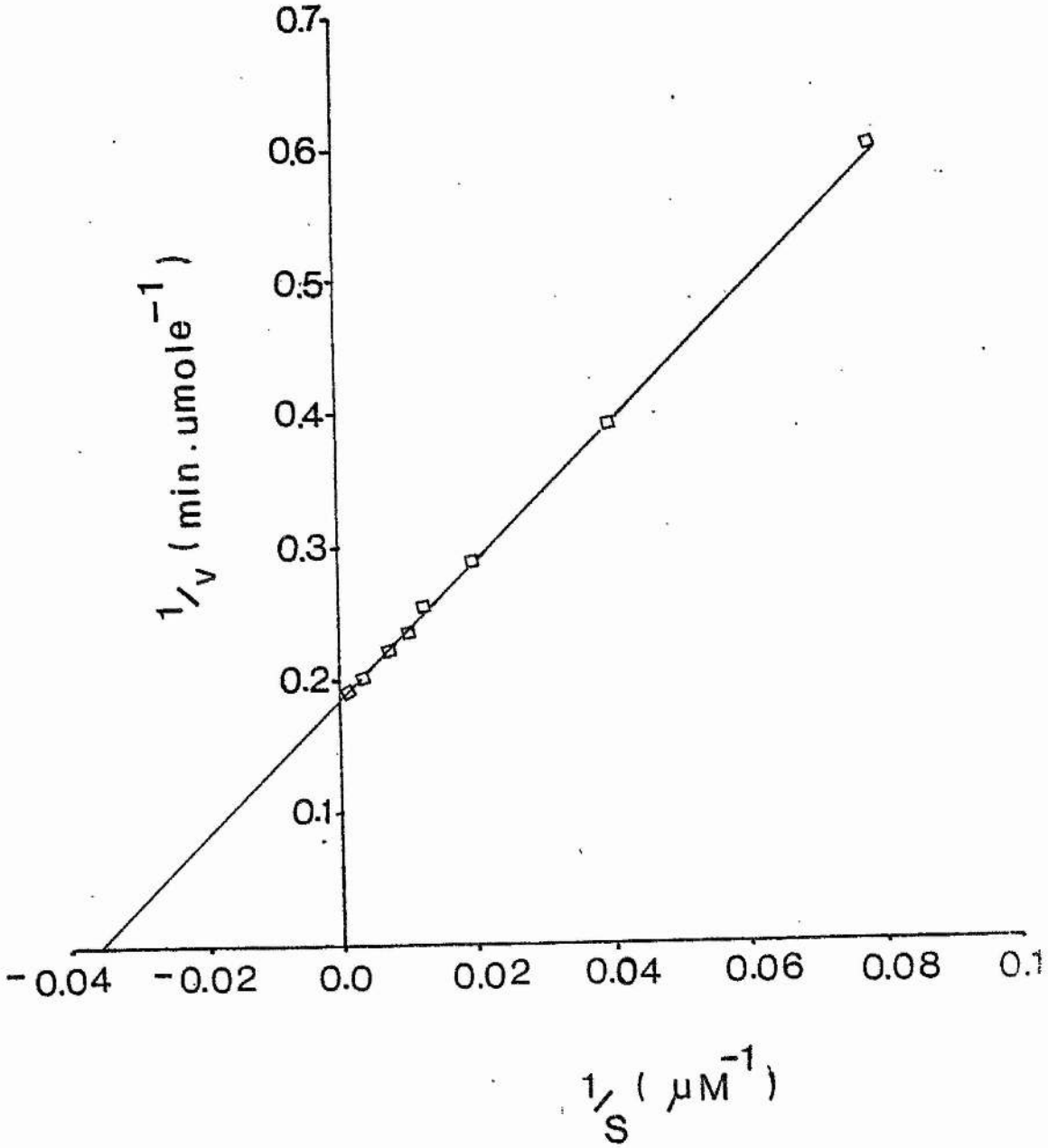

3.2
2.8
2.4
2.0
1.6
1.2
0.8
0.4
1/v (min. umole^{-1})
- 0.02
0.0
0.02
0.04
0.06
0.08
0.1
1/S (mM^{-1})

Fig. 4.11.3.3 Double reciprocal plot (Lineweaver Burk) for SDH. Inverse initial velocity plotted against inverse NADH concentration in the presence of 0.5 M fructose in 20 mM Tris-HCl buffer pH 7.0 at 30° (NADH concentration range 0.015 mM to 10 mM).


0.7
0.6
0.5
0.4
0.3
0.2
0.1
1/v (min.umole-1)
-0.04
-0.02
0.0
0.02
0.04
0.06
0.08
0.1
1/S (μM-1)

where I is the concentration of inhibitor, therefore,

$$1/v = \frac{K_m}{V_m(s)} + \frac{1}{V_m} + \frac{K_m}{V_m(s)} \cdot \frac{I}{K_i} \quad \ldots\ldots\ldots(6)$$

If 1/v is plotted against I and a concentration value of S (equation 6), a straight line is obtained.

If the velocity of the reaction is measured in the presence of inhibitor at two substrate concentrations, S_1 and S_2, the lines obtained by plotting 1/v against I will intercept at a point above the abscissa for competitive inhibition, so that

$$\frac{K_m}{(S_1)} + 1 + \frac{K_m}{(S_1)} \cdot \frac{I}{K_i} = \frac{K_m}{(S_2)} + 1 + \frac{K_m}{(S_2)} \cdot \frac{I}{K_i}$$

or

$$\frac{1}{(S_1)} \cdot \frac{(1 + I)}{K_i} = \frac{1}{(S_2)} \cdot \frac{(1 + I)}{K_i}$$

which is true only if $S_1 = S_2$ or $I = -K_i$

Therefore, the intersection of the two lines occurs at a point equal to $-K_i$.

In the case of uncompetitive inhibitor, the lines do not intercept, but remain parallel.

When S = ∞ the line will meet the abscissa at a point which again gives $-K_i$. This can be seen on plotting 1/v = 0 and S = ∞ in the reciprocal uncompetitive equation.

$$\frac{1}{v} = \frac{1}{V_m K_i} \cdot I + \frac{1}{V_m} \cdot \frac{(1 + K_m)}{(s)} \quad \ldots\ldots\ldots(7)$$

4.11.4.1 Inhibition by sorbitol

The K_i value for sorbitol was determined by incubating SDH (1.58 U) at 30° with 3 ml aliquots of 0.173M and 0.26M fructose solution containing 0.25 mM NADH in 20 mM Tris-HCl buffer pH 7.0. A range of sorbitol concentration (8-104mM) was employed and the rate of decrease in absorbance at 340 nm was measured spectrophotometrically as described in section 3.7.1. The results were plotted in Fig. 4.11.4.1.1 as sorbitol concentration against the reciprocal of the velocity. The intercepts of each line with the ordinate and abscissa were computed using a FORTRAN IV bivariance program for correlation analysis and discussed in the appendix.

The lines were found to meet above the abscissa, Dixon (79), indicating competitive inhibition by sorbitol, with a K_i of 19 mM.

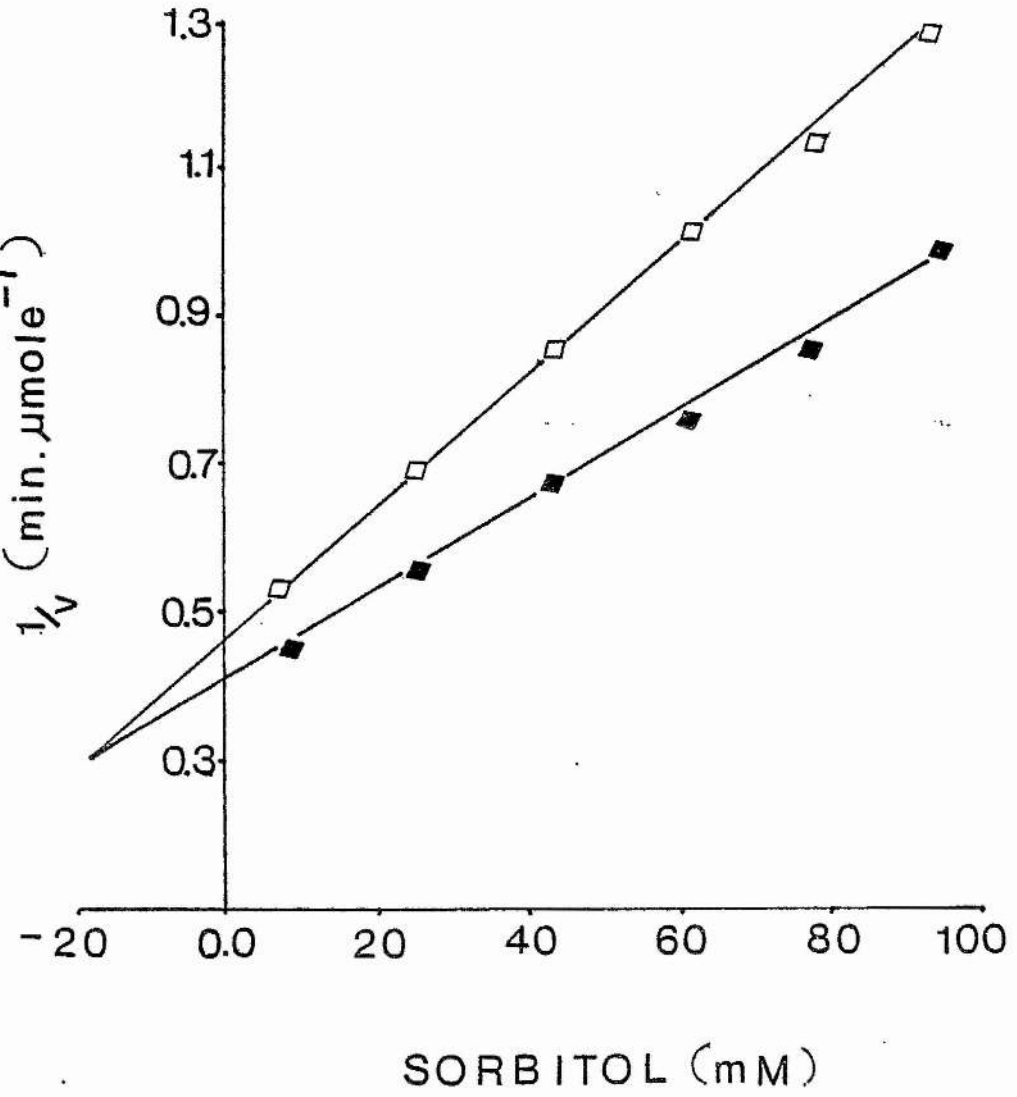


Fig. 4.11.4.1.1 Dixon plot of sorbitol concentration against the reciprocal of the velocity for SDH using 0.173 M (◇) and 0.26 M (◆) fructose with 0.25 mM NADH in 20 mM Tris-HCl buffer pH 7.0 (sorbitol concentration range 8 - 104 mM).

4.11.4.2 Inhibition by mannitol

The K_i value for mannitol was determined as described in 4.11.4.1 using 1.99 U enzyme. The results were plotted in Fig. 4.11.4.2.1 as mannitol concentration against the reciprocal of the velocity. The intercepts of each line with the ordinate and abscissa were computed as in 4.11.4.1. The lines were found to meet above the abscissa Dixon (79), indicating competitive inhibition by mannitol, with a K_i of 600 mM.

4.11.4.3 Inhibition by sorbose

The K_i value for sorbose was determined as in 4.11.4.1 by incubating SDH (1.99 U) at 30° with 3 ml aliquots of 0.173 M and 0.26 M fructose solution containing 0.25 mM NADH in 20 mM Tris-HCl buffer pH 7.0. A range of sorbose concentration (0.086- 0.78 mM) were used and the rate of decrease of absorbance was assayed at 340 nm spectrophotometrically as in 3.7.1. The results were plotted in Fig. 4.11.4.3.1 as sorbose concentration against the reciprocal of the velocity. The intercepts of each line with the ordinate and abscissa were computed as in 4.11.4.1.

The lines were found to remain parallel Dixon (79), indicating uncompetitive inhibition by sorbose with a K_i of 13 mM.

4.11.4.4 Inhibition by p-chloromercuribenzoate

The K_i value for p-chloromercuribenzoate was determined as in 4.11.4.1 by incubating SDH (3.1 U) with 3 ml aliquots of 0.173 M and 0.26 M fructose solution containing 0.25 mM NADH in 20 mM Tris-HCl buffer pH 7.0. A range of p-chloromercuribenzoate concentrations (3 - 8 mM) were used and the rate of decrease in absorbance was assayed at 340 nm spectrophotometrically as in 3.7.1.

The results were plotted in Fig. 4.11.4.4.1 as p-

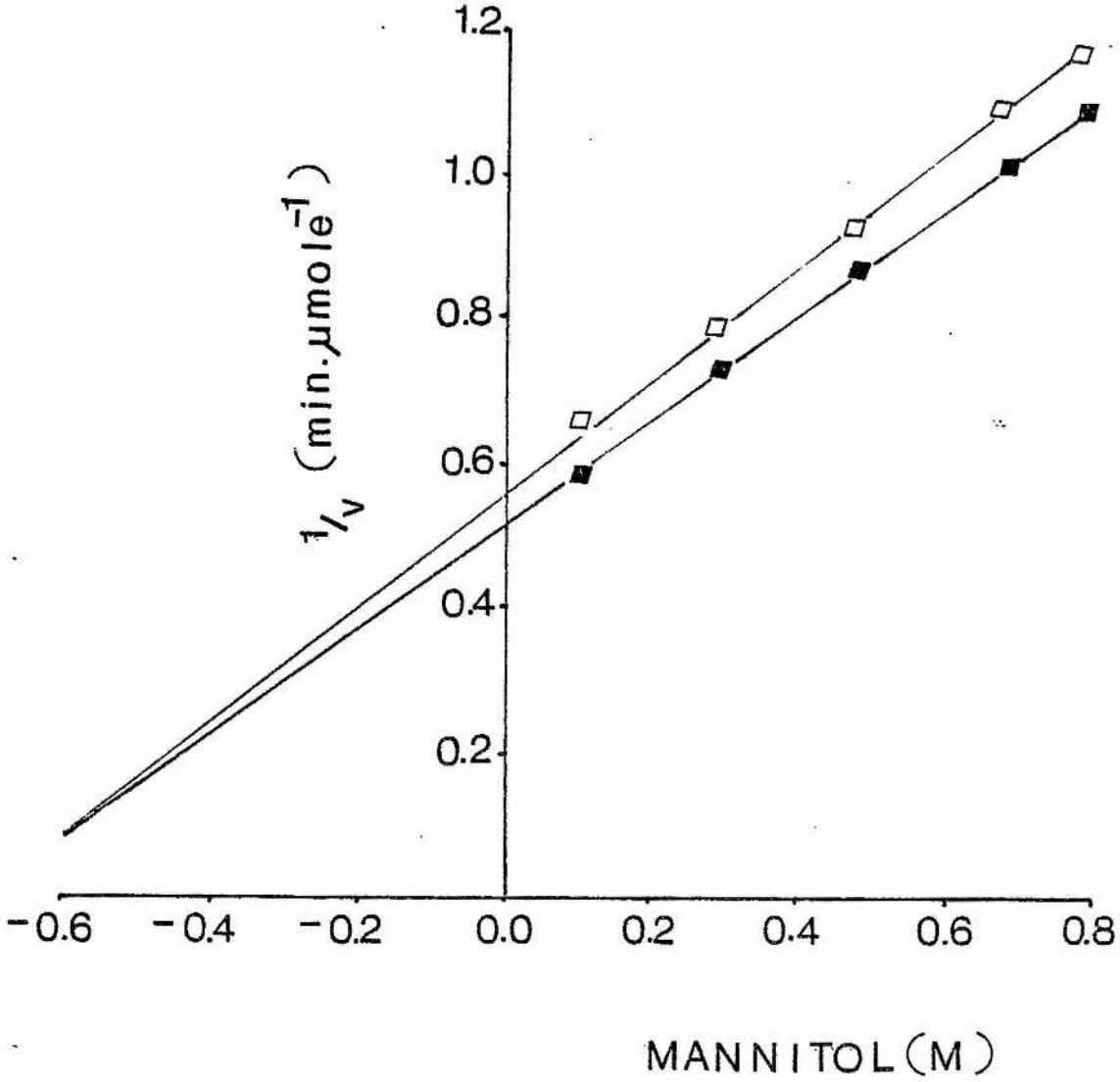


Fig. 4.11.4.2.1 Dixon plot of mannitol concentration against the reciprocal of the velocity for SDH using 0.173 M (◇) and 0.26 M (◆) fructose with 0.25 mM NADH in 20 mM Tris-HCl buffer pH 7.0 (mannitol concentration range 0.086 - 0.8 M).

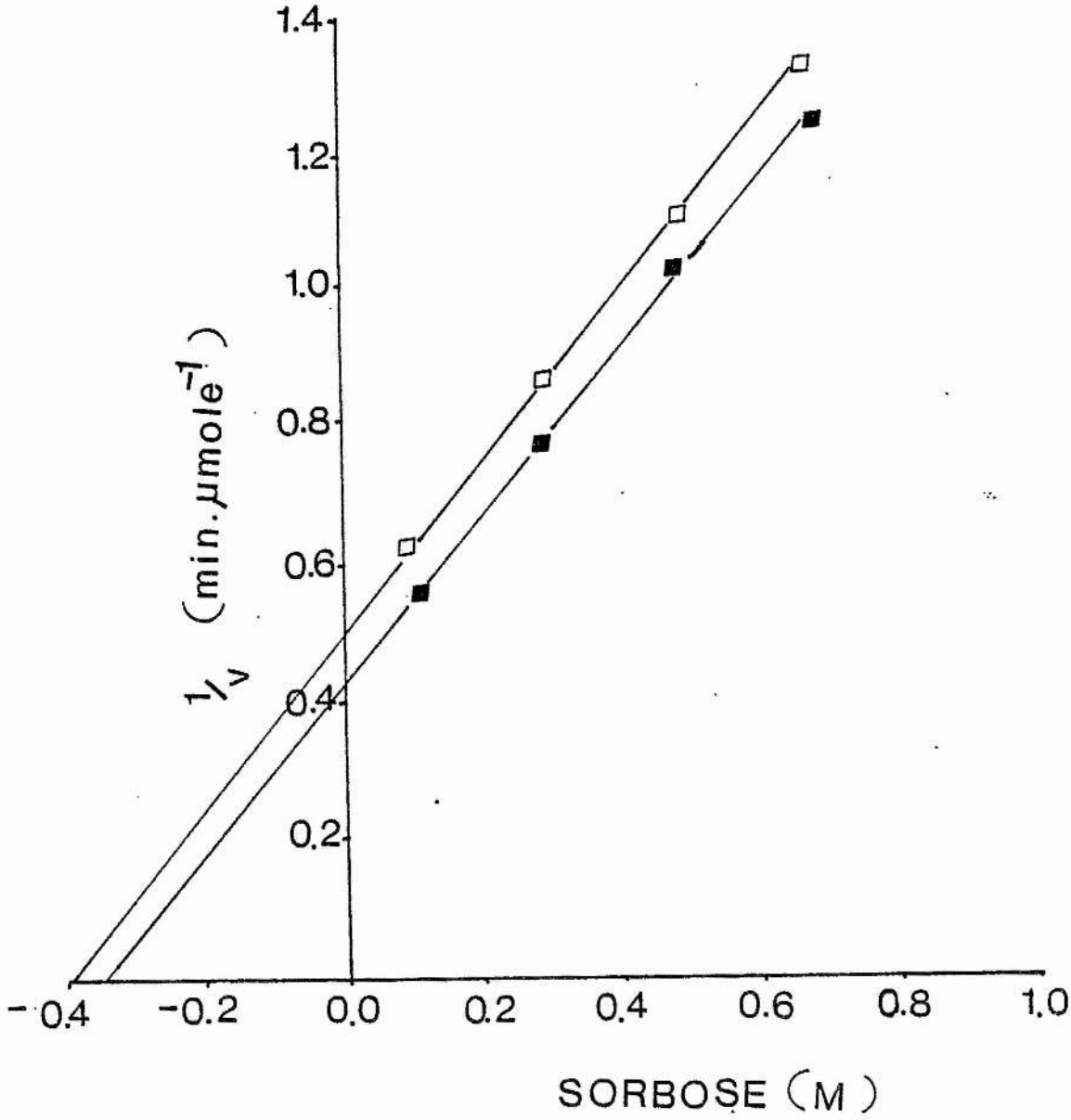


Fig. 4.11.4.3.1 Dixon plot of sorbose concentration against the reciprocal of velocity for SDH using 0.173 M (◇) and 0.26 M (◆) fructose with 0.25 mM NADH in 20 mM Tris-HCl buffer pH 7.0 (sorbose concentration range 0.086 - 0.78 M).

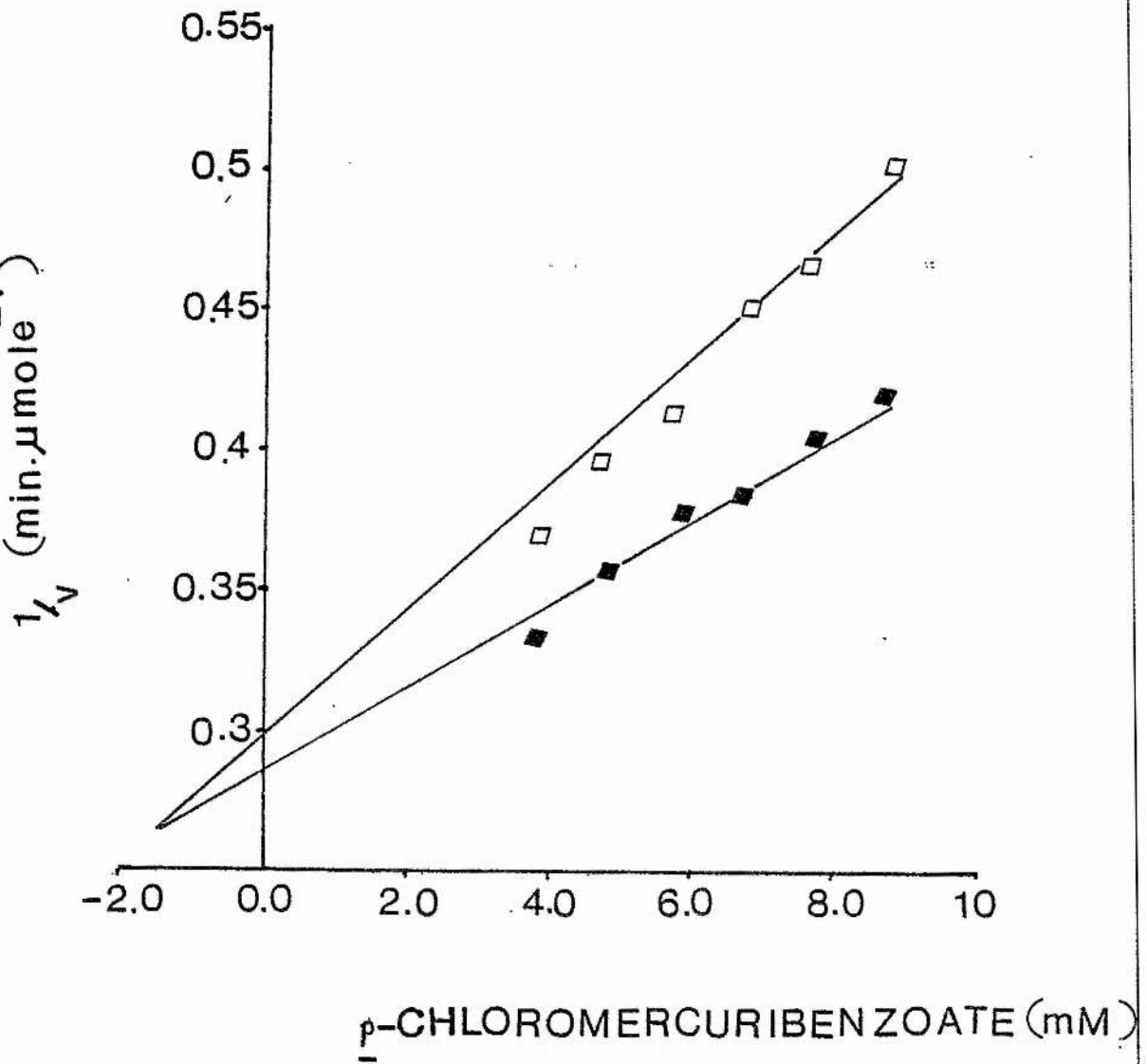


Fig. 4.11.4.4.1 Dixon plot of p-chloromercuribenzoate concentration against the velocity of reciprocal for SDH using 0.173 M (◇) and 0.26 M (◆) fructose with 0.25 mM NADH in 20 mM Tris-HCl buffer pH 7.0 (p-chloromercuribenzoate concentration range 3 - 8 mM).

chloromercuribenzoate concentration against the reciprocal of the velocity. The intercepts of each line with the ordinate and abscissa were computed as in 4.11.4.1. The lines were found to intersect above the abscissa, Dixon (79), indicating competitive inhibition by p-chloromercuribenzoate with a K_i of 1.3 mM.

4.11.4.5 Inhibition by o-iodosobenzoate

The K_i value for o-iodosobenzoate was determined as in 4.11.4.4. The results were plotted in Fig. 4.11.4.5.1 as o-iodosobenzoate concentration against the reciprocal of velocity and the intercepts of each line with the ordinate and abscissa were computed as in 4.11.4.1. The lines were found to intersect above the abscissa, Dixon (79), indicating competitive inhibition by o-iodosobenzoate, with a K_i of 3.6 mM.

4.11.4.6 Inhibition by nicotinamide

The K_i value for nicotinamide was determined by incubating SDH (3 U) at 30° with 3 ml aliquots of 0.15 mM and 0.25 mM NADH solution containing 0.25 M fructose in 20 mM Tris-HCl buffer pH 7.0. A range of nicotinamide concentrations (8 - 90 mM) were used and the rate of decrease in absorbance was assayed at 340 nm spectrophotometrically as in 3.7.1. The results were plotted in Fig. 4.11.4.6.1 as nicotinamide concentration against the velocity. The intercepts of each line with the ordinate and abscissa were computed as in 4.11.4.1.

The lines were found to intersect above the abscissa, Dixon (79), indicating competitive inhibition by nicotinamide, with a K_i of 5 mM.

4.11.4.7 Inhibition by salicylic acid

The K_i value for salicylic acid was determined by

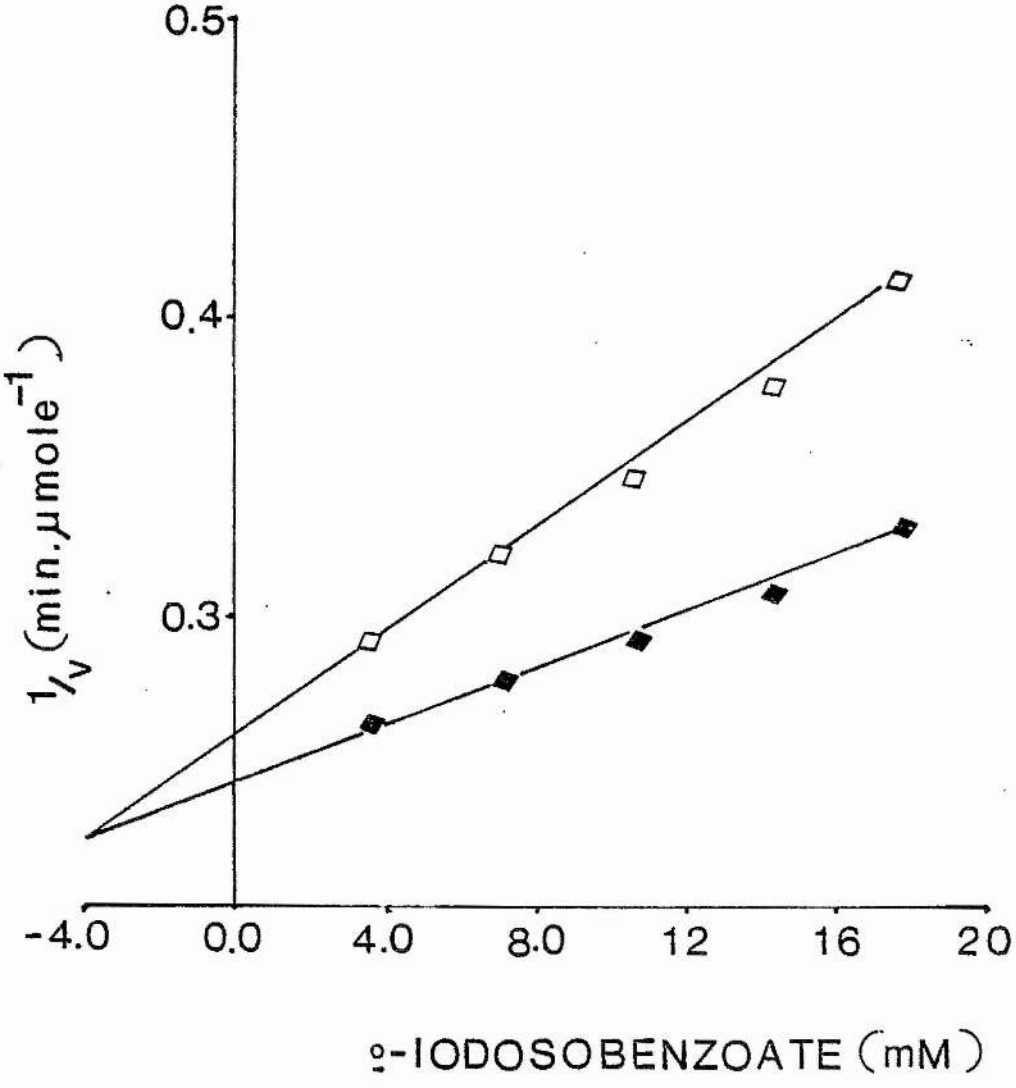


Fig. 4.11.4.5.1 Dixon plot of o-iodosobenzoate concentration against the reciprocal of velocity for SDH using 0.173 M (◇) and 0.26 M (◆) fructose with 0.25 mM NADH in 20 mM Tris-HCl buffer pH 7.0 (o-iodosobenzoate concentration range 2 - 20 mM).

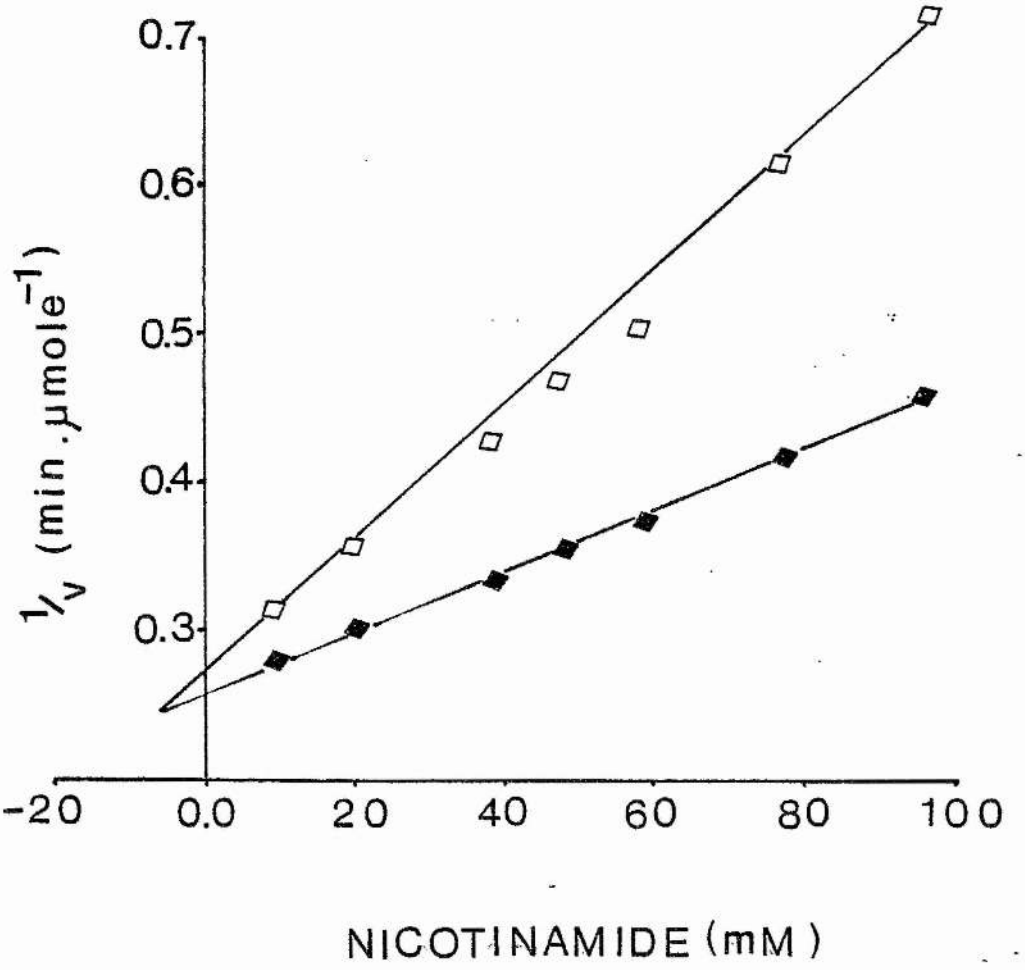


Fig. 4.11.4.6.1 Dixon plot of nicotinamide concentration against the reciprocal of velocity for SDH using 0.15 mM (◆) and 0.25 mM (◇) NADH with 0.25 M fructose in 20 mM Tris-HCl buffer pH 7.0 (nicotinamide concentration range 8 - 90 mM).

incubating SDH (1.85 U) at 30° with 3 ml aliquots of 0.1 mM and 0.2 mM NADH in 20 mM Tris-HCl buffer pH 7.0. A range of salicylic acid concentrations (1 - 19 mM) were used and the rate of decrease in absorbance was assayed at 340 nm spectrophotometrically as in 3.7.1. The results were plotted in Fig. 4.11.4.7.1 as salicylic acid concentration against the reciprocal of the velocity. The intercepts of each line with the ordinate and abscissa were computed as in 4.11.4.1. The lines were found to meet above the abscissa, Dixon (79), indicating competitive inhibition by salicylic acid, with a K_i of 10.4 mM.

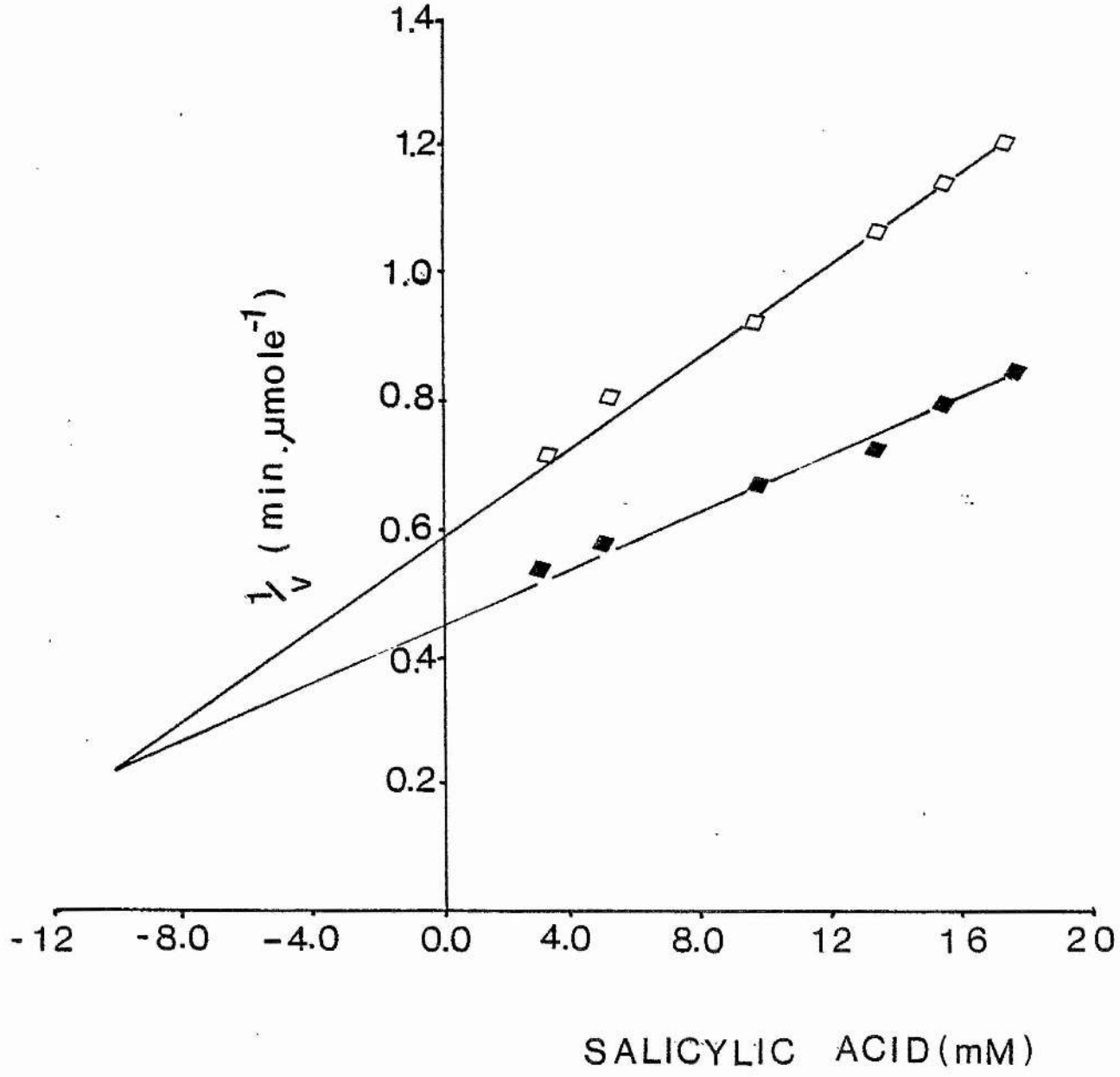


Fig. 4.11.4.7.1 Dixon plot of salicylic acid concentration against the reciprocal of velocity for SDH using 0.1 mM (◇) and 0.2 mM (◆) NADH with 0.25 M fructose in 20 mM Tris-HCl buffer pH 7.0 (salicylic acid concentration range 1 - 19 mM).

Table 4.11.4.7.3 is a summary of the inhibition studies showing the K_i values and the type of inhibition obtained.

Table 4.11.4.7.3

Summary of inhibition studies showing K_i and type of inhibition.

Inhibitor	K_i (mM)	Type of inhibition
Sorbitol	19	Competitive
Mannitol	600	"
Sorbose	13	Un-competitive
p-Chloromercuribenzoate	1.3	Competitive
o-Iodosobenzoate	3.6	"
Nicotinamide	5	"
Salicylic acid	10.4	"

5. DISCUSSION

Studies of the nutritional requirements of Acetobacter suboxydans (A. suboxydans) are largely confined to qualitative data relating to carbon sources which support growth (35). There have been no reports to date of kinetic data relating specific growth rates to nutrient concentration. Bacterial growth can be defined in terms of the rate of increase of either mass of cellular material (biomass) or the rate of increase of number of cells.
The two remain proportional under conditons of steady-state growth, but their ratio can vary with growth conditons.
The cell mass can be determined directly in terms of dry weight or indirectly in terms of packed cell volume or nitrogen content. A useful index of bacterial growth used in this work is turbidimetric measurement. Measurement of culture turbidity at 610 nm allows rapid and simple assessment of the bacterial growth rate.

The effect of batch culture design on the specific growth rate of A. suboxydans was investigated. A 60% increase in the specific growth rate (on a complex medium) resulted when the growth vessel was a vortex-stirred batch apparatus as compared with growth in the New Brunswick controlled environmental orbital shaker. An apparent 60% increase in the specific growth rates (μ), (table 4.2.1) is due to oxygen limitation, these rates being less than maximum specific growth rates (μ_{max}). These results medonstrate clearly that dissolved oxygen tension can be rate limiting to growth, therefore, oxygen must be

thought of as a substrate in no way different to other nutrients required for the growth of aerobes. Due to the low solubility of oxygen (water saturation is 0.235 mM with respect to oxygen), dissolved oxygen is rapidly consumed and must be continuously replaced by solution from air space.

Control of aeration/agitation is therefore important to keep the dissolved oxygen level above the rate limiting values. In this respect the small batch vessel with vortex stirring is superior to orbital shaker techniques. Optimal parameter values (vessel geometry, culture volume and eccentricity of rotation) are not easy to achieve with the latter apparatus. Further investigation into the mass transfer of oxygen in small scale culture vessels could improve the growth rate of aerobes.

Growth experiments on carbohydrate/salts medium demonstrate clear evidence for growth of A. suboxydans NCIB 9108 in the absence of amino acids added to the culture medium.

These experiments suggest that the organism has a limited biosynthetic ability in being able to make its complement of nitrogenous compounds from ammonia and carbon source. Similar findings, although no specific growth rates quoted, were obtained by several workers (31,49,50,51).

The rate of growth appears to depend on the carbon source, higher specific growth rates are achieved in the presence of sorbitol, mannitol and xylitol, lower values are found in the presence of glucose and fructose.

Similar findings, although no specific growth rates were quoted, were obtained by Fewster (35), who reported that a number of polyols investigated were found to be satisfactory as an energy source for growth of A. suboxydans and of the aldoses tested, glucose, in the presence of an adequate neutralising agent, allowed good growth while fructose, as a carbon source, resulted in poor growth. These results suggest that the carbon source is called upon to meet requirements not only for energy but also for part at least of the available carbon for biosynthetic purposes.

The addition of vitamins to the sorbitol/salts medium has, in general, a stimulatory effect on the growth rates of A. suboxydans.

The addition of either nicotinic acid, pantothenic acid, pyridoxine hydrochloride or p-aminobenzoic acid to sorbitol/salts medium stimulated the growth of the organism by 2.4 to 3.5 fold. Biotin, thiamine and inositol stimulated growth by 1.6 to 2.3 fold. Similar results, although no specific growth rates quoted, were obtained by Underkolfler et al. (38), who reported that pantothenic acid, nicotinic acid and p-aminobenzoic acid were required for the growth of A. suboxydans. These results suggest that the relationship of the vitamins to the nutrition of A. suboxydans NCIB 9108 is stimulatory rather than essential. The specific growth rate of 0.043 h^{-1} obtained with sorbitol/salts medium in table 4.5.1.1 and table 4.5.2.1.1 is due to limitation of nitrogen assimilation by the organism.

Yeast extract has been shown to be an important source of growth factors (80), and its inclusion, a series of concentrations in a sorbitol/salts medium resulted in a

parallel increase in the specific growth rate of the organism. The inclusion of yeast extract in a sorbitol/salts medium plus peptone give a similar type of response. Naturally in the latter case, specific growth rates are higher due to the supply of nitrogenous growth substrate in the presence of fixed concentration of peptone.

These two experiments suggest a role for yeast extract as a supplier of growth factors not found in peptone. It suggests also that the chief source of nitrogen is peptone which supplies nitrogen in the form of a protein hydrolysate (amino acids and peptides). A secondary source of nitrogen is yeast extract but the nitrogen content in yeast extract is lower than that of the peptone.

The higher specific growth rates obtained in the presence of peptone may be due to the stimulatory effect of certain amino acids reported by Belly and Claus (49) who found that L-glutamate, L-glutamine, α-ketoglutarate, L-proline and L-histidine greatly stimulated the growth of A. suboxydans.

The induction of sorbitol dehydrogenase activity in response to various carbon sources was investigated. It was shown that a 5-fold increase in specific activity of sorbitol dehydrogenase (SDH) followed growth on sorbitol as a carbon source compared with growth on fructose or sorbose. It must be stated that this corresponds to very poor induction compared with findings of other workers albeit with different systems (81), for β-galactosidase from Escherichia coli, yielded 100-fold or more .

Production of SDH during the growth of A. suboxydans on any of the carbon sources investigated was found to be maximal in the late log phase.

Experiments on medium formulation to maximise SDH activity show saturation type kinetics. A typical saturation kinetic (82,83), response is obtained with either carbon or nitrogen source. It must be stated that maximum SDH specific activity is obtained with yeast extract in the presence of fixed concentration (0.5%) of peptone and fixed concentration of sorbitol (2%) suggesting again that peptone is the chief source of nitrogen while yeast extract serves as a secondary nitrogen source.

Sorbitol dehydrogenase (SDH) [L-iditol dehydrogenase E.C. 1.1.1.14] has been purified from rat liver (25,54,58, 59), sheep liver (57) and from Acetobacter melanogenum (62), while SDH of A. suboxydans had been purified by Cummins et al. (20). Chromatography on DEAE-Cellulose (DE 32) and 5' AMP-Sepharose 4B show that SDH and GI are co-chromatographed with constant ratio of activities.

Attempts to separate SDH and GI activities by thermal or acid denaturation of one component proved unsuccessful, both activities suffered parallel decreases when subjected to these treatments.

The purified SDH/GI complex on 5' AMP-Sepharose 4B bioaffinity chromatography, resolve into two bands upon being subjected to gel electrophoresis. This separation is due to the effect of urea (at a concentration of 6 M) present in the gel buffer. This separation proved unsuccessful, since both activities are lost due to urea denaturation.

In view of these findings, it appears that SDH/GI

activities are confined to two proteins possibly bound by hydrogen bonding and this bonding is dissociated by urea.

The observed tight binding of SDH and GI activities may possibly be related to their joint involvement in the conversion of sorbitol to glucose (probably for further metabolism) in whole growing cells of A. suboxydans as reported by Embden and Griesbach (22), who found that sorbitol was converted into fermentable reducing sugars. On the basis of changes in optical rotation, they considered that the primary product of sorbitol oxidation was fructose. Fructose was subsequently transformed into glucose. However, the present work demonstrated clearly that the primary product of sorbitol oxidation by growing whole cells of A. suboxydans NCIB 9108 is sorbose. In view of these findings, it appears that either the organism used by these workers was a different strain or their observation based solely on optical rotation evidence was misleading.

The pH activity profile of SDH shows that the enzyme has a broad optimum pH ranging from 6.5 to 7.0. This observation differs from that obtained by Smith (57), who reported that pH 7.0 was found to be optimum for the reduction of fructose by SDH from sheep liver in the presence of reduced nicotinamide-adenine dinucleotide (NADH). This difference could be due to the source of enzyme suggesting that bacterial SDH has a broader optimum pH than animal SDH.

Temperature activity profile demonstrates that optimum temperature of SDH activity lies between 28° and 32°, however, temperature activity of this kind is not very meaningful, since the optima are due to two competing effects, namely the normal increase in rate with increasing temperature and the inactivation of the enzyme at elevated temperatures. Thus it can be seen that such determinations indicate the region of optimum activity, and when used together with the thermal stability data (Fig. 4.10.5.2.1), a compromise temperature optimum for SDH activity can be said to be 30°.

A plot of initial velocity (v) versus substrate concentration (s) results in a hyperbolic curve; from this curve it is rather difficult to determine the maximum velocity (V_m) and also the Michaelis constant (K_m).

Without resorting to a computer-aided method, the Lineweaver-Burk double reciprocal plot is the most widely used primary diagnostic plot. However, this plot has two disadvantages. Firstly, equal increments of substrate concentrations that yield equally spaced points on the v versus (s) hyperbolic plot do not yield equally spaced points on the reciprocal plot, which puts greater weight on those points further from the ordinate points that are obtained at the lower values of the substrate concentrations. Secondly, small errors in the determination of v are magnified when reciprocals are taken, such errors are most likely to be significant at low substrate concentrations. These disadvantages can contribute to inaccurate estimation for V_m and K_m.

Statistical treatment of enzyme kinetic data has permitted more accurate estimation of kinetic parameters (84). Data points can be weighted and fitted by computer techniques to different forms of the Michaelis-Menten equation (see appendix).

Inhibition studies by p-chloromercuribenzoate (p-CMB) demonstrate a competitive inhibition with an inhibition constant (K_i) value of 1.3 mM.

Cummins et al. (20) reported a K_i value of 1 mM, a difference of 0.3 mM is probably related either to the method used for data analysis, a computer-aided method used in this work, although not direct is likely to be more accurate, or the source of enzyme being sheep liver which suggests that animal SDH could be more susceptible to p-CMB than bacterial SDH. Unfortunately, the type of inhibition was not reported for comparison, but a competitive inhibition observed appears to be common. Webb (85), reported that a competitive behaviour by mercurials has been observed in a large number of instances, and suggested that the inhibition is probably related to mercaptide formation in pure enzyme preparations.

The reaction scheme describing "dead-end" competitive inhibition is:

$$E + S \underset{}{\overset{k_s}{\rightleftharpoons}} ES \xrightarrow{k_p} E + P$$

$$+$$

$$I$$

$$K_i \updownarrow$$

$$EI$$

Where E is the enzyme, k_s is the substrate constant, p is the product and k_p is the product constant.

The initial velocity of the reaction is proportional to the steady-state concentration of the enzyme-substrate complex, ES.

The actual mechanism involved in the inhibition of SDH by p-CMB is not known, yet, there are three possibilities:

a) binding with sulphydryl (-SH) group at the active centre, preventing complexing of the apoenzyme with any of the other components.
b) binding with an -SH group vicinal to the active centre and interference with the catalysis sterically or electrostatically.
c) secondary disruption of the protein structure which alters the normal configuration of the active centre.

In view of these possibilities, it is possible that one or more -SH groups are sufficiently near the active centre to interfere with the catalysis, either directly or by structural changes. It must be admitted that such a conclusion is not very informative, especially when it is considered that most enzymes contain 5 - 30 -SH groups per molecule and that statistically one would expect one or more of these to be near the active centre.

Inhibition by o-iodosobenzoate also exhibits competitive inhibition with a K_i of 3.6 mM.

Cummins et al. (20), reported a K_i value of 5 mM, a difference of 1.3 mM is probably related again to either the accuracy of the method used for data analysis or that bacterial SDH is more susceptible to o-iodosobenzoate than animal SDH.

As in the case of p-CMB, the type of inhibition was not reported for comparison, but a competitive inhibition seems to be common according to Webb (85), who reported that inhibition by o-iodosobenzoate, and probably most -SH reagents were usually competitive. Webb (85), also reported that most -SH enzymes were inhibited by o-iodosobenzoate while enzymes without -SH groups at or near their active centres were not affected.

o-iodosobenzoate is the most common oxidant for free sulphydryl groups in enzyme molecules, probably due to its selectivity for these groups. The primary action of o-iodosobenzoate is generally believed to be an oxidation of the -SH groups to the disulphide state.

The specific oxidation of the protein -SH groups to disulphide appears to be accompanied by changes in the protein structure, which could have important bearing on the mechanism of enzyme inhibition. Evidence for such structural alteration is given by the increased water-binding capacity of gels formed from serum protein previously treated with o-iodosobenzoate according to Jensen et al. (86), who reported that at equilibrium ratios, o-iodosobenzoate changes the nature of the clots from soft and opaque to firm, elastic, and almost transparent, with a simultaneous increase in water binding from 14.3 to 36.5 g/g.

Again as in the case of p-CMB, it appears that one or more -SH groups are sufficiently near the active centre to interfere with the catalysis, either directly or by structural changes. It must be also admitted that such a conclusion is not very informative, for the same reasons

discussed previously.

Competitive inhibition is also observed with nicotinamide where a K_i value of 5 mM is calculated.

A K_i value of 5 mM appears to be consistent with results quoted by McIlwain and Rodnight (87), who reported that nicotinamide-adenine dinucleotide (NAD^+)-linked enzymes were almost completely inhibited by nicotinamide at concentrations between 2 and 10 mM.

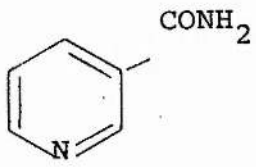
This observation indicates that NAD^+-linked enzymes of various organisms and tissues have marked differences in their susceptibilities to inhibition by nicotinamide.

Inhibition by salicylic acid is found to be competitive with a K_i value of 10.4 mM, suggesting that salicylic acid combines with the free enzyme in a manner that prevents the binding of the coenzyme (NADH). In other words, the inhibitor (I) and the coenzyme (NADH) are mutually exclusive, probably because of true competition for the same site.

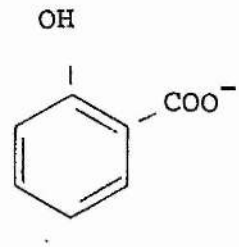
Webb (85), reported that nicotimate was the precursor in the major route of NAD^+ formation in mammalian tissues and probably in microorganisms, while nicotinamide was a secondary precursor in an alternative pathway. The latter was reported to be kinetically and thermodynamically unfavourable. Since salicylic acid is an analogue of nicotinamide, Webb (85), suggested that salicylic acid could either inhibit the reactions of the NAD^+ formation or enter into the reactions to form abnormal intermediates or perhaps an analogue of NAD^+ which might be inhibitory.

Von Eular (88), made the initial investigation of dehydrogenase inhibition by salicylic acid and found that lactate dehydrogenase (LDH) and glucose dehydrogenase (GDH) were inhibited with a K_i value of 7.7 mM for LDH but, the K_i for GDH as well as the type of inhibition was not reported.

Webb (85), reported that substitution in the 3-position of the pyridine ring is necessary for significant inhibition and that the nature of this group could vary considerably, thus, it would appear that no marked electrostatic interaction is involved.



Nicotinamide



salicylic acid

Webb (85), also reported that the pyridine nitrogen (N) appeared not to be of much importance in binding benzamide which was found to be as inhibitory as nicotinamide.

A K_i value of 10.4 mM appears to be relatively high, suggesting that the major binding energy of NAD^+ is contributed by the adenine nucleotide portion of the molecule, so that the pyridine derivatives might not be expected to be potent inhibitor.

Inhibition by sorbitol and mannitol exhibit competitive inhibition with a K_i value of 19 mM and 600 mM respectively, suggesting again that the inhibitor (I) combines with the

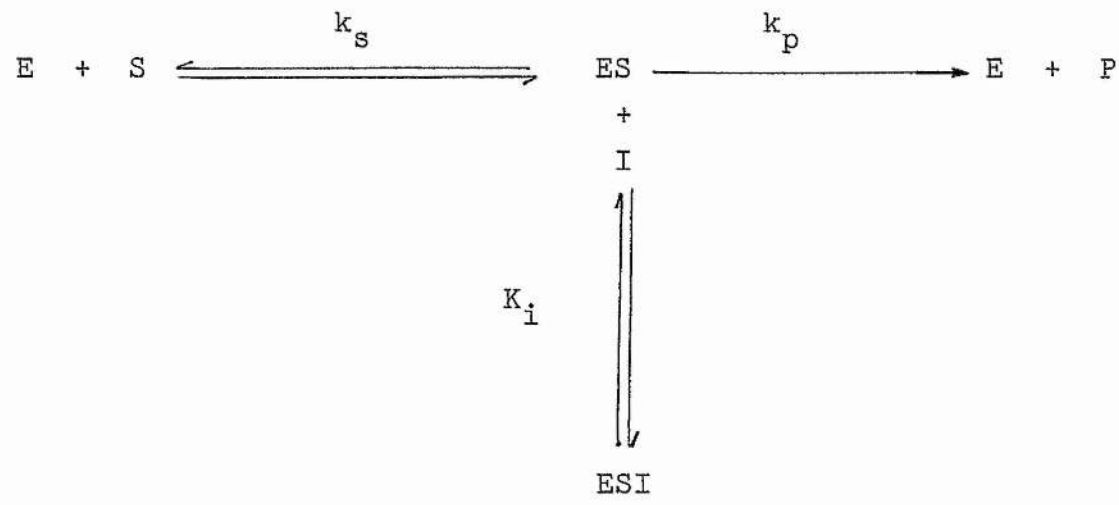
free enzyme in a manner that prevents substrate binding. That is, the inhibitor and the substrate are mutually exclusive, as discussed previously, probably because of true competition for the same site.

The affinity for sorbitol is greater than mannitol suggesting that the binding site could be specifically for the purpose of allowing feed-back inhibition by sorbitol since its formation is a product of fructose in the reaction. The high value obtained for mannitol suggests that SDH has a low affinity for mannitol, probably because mannitol is an isomer of the product (sorbitol).

Inhibition by sorbose illustrates uncompetitive inhibition with a K_i value of 13 mM, suggesting that the inhibitor binds reversibly to the enzyme-substrate complex, resulting in an inactive enzyme-substrate-inhibitor complex, ESI, and that the inhibitor does not bind to the free enzyme.

This type of inhibition appears to have the characteristic of sequential process of the two enzyme ligands in an obligate order.

Classical uncompetitive inhibition is described by the following equilibria:



Uncompetitive inhibition has been reported to be rare in unireactant systems, but common in a multireactant system, because the inhibitor (I) is uncompetitive with respect to a given substrate(S) when the inhibitor (I) binds to the enzyme (E) only after the substrate binds, although the inhibitor (I) rarely binds to a central complex where all the substrate binding sites are filled.

6. SUMMARY

1. The effectiveness of various carbohydrates as carbon sources for the growth of A. suboxydans in a carbohydrate/salts medium was investigated. Higher specific growth rates are obtained in the presence of sorbitol, mannitol and xylitol, lower values are found in the presence of glucose and fructose. Growth of the organism on this medium demonstrates clear evidence that A. suboxydans has a limited biosynthetic ability to make its complement of nitrogenous compounds from ammonia and carbon source.

2. The addition of vitamins to a sorbitol/salts medium has in general a stimulatory effect on the growth rates of A. suboxydans. The role of yeast extract as a supplier of growth factors is described.

3. The product of sorbitol oxidation by whole growing cells of A. suboxydans was found to be sorbose.

4. The induction of sorbitol dehydrogenase activity by A. suboxydans in the presence of various carbon sources was investigated. Production of sorbitol dehydrogenase during the growth of A. suboxydans on all the carbon sources investigated was found to be maximal in the late log phase.

5. Sorbitol dehydrogenase from A. suboxydans is purified by ammonium sulphate fractionation, followed by chromatography on DEAE-Cellulose and bioaffinity chromatography on 5' AMP-Sepharose 4B. The specific activity of the partially purified enzyme is 17 fold (after DEAE-Cellulose chromatography) and 80 fold

(after 5' AMP-Sepharose 4B bioaffinity chromatography) of that of the crude extract.

6. The Michaelis constant (K_m) and maximum velocity (V_m) have been determined for fructose and reduced nicotinamide adenine dinucleotide (NADH). Data are analysed by two computer-aided methods.
7. Inhibition studies involving sorbitol, mannitol, sorbose, nicotinamide, salicylic acid, p-chloromercuribenzoate and o-iodosobenzoate were carried out. The inhibition constants (K_i) and type of inhibition were determined.

7. Appendix

7. 1 Evaluation of V_m, K_m from substrate and velocity data sets.

This program, written in ALGOL-W, computes V_m and K_m together with standard error determinations from data sets of substrate concentrations and corresponding initial velocity measurements.

(Program listing)

```
0000 1-      BEGIN COMMENT THIS PROGRAM CALCULATES KM AND V MAX. FIRSTLY  PROVISIONALLY
0001 --      AND THEN WITH GREATER PRECISION. THE PROVISIONAL ESTIMATES ARE MADE BY
0001 --      FITTING VELOCITY AND SUBSTRATE CONCENTRATION DATA SETS TO A LINEAR  EQUATION
0001 --       BY THE METHOD OF LEAST SQUARES REGRESSION WHILE THE FINE ESTIMATES ARE
0001 --      MADE BY FITTING ONE OF THE PROVISIONAL ESTIMATES TO THE HYPERBOLIC
0001 --      MICHAELIS MENTEN FUNCTION EXPRESSED IN APPROX. LINEAR FORM OF A
0001 --      TAYLOR  EXPANSION
0001 --      INTEGER N;
0002 --      REAL CODING;
0003 --      READ(CODING,N);
0004 --      WRITE("CODE NUMBER IS",CODING);
0005 2-      BEGIN REAL ARRAY S,V,F,F1(1::N);
0007 --      REAL ALPHA,BETA,GAMMA,DELTA,EPSILON,THETA,KM,V1,A,B,C,D,E,B1,B2,
0007 --      S1,VO,KOM,P2,EKOM,EVO,VSQ;
0009 --      INTEGER I;
0009 --      ALPHA:=BETA:=GAMMA:=DELTA:=EPSILON:=VSQ:=0;
0010 --      FOR I:= 1 UNTIL N DO
0010 3-      BEGIN READ(S(I),V(I));
0012 --      VSQ:=VSQ+V(I)**2;
0013 --      ALPHA:=ALPHA+V(I)**3;
0014 --      BETA :=BETA+V(I)**4;
0015 --      GAMMA:=GAMMA+V(I)**3/S(I);
0016 --      DELTA:=DELTA+V(I)**4/S(I);
0017 --      EPSILON:=EPSILON+V(I)**4/S(I)**2;
0018 -3      END;
0019 --      THETA:=ALPHA*EPSILON -GAMMA*DELTA;
0020 --      KOM:=(BETA*GAMMA-ALPHA*DELTA)/THETA;
0021 --      VO:=(BETA*EPSILON-DELTA**2)/THETA;
0022 --      WRITE("PROVISIONAL ESTIMATE OF KM=",KOM);
0023 --      WRITE("PROVISIONAL ESTIMATE OF V MAX =",VO);
0024 --      A:=B:=C:=D:=E:=0;
0025 --      FOR I:= 1 UNTIL N DO
0025 3-      BEGIN F(I):=(VO*S(I))/(S(I)+KOM);
0027 --      F1(I):=(-VO*S(I))/((S(I)+KOM)**2);
0028 --      A:=A+F(I)**2;
0029 --      C:=C+F(I)*F1(I);
0030 --      D:=D+V(I)*F(I);
0031 --      B:=B+F1(I)**2;
0032 --      E:=E+V(I)*F1(I);
0033 -3      END;
0034 --      S1:=(A*B)-C**2;
0035 --      B1:=(B*D-C*E)/S1;
0036 --      B2:=(A*E-C*D)/S1;
0037 --      V1:=B1*VO;
0038 --      KM:=KOM+B2/B1;
0039 --      WRITE("FINE ESTIMATE OF V MAX=",V1);
0040 --      WRITE("FINE ESTIMATE OF KM =",KM);
0041 --      P2:=(VSQ-B1*D-B2*E)/(N-2);
0042 --      EKOM:=(SQRT(P2)/B1)*SQRT(A/S1);
0043 --      EVO:=V1*SQRT(P2)*SQRT(B/S1);
0044 --      WRITE("THE STANDARD ERROR OF KM =",EKOM);
0045 --      WRITE("THE STANDARD ERROR OF V MAX=",EVO);
0046 -2      END;
0047 -1      END.
```

(Typical Data Input)

```
0047 --        1  10
0047 --      0.015  2.003
0047 --      0.025  2.554
0047 --      0.05  3.416
0047 --      0.1  3.45
0047 --      0.15      4.499
0047 --      0.2      4.669
0047 --      0.25     4.431
0047 --      0.5      5.635
0047 --      0.75   4.935
0047 --      1.00     5.03
```

Note. In the first line of the data input 1 is simply a code number (i.e. to identify the experiment and 10 is the number of data pairs (S,V) entered.

(Typical Output)

```
CODE NUMBER IS        1.000000
PROVISIONAL ESTIMATE OF KM=      0.02835648
PROVISIONAL ESTIMATE OF V MAX =        5.334785
FINE ESTIMATE OF V MAX=        5.250582
FINE ESTIMATE OF KM =     0.02852988
THE STANDARD ERROR OF KM =    0.005534533
THE STANDARD ERROR OF V MAX=      0.1947537
```

7. 2 Linear regression program coupled with off-line graphical plotting.

A number of investigations during the course of this work required drawing a best fit line through a set of data points to give accurate information of the intercepts of the live at the ordinate and abscissa (section 4.11.3) and also to allow evaluation of the intersections of two lines (section 4.11.4.1, 4.11.4.2, 4.11.4.3, 4.11.4.4, 4.11.4.5, 4.11.4.6 and 4.11.4.7).

A standard FORTRAN IV bivariance statistical program coupled with sophisticated graphics software was used (ESBIVAR: author, W. E. Stephens, Department of Geology, University of St. Andrews) stored in the University of St. Andrews computer was used for correlation analysis. Output was by line printer (regression data) and CIL 6011 digital plotter (plotted graph).

8. BIBLIOGRAPHY

1. Policy of the Journal and Instructions to Authors (1975) Biochem. J. 145, 1
2. Bergey's Manual of Determinative Bacteriology, 8th edn. Bailliere, Tindall and Cox, London.
3. Kluyver, A. J. and Leeuw, F. J. de (1924) Tijdschr. Vergelijk Geneesk, 10, 170.
4. White, J. (1966) Process Biochemistry. 1, 139.
5. Leisinger, Th. and Muller, J. (1967) Process Biochemistry, 4, 10
6. Cheldelin, V. H. (1961) Metabolic Pathways in Microorganisms, New York : J. Wiley & Sons.
7. William, P. J. le B., and Rainbow, C. (1964) J. Gen. Microbiol., 35, 237.
8. King, T.E. and Cheldelin, V. H. (1954) Biochem. Biophys. Acta, 14, 108.
9. King, T.E. and Cheldelin, V. H. (1956) J. Biol. Chem. 220, 177.
10. Kondo, K. and Ameyama, M. (1957) The Reports of the Department of Agr. Shizuoka Univ., Japan. 7, 118, 132, 136.
11. Stouthamer, A. H. (1960) Thesis Univ. Utrecht.
12. Rao, M. R.R. (1955) Ph. D. Thesis, Univ. Illinois, Urbana.
13. King, T.E. and Cheldelin, V. H. (1952) J. Biol. Chem. 198, 127.
14. Sekizawa, Y., Maragoudakis, M. E. and Kerwar, S. S. (1962) Biochem. Biophys. Res. Commun., 9, 361.
15. Greenfield, S. and Claus, G. W. (1972) J. Bacteriol., 112, 1295.

16. Edson, N.L. and McCorkindale, J. (1954) Biochem. J. 57, 518.

17. Arcus, A.C. and Edson, N.L. (1956) Biochem. J. 64, 385.

18. Bertrand, D. (1904) Ann. Chem. Phys. (8), 3, 181,195,227.

19. Hudson, C.S., Hann, R.M. and Tilden, E.B. (1938) J. Am. Chem. Soc. 60, 1201.

20. Cummins, J.T., Cheldelin, V.H. and King, T.E. (1957) J. Biol. Chem., 226, 301.

21. Shaw, D.R.D. and Bygrave, F.L. (1966) Biochem. Biophys. Acta 113, 608.

22. Embden, G. and Griesbach, W. (1914) Hoppe-Seyl.Z. 91, 251.

23. Anschel, N. (1930) Klin.Wachr., 9, 1400.

24. Thaunhanser, S.J. and Meyer, K.H. (1929) Munch. Med., 178, 597.

25. Blakley, R.L. (1951) Biochem. J., 49, 257.

26. Edson, N.L. (1936) Biochem. J. 30, 1862.

27. Hoyer, D.P. (1898) Dissert. Univ. Leiden, Waltmann, Delft.

28. Frateur, J. (1950) La Cellule, 53, Fasc. 3, 333.

29. Shimwell, J.L. (1957) J. Inst. Brew., 63, 44.

30. Gray, C.H. and Tatum, E.L. (1944) Proc. Natl. Acad. Sci., 30, 404.

31. Rao, M.R.R. and Stokes, J.L. (1952) J. Bacteriol., 65, 405.

32. Rao, M.R.R. and Stokes, J.L. (1953) J. Bacteriol. 66, 371.

33. Rainbow, C. (1966) Wallerstein Lab. Commun., 29, 5

34. Rainbow, C. and Mitson, G.W. (1953) J. Gen. Microbiol., 9, 371.

35. Fewster, J.A. (1958) Biochem. J. 69, 582.

36. King, T.E. and Cheldelin, V.H. (1953) J. Bacteriol. 66, 581.

37. Lampen, J.O., Underkolfer, L.A. and Peterson, W.H. (1942) J. Biol. Chem., 146, 277.

38. Underkolfer, L.A., Bantz, A.C. and Peterson, W.H. (1943) J. Bacteriol., 45, 183.

39. Landy, L. and Dicken, D.M. (1942) J. Biol. Chem. 146, 109.

40. Landy, L. and Streightoff, F. (1943) Proc. Soc. Exp. Biol. Med., 52, 127.

41. Marshall, J.H. and Postage, J.R. (1949) International Congr. Biochem., Cambridge, Abstr. Commun., p.338.

42. Litsky, W., Esselen, W.R., Tepper, B.S. and Miller, G. (1953) J. Food Res. 18, 250.

43. Karabinoos, J.V. and Dicken, M. (1944) Arch. Biochem. 4, 211.

44. Hall, A.N., Tiwari, K.S., Thomas, G.A. and Walker, T.K. (1953) Arch. Biochem. Biophys. 46, 485.

45. Sarette, H.P. and Cheldelin, V.H. (1945) J. Biol. Chem., 159, 311.

46. Stokes, J.L. and Larsen, A. (1945) J. Bacteriol., 49,495.

47. Kerwar, S.S., Cheldelin, V.H. and Parks, L.W. (1964) J. Bacteriol. 88, 179.

48. Yamada, Y., Tsuchiya, K., Aida, K. and Uemura, T.(1965) Proceedings of the Annual Meeting of the Agr. Chem. Soc. Japan, Tokyo.

49. Belly, R. T. and Claus, G. W. (1972) Arch. Microbiol. 83, 237.

50. Asai, T. (1968) Acetic Acid Bacteria; classification and chemical activities : University of Tokyo press.

51. Rao, M. R. R. (1957) Ann. Rev. Microbiol., 11, 317.

52. Maragoudakis, M. E. and Strassman, M. (1967) J. Bacteriol. 94, 512.

53. Greenfield, S. and Claus, G. W. (1969) J. Bacteriol. 100, 1264.

54. Banks, J. and William-Ashman, H. G. (1954) Arch. Biochem. Biophys., 50, 513.

55. Banks, J. and William-Ashman, H. G. (1957) Arch. Biochem. Biophys., 72, 185.

56. Widmer, C., King, T.E. and Cheldelin, V. H. (1956) J. Bacteriol., 71, 737.

57. Smith, A. G. (1962) Biochem. J. 82, 135.

58. Chida, K., Yamamoto, N. and Yasuda, K. (1976) Acta Histochem. Cytochem., 9, 144.

59. McCorkindale, J. and Edson, N. L. (1954) Biochem. J. 57, 523.

60. Cohen, R. B. (1961) Lab. Invest. 10, 459.

61. Chida, K., Yamamoto, N. and Yasuda, K. (1975) Acta Histochem. Cytochem. 8, 234.

62. Sasajima, K. and Isono, M. (1968) Agr. Biol. Chem. 32, 161.

63. Kersters, K., Wood, W. A. and De Ley, J. (1965) J. Biol. Chem. 240, 965.

64. Englard, S. and Avigad, G. (1965) J. Biol. Chem. 240, 2297.

65. King, T.E. (1958) J. Biol. Chem. 233, 1295.

66. Herr, H.G. (1956) Biochem. Biophys. Acta 22, 202.

67. Myers, J.S. and Jakoby, W.B. (1973) Biochem and Biophys. Research Commun., 51, 631.

68. Hollman, S. (1964) In: Hoppe-Seyler/Thierfelder: Handbuch der physiologisch und pathologisch-chemischen. Analyse Springer Verlag, Berlin-Heidelberg, N.Y. Vol. VI part A p.704.

69. Charkavorty, M., Veiga, L.A., Bacila, M. and Horecker, B.L. (1962) J. Biol. Chem. 237, 1014.

70. Leon, M. and Marr, A.G. (1961) J. Bacteriol. 82, 224.

71. Elsaesser, Th., Huber, J. and Hirscher, H.Z. (1962) Allgem. Mikrobiol., 2, 249.

72. Schnarr, J.W., Szarek, W.A. and Jones, J.K.N. (1977) Appl. and Environm. Microbiol., 33, 732.

73. Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265.

74. Llayne, E. (1957) In Methods in Enzymology (Collowick, S.P. and Kaplan, N.O. Eds.) Vol. 3, p.450, Academic Press, London & N.Y.

75. Holligan, P.M. (1971) New Phytol., 70, 239.

76. Weber, K., Pringle, J.R. and Osborn, M. (1972) Method Enzymol., 26, 3.

77. Mosbach, K., Guilford, H., Ohlsson, R. and Scott, M. (1972) Biochem. J. 127, 627.

78. Lineweaver, H. and Burk, D. (1934) J. Am. Chem. Soc. 56, 658.

79. Dixon, M. (1953) Biochem. J. 55, 170.

80. Bridson, E. Y. and Breaker, A. (London 1970) In "Methods in Microbiology" eds. Morris, J. R. and Ribbons, D. W. Vol. 3A Acad. Press.

81. Rickenberg, H. V., Cohen, G. N., Buttin, G. and Monod, J. (1956) J. Am. Inst. Pasteur. 91, 829.

82. Monod, J. (1942) Récherches sur da croissance des Cultures bacteriennes, Paris: Herman et Cie.

83. Monod, J. (1949) Ann. Rev. Microbiol. 3, 371.

84. Wilkinson, G. N. (1961) Biochem. J. 80, 324.

85. Webb, J. L. (1966) In "Enzyme and Metabolic Inhibitors", Vol. II, Academic Press, N.Y. & London.

86. Jensen, E. V., Hospelhorn, V. D., Tapley, D. F. and Huggins, C. (1950) J. Biol. Chem. 185, 411.

87. McIlwain, H. and Rodnight, R. (1949) Biochem. J. 44, 470.

88. Von Eular, H. (1942) Ber. 75, 1876.