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**ACETALDEHYDE METABOLISM IN MAMMALS**

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fulfilment of the requirements  
for the degree of

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### ABSTRACT

The metabolism of acetaldehyde in mammalian systems has been investigated both with in vitro studies on sheep liver aldehyde dehydrogenase, and by following changes in blood acetaldehyde levels in human volunteers.

The intracellular localisation of aldehyde dehydrogenase was examined, using concurrent assays for marker enzymes. Average results for the distribution of the enzyme showed 30% of the total activity to be mitochondrial, 42% cytosolic, and 10% microsomal. The presence of activity in the microsomes was confirmed in studies where the enzyme was solubilised using sonication and Triton X-100 treatments. The cytoplasmic enzyme was purified by ammonium sulphate fractionations, ion exchange chromatography, and gel filtration, to a reproducible purity of 95%. Molecular weights of the native protein (205,000 - 220,000), subunit molecular weights (51,000 - 55,000) and behaviour during gel electrophoresis have been determined for both the cytoplasmic and mitochondrial sheep liver aldehyde dehydrogenases.

An enzymic assay was developed for measuring very small amounts of acetaldehyde (down to 0.25 nmoles), using purified cytoplasmic sheep liver aldehyde dehydrogenase preparations. This assay has been applied to the determination of levels of acetaldehyde in the blood of human volunteers following ethanol ingestion. Untreated whole blood containing ethanol formed acetaldehyde, and the levels of acetaldehyde determined in either perchlorate-treated or untreated plasma were lower than those in whole blood. Acetaldehyde levels determined enzymically using perchloric acid supernatants of whole blood were reasonably close to the levels determined by gas chromatography, and the range of results (0.05 - 0.25 mg%) correlated well with similar results reported recently in the literature.

A new ion exchange resin, DEAE Protion, was used during this investigation and separations of aldehyde dehydrogenase on forms of this resin and DEAE cellulose have been compared in an Appendix to the thesis.

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## SECTION 1

INTRODUCTION

Acetaldehyde is the first product of ethanol metabolism in mammalian systems. Ethanol is oxidised to acetaldehyde primarily by alcohol dehydrogenase, and to a lesser extent by microsomal oxidising systems (Lieber and DeCarli, 1970 b; Orme-Johnson and Ziegler, 1965) and by catalase (Lundquist et al., 1963). There is still debate on the relative importance of the latter two significant systems of ethanol oxidation. Alcohol dehydrogenase, however, has been studied in detail since 1909, when Battelli and Stern investigated the properties of the enzyme in animal tissues. It has been established as the major ethanol-removing enzyme by studies using pyrazole (Lester et al., 1968; Bustos et al., 1970), by studies on the changes in redox ratios of tissues following ethanol intake (Tygstrup et al., 1965) and by comparison of  $K_M$  values for ethanol in vivo and in vitro (Lundquist and Wolthers, 1958). The extensive literature on alcohol dehydrogenase is covered in reviews by Sund and Theorell (1963), Wallgren and Barry (1970) and von Wartburg (1971).

By contrast, enzyme systems removing acetaldehyde have only been studied more recently. In 1937, Dixon and Lutwak-Mann demonstrated that aldehyde mutase activity, which catalysed the dismutation of acetaldehyde to ethanol and acetic acid, was distinct from aldehyde oxidase activity. It was subsequently shown (Racker, 1949) that "aldehyde mutase" activity in bovine liver was in fact due to two separable enzymes, the already known alcohol dehydrogenase, and a previously unknown enzyme, aldehyde dehydrogenase, which catalysed the oxidation of acetaldehyde to acetate using  $NAD^+$  as a cofactor.

Both aldehyde oxidase and aldehyde dehydrogenase have now been isolated from and studied in mammalian tissues. Aldehyde oxidase, which catalyses the reaction



has been established as distinct from xanthine oxidase (Gordon et al., 1940), which also catalyses acetaldehyde oxidation, and has been purified to homogeneity (Rajagopalan et al., 1962) from rabbit liver.

The substrate binding site, metal content, and electron transfer properties of the rabbit liver enzyme have been studied (Rajagopalan and Handler, 1964 a; 1964 b). Recently, in this department, sheep liver aldehyde oxidase has been isolated and its metal content and other properties examined (Hendtlass, 1973). Work carried out on aldehyde dehydrogenase (aldehyde: NAD<sup>+</sup> oxidoreductase, EC 1.2.1.3) since it was first studied in 1949 (Racker) is described fully in the introductions to Sections 2, 3, 4 and 5. Until 1972, the enzyme had not been purified to homogeneity from any mammalian source, but sufficient kinetic data had been obtained (Buttner, 1965; Deitrich et al., 1962) to show that aldehyde dehydrogenase exhibited an extremely low  $K_M$  for acetaldehyde. Since the affinity of aldehyde and xanthine oxidases for acetaldehyde (Rajagopalan and Handler, 1964 b; Mackler et al., 1954) was not nearly as high as that for aldehyde dehydrogenase, the latter enzyme has been assumed to be of more importance in the removal of acetaldehyde in mammalian systems (Hawkins and Kalant, 1972). Evidence for the importance of the NAD<sup>+</sup>-dependent aldehyde dehydrogenase has also been reported from studies using molybdenum-deficient rats (Richert and Westerfeld, 1957). Recent result using NAD<sup>+</sup>-depleted sheep liver homogenates have shown that aldehyde oxidase may assume an increasing role in acetaldehyde removal when NAD<sup>+</sup> is not readily available (Hendtlass, 1973). The latter findings are of interest because there is much evidence that the oxidation of ethanol results in a decrease in the NAD<sup>+</sup>/NADH ratio in the liver cell (Smith and Newman, 1959; Raiha and Oura, 1962; Slater et al., 1964; Forsander, 1970). The apparent rate of alcohol removal in vivo does not correlate well with measured levels of hepatic alcohol dehydrogenase (Wallgren and Barry, 1970) and it is now accepted that this is probably due to the rate of ethanol removal being limited by the rate of NADH reoxidation (Hawkins and Kalant, 1972).

Since the main enzyme involved in acetaldehyde removal, aldehyde dehydrogenase, is dependent on NAD<sup>+</sup>, it would seem likely that acetaldehyde removal might also be limited by the rate of NADH reoxidation. The fact that very little acetaldehyde accumulates during ethanol breakdown (Lundquist and Wolthers, 1958; Freund and O'Hollaren, 1965; Ridge, 1963) indicates that the removal of acetaldehyde is not prevented by NAD<sup>+</sup> depletion. One of the reasons for this could be that the NAD<sup>+</sup> independent aldehyde oxidase assumes a greater role in acetaldehyde

removal when the availability of  $\text{NAD}^+$  is limited. Another possible explanation arises from the intracellular localisation of aldehyde oxidation. Although the intracellular distribution of aldehyde dehydrogenase is still in doubt (see Section 2.1) experiments using perfused rat liver (Lindros et al., 1972; Hassinen et al., 1970) have indicated that most acetaldehyde oxidation may occur in the mitochondria. An intramitochondrial location of aldehyde dehydrogenase could possibly allow a more ready access for this enzyme to reoxidised NADH, which may prevent the accumulation of acetaldehyde. Alcohol dehydrogenase, in contrast to aldehyde dehydrogenase, is localised solely in the cytoplasm (Nyberg et al., 1953). On the other hand, studies of the metabolism of ethanol in rat liver suggest that the shuttle system for transporting reducing equivalents into and out of the mitochondria is not the primary rate limiting factor in ethanol oxidation. Uncoupling agents, which increase the rate of  $\text{NAD}^+$  regeneration, also increase the rate of ethanol metabolism (Israel et al., 1970; Videla and Israel, 1970), which suggests that the rate limiting step is the reoxidation of NADH via the cytochrome system. If the shuttle system is not rate limiting at all, then the proximity of intramitochondrial aldehyde dehydrogenase to the site of NADH reoxidation would be of less significance.

The low  $K_M$  of aldehyde dehydrogenase for acetaldehyde (Deitrich et al., 1962; Buttner, 1965; Kraemer and Deitrich, 1968; Sheppard et al., 1970), combined with a constant rate of ethanol breakdown to give acetaldehyde (Kalant, 1971) and the equilibrium position for the alcohol dehydrogenase reaction, which favours ethanol formation (Sund and Theorell, 1963) may be sufficient to explain the lack of acetaldehyde accumulation following ethanol intake. However, more kinetic data for the two enzymes, alcohol and aldehyde dehydrogenases, might prove useful in determining the reasons for lack of acetaldehyde accumulation. It may be found, for example, that the relative turnover rates of the enzymes are of equal or more significance than their  $K_M$  values for acetaldehyde.  $K_{\text{cat}}$  values for alcohol and aldehyde dehydrogenases, and their  $K_M$  values for  $\text{NAD}^+$  should have some bearing on their relative abilities to remove the two substrates ethanol and acetaldehyde in situations where the availability of  $\text{NAD}^+$  is limited.  $K_M$  values of the two enzymes for  $\text{NAD}^+$  reported in the literature (alcohol dehydrogenase, Sund and Theorell, 1963; aldehyde dehydrogenase, Blair and Bodley, 1969; Kraemer and Deitrich, 1968) appear to be similar, but these values should be

compared reservedly, since the enzymes were from different species, and different assay conditions were used for each determination. There is a need for more kinetic data for alcohol and aldehyde dehydrogenases obtained under conditions closely approximating the physiological situation.

While there is general agreement in the literature that acetaldehyde does not accumulate during ethanol metabolism, the levels which have been reported in the blood of mammals following ethanol intake vary greatly. Table 1.1 gives some of the values reported in the literature for acetaldehyde levels found in the blood of human subjects following ethanol intake. There is at least a 100 fold variation in these levels, although the more recent values tend to be more of the same order. Although the amount of acetaldehyde in the blood is low compared with the levels of ethanol that may be reached, the exact determination of levels of this substance may prove to be critical in elucidating the mechanisms involved in the effects of acute and chronic ethanol intake, and in the development of addiction to ethanol.

Acetaldehyde has some effects on mammalian systems that cannot be reproduced by ethanol alone. It interferes with respiration and oxidative phosphorylation in rat brain cells (Truitt et al., 1956; Beer and Quastel, 1958; Kiessling, 1962; Majchrowicz, 1965), reacts with acetyl CoA (Ammon et al., 1971), causes the release of catecholamines (Eade, 1959; Nelson, 1943; Walsh et al., 1969) and has been implicated in the shift to a reductive pathway for the metabolism of biogenic amines following ethanol consumption (Lahti and Majchrowicz, 1969; Truitt and Walsh, 1971). Acetaldehyde can also undergo direct condensation reactions with biogenic amines (Cohen and Collins, 1970; Heikkila et al., 1971) and by virtue of its inhibition of the breakdown of the metabolic products of dopamine, it may cause the formation of tetrahydropapaveroline, a morphine-like alkaloid (Davis et al., 1970; Davis and Walsh, 1970 a).

Of the above effects of acetaldehyde, reaction with acetyl CoA (Martin, 1965), production of alcohols from biogenic amines by shifting to a reductive pathway (Truitt and Walsh, 1971) direct condensation with biogenic amines (Cohen and Collins, 1970) and formation of tetrahydropapaveroline (Davis and Walsh, 1970 a) have all been suggested as possible causes of alcoholism. The proposed formation and the possible addictive potential of tetrahydropapaveroline, in particular, have generated much discussion in the literature (Seevers, 1970; Davis and Walsh, 1970 b; Anon., 1972).

TABLE 1.1

BLOOD LEVELS OF ACETALDEHYDE IN HUMAN SUBJECTS FOLLOWING  
ETHANOL ADMINISTRATION

Acetaldehyde level after ethanol ( $\mu\text{g}/\text{cm}^3$ )	Reference
16.06	Stepp, 1920
1.05	Hald and Jacobsen, 1948
6.9 - 9.6	Furtado <u>et al.</u> , 1951
3.61	Hine <u>et al.</u> , 1952
5.9 - 17	Raby, 1954
30.0	Forster, 1956
0.30	Lundquist and Wolthers, 1958
1.0	Alabane, 1960
13.75	Hulpieu <u>et al.</u> , 1965, see Forney and Hager, 1965
2.5	Krantz and Carr, 1965, no reference
0.4 - 1.5	Majchrowicz and Mendelson, 1970*
0.1 - 0.56	Truitt and Walsh, 1971
0.05 - 0.10	Truitt, 1971*

The above table, with the exception of those references marked (\*) is as given by Truitt and Walsh (1971). The results of Majchrowicz and Mendelson (1970) were obtained using alcoholics, and Truitt (1971) studied both alcoholics and non-alcoholics.

Results of a completely different type, relating acetaldehyde to the development of a preference for ethanol, have been published by Sheppard et al., (1970). These workers found that a strain of mice which preferred ethanol to water had a more active form of aldehyde dehydrogenase, and lower levels of acetaldehyde in their blood following ethanol intake, than another strain of mice which avoided ethanol. This suggested that the preference for ethanol possibly arose through a lack of the ill effects normally associated with acetaldehyde. Although such results for mice are far removed from the situation of ethanol addiction in human subjects, the genetic aspects of this study are interesting in view of a possible genetic pattern in the occurrence of alcoholism (Cruz-Coke and Mardones, 1971).

Attempts have therefore been made to explain addiction to ethanol on the one hand by the effects of acetaldehyde on metabolic processes, where increased acetaldehyde levels would give an increasing possibility of addiction, and on the other, it could be suggested that a low level of acetaldehyde might promote addiction by avoiding the unpleasant effects normally associated with ethanol intake. The validity of such hypotheses can only be ascertained by further research into (a) the mechanisms available to mammalian systems for the formation and removal of acetaldehyde and (b) the levels of this compound which result from ethanol intake.

The aims of this study were to examine mechanisms in mammalian liver for the removal of acetaldehyde, and the levels of this compound that would result following ethanol administration. Sheep were used for initial studies to continue work already in progress in the department (Hendtlass, 1973) and because of the ready availability of sheep livers. Since alcohol dehydrogenase has been extensively studied, and aldehyde oxidase from sheep liver had been purified and characterised (Hendtlass, 1973) the second, and major, enzyme involved in acetaldehyde removal, aldehyde dehydrogenase, was chosen for detailed examination.

## SECTION 2

INTRACELLULAR LOCALISATION OF ALDEHYDE DEHYDROGENASE2.1 INTRODUCTION

Although mammalian aldehyde dehydrogenase has been purified to homogeneity from one source (Feldman and Weiner, 1972) and partially purified with detailed characterisation from others, there is still debate as to its intracellular localisation.

Aldehyde dehydrogenase has been studied in a "soluble fraction" of human liver (Kraemer and Deitrich, 1968; Blair and Bodley, 1969), horse liver (Feldman and Weiner, 1972), rabbit liver (Maxwell and Topper, 1961), and rat liver (Shum and Blair, 1972). Low levels of activity have been detected in supernatants of rat kidney and rat brain (Deitrich et al, 1972).

Mitochondrial aldehyde dehydrogenase has been reported in rabbit brain (Rutledge and Deitrich, 1971), pig brain (Duncan and Tipton, 1971), pigeon liver (McGuire, 1965), bovine brain (Erwin and Deitrich, 1966), and rat liver (Glenn and Vanko, 1959).

The presence of both mitochondrial and cytoplasmic enzymes has been observed in rat liver (Deitrich, 1971), rat brain (Erwin and Deitrich, 1966), and mouse liver (Sheppard et al, 1968).

In a majority of these studies, intracellular fractions were described on the basis of centrifugation alone, without the use of any marker enzymes. The results must therefore be noted with reservations, particularly in respect to descriptions of supernatant enzyme. Activity could appear in supernatant fractions through leakage from broken mitochondria, and is thus not necessarily of cytoplasmic origin. Experiments so far reported where marker enzymes have been assayed have used primarily rat liver. One exception has been a fractionation of rat brain aldehyde oxidising capacity (Erwin and Deitrich, 1966) where succinic dehydrogenase and monoamine oxidase were used as mitochondrial marker enzymes.

Results of fractionation experiments with rat liver have differed

considerably. Deitrich (1966) found that 13.4% of rat liver aldehyde oxidising capacity was present in the mitochondria, and 86.6% in the supernatant fraction. The latter figure included microsomal activity, which amounted to 3.7% of the total aldehyde oxidising capacity. Succinic dehydrogenase was used as a mitochondrial marker. Marjanen (1972), using zonal centrifugation of an homogenate in a sucrose gradient, found 80% of the aldehyde dehydrogenase activity in the mitochondrial fraction, and 20% in the cytoplasm. No microsomal activity was detected. Cytochrome oxidase was used to mark the position of mitochondria in the gradient.

The work reported in this section was carried out in an attempt to define the intracellular localisation of aldehyde dehydrogenase in sheep liver, and compare results with those obtained by other workers using rat liver.

Marker enzymes were used to identify the subcellular fractions. Glucose-6-phosphatase (G-6-Pase), which was chosen as a microsomal marker (Hers et al., 1951), may occur in the mitochondria, but none, however, should be present in cytoplasm completely free of microsomes. Glutamate dehydrogenase (GDH) was used as a mitochondrial marker. It occurs in the mitochondrial matrix, and is readily released into the cytoplasm with mitochondrial damage (Hogeboom and Schneider, 1953; Beaufay et al., 1959). Lactate dehydrogenase (LDH) occurs almost exclusively in the cytoplasm (Dixon and Webb, 1964), and was chosen as a marker because (a) it could be readily assayed, and (b) it has a comparatively high level of activity in crude homogenates.

When this work was carried out, no reports had appeared with results for aldehyde dehydrogenase intracellular localisation confirmed by marker enzyme levels for each intracellular fraction. A paper on the intracellular localisation of rat liver aldehyde dehydrogenase (Tottmar et al., 1973) appeared while the results described here were being published.

## 2.2 METHODS

### 2.2.1 Homogenisation.

A 20% w/v homogenate of sheep liver was made using a Potter-Elvehjem homogeniser with a teflon head. The homogenising buffer was 0.0025M phosphate buffer, pH 7.3, containing sucrose (0.25M) and mercaptethanol (0.1%).

### 2.2.2 Fractionation procedure.

The above homogenate was fractionated by centrifuging in a Sorvall RC-2B centrifuge for 5 minutes at 500g, 30 minutes at 20,000g, and 2.5 hours at 34,000g. All homogenisation and fractionation steps were carried out at 4°C.

The homogenate and all fractions were assayed immediately for aldehyde dehydrogenase and marker enzymes. The precipitate from the first centrifugation should contain unbroken cells and nuclei, and is referred to as the "500g" precipitate. The second precipitate should contain mitochondria, the third, microsomes, and the final supernatant, cytoplasm free of other cell fractions.

### 2.2.3 Assay procedures.

#### (a) Aldehyde dehydrogenase.

The assay of aldehyde dehydrogenase was carried out by following the increase in absorbance at 340nm with the formation of NADH.

Reagents used in the assay were:

pyrophosphate buffer pH 9.3	33.0 mM
NAD <sup>+</sup>	1.67 mM
acetaldehyde	1.67 mM

The final volume of this assay mix was adjusted to 3cm<sup>3</sup> with water or other additions as required. Pyrazole, which is a potent inhibitor of alcohol dehydrogenase, was added to all assays where alcohol dehydrogenase would be present and might interfere with estimations. The amount of pyrazole used was 0.5cm<sup>3</sup> of a 0.02% solution, in the assay volume of 3cm<sup>3</sup>. The aldehyde dehydrogenase assay was carried out at room temperature.

Units of activity for aldehyde dehydrogenase were calculated as units of absorbance, where an increase of 0.001 absorbance units/minute was taken as equivalent to 1 unit of enzyme activity, or as moles of NADH produced/minute (0.001 absorbance units/minute  $\equiv$  0.483 nmoles of NADH/minute).

#### (b) Lactate dehydrogenase.

LDH was assayed by following the oxidation of NADH at 340nm, when samples containing enzyme were added to buffer, NADH and sodium pyruvate (Wroblewski and LaDue, 1955).

#### (c) Glucose-6-phosphatase.

G-6-Pase was assayed by measuring the production of inorganic phosphate after incubation of samples with glucose-6-phosphate (Swanson, 1955).

Reagents were used, and procedures followed, exactly as in the reference quoted, except that a 20% w/v homogenate of liver was used, instead of the 5-10% described.

Linearity of the assay with time was checked using a microsomal sample. The reaction was linear for 30 minutes, when the rate began to decrease. A standard 15 minute incubation was used for all further assays. Units of activity were calculated as  $\mu\text{g}$  inorganic phosphate formed in a 15 minute incubation.

Calculation of the amount of phosphate released per g of sheep liver in 15 minutes gave values of 7.8mg/g and 7.7mg/g from two liver homogenates. The reference followed quoted a value of about 7mg of phosphate released per g of rat liver in 15 minutes.

(d) Glutamate dehydrogenase.

The assay for GDH was adapted from a method for assaying this enzyme in brain tissue (Robins *et al.*, 1956), using a microdetermination of  $\alpha$ -ketoglutarate formed from sodium glutamate during incubation of this substrate with tissue preparations. The simpler assay with  $\text{NAD}^+$  (Hogeboom and Schneider, 1953) was not used because of probable interference from other  $\text{NAD}^+$ -coupled enzymes in crude homogenates.

The major modification used was an increase in the final volume of the assay from 0.08-0.12 $\text{cm}^3$  to a final volume of 3.5 $\text{cm}^3$ , which was more appropriate with normal spectrophotometer cuvettes. All solutions were prepared as described in the reference, with the following final assay volumes:

sample	0.5 $\text{cm}^3$
buffer/substrate solution	0.5 $\text{cm}^3$
3-quinoyl-hydrazine	0.5 $\text{cm}^3$
0.01M HCl	2.0 $\text{cm}^3$

According to the reference, blanks contained water in place of sample, and were treated identically to sample tubes. In this case, as the samples contributed significant non-specific absorbance at 305nm, water was used in the blanks to zero the spectrophotometer, and sample-containing blanks were also prepared by adding quinoyl-hydrazine solution directly to the homogenate/buffer/substrate solution, in ice, leaving 1 hour at room temperature, and completing the final stage of HCl addition as for incubated samples. The absorbance at 305nm of sample blanks was subtracted from the absorbance of samples, to give a final value which was used in calculation of glutamate dehydrogenase activity as described in the reference.

A range of dilutions of the 20% liver homogenate and the fractions were tried, and the following dilutions were found to give satisfactory readings:

20% homogenate	1000 fold
"500g" precipitate	1000 fold
mitochondria	1000 fold
microsomes	100 fold
cytoplasm	10 fold

To check linearity and accuracy of the modified method,  $\alpha$ -keto-glutarate standards of 0.5, 1.0 and 2.0mM were prepared, and 0.05cm<sup>3</sup> of these solutions was used in place of an aliquot of sample. Water was used to make the same total volume as for assay mixtures containing samples. A linear graph of absorbance at 305nm against concentration of  $\alpha$ -ketoglutarate was obtained. Values of  $\epsilon_{305}$  for  $\alpha$ -ketoglutarate quinoylhydrazone were calculated from the values of absorbance at 305nm obtained for these standards. Each estimation was an average of two standard determinations, and the values obtained were 20,800, 21,500, and 21,500 which are very close to the value quoted by Robins et al. (1956) of 21,700.  $\epsilon_{305}$  for QH/NADH was not determined. All samples were read 10 minutes after the final dilution, and the value quoted by Robins et al. (1956) for  $\epsilon_{305}$  QH/NADH at 10 minutes was used. Units of activity used for the enzyme were moles of glutamate oxidised/kilogram of protein/hour.

#### 2.2.4 Protein estimations.

Protein concentrations were determined by the method of Lowry et al. (Lowry et al., 1951).

### 2.3 RESULTS

Tables 2.1 and 2.2 show the results of assaying subcellular fractions for G-6-Pase, LDH, GDH, and aldehyde dehydrogenase. Percentage of recovered activity, and specific activity, in each intracellular fraction are shown for each enzyme. Presentation of these results in histogram form (Figures 2.1 and 2.2) gives a direct comparison between the distribution of aldehyde dehydrogenase and the marker enzymes. The aldehyde dehydrogenase distribution pattern does not appear to correspond to any of the typical marker enzyme patterns, and suggests that the enzyme occurs in more than one fraction. The marker enzyme and aldehyde

TABLE 2.1

DISTRIBUTION OF ACTIVITIES OF MARKER ENZYMES AND  
ALDEHYDE DEHYDROGENASE IN INTRACELLULAR FRACTIONS

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The level of each enzyme in each intracellular fraction is expressed as a percentage of the total activity recovered after fractionation. "Percentage recovery" indicates the percentage of activity of the original homogenate recovered after fractionation.

---

Cell fraction	Activities (% of total recovered activity)			
	Lactate Dehydrogenase	Glutamate Dehydrogenase	Glucose-6- Phosphatase	Aldehyde Dehydrogenase
"500g" precipitate	6.4	31.0	27.0	9.0
mitochondria	2.0	61.0	24.0	26.0
microsomes	1.2	5.0	45.0	17.0
cytoplasm	90.4	2.5	4.9	48.0
percentage recovery	81%	75%	126%	96%

---

TABLE 2.2

SPECIFIC ACTIVITIES OF MARKER ENZYMES AND ALDEHYDE  
DEHYDROGENASE IN INTRACELLULAR FRACTIONS

---

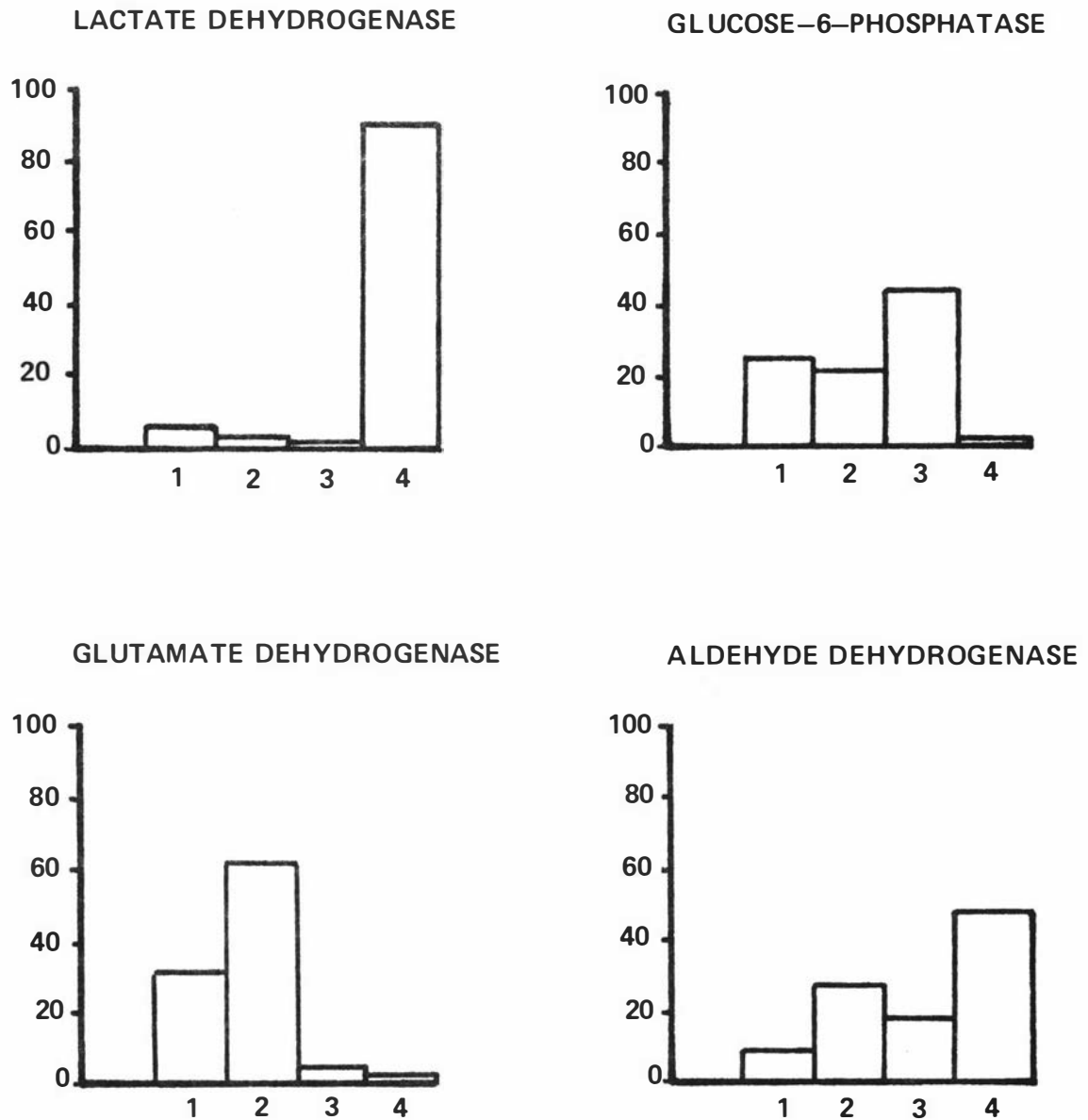
The specific activities of each enzyme in each cell fraction are shown. Units of specific activity are: lactate dehydrogenase, nmol-  
es NADH oxidised/minute/mg protein; glutamate dehydrogenase, moles  
glutamate oxidised/hour/Kg protein; glucose-6-phosphatase,  $\mu$ g P  
produced/mg protein; aldehyde dehydrogenase, nmoles NADH produced/min/mg  
protein.

---

Cell fraction	Specific Activities			
	Lactate Dehydrogenase	Glutamate Dehydrogenase	Glucose-6- Phosphatase	Aldehyde Dehydrogenase
initial homogenate	50	3.6	16.9	5.9
"500g" precipitate	8.3	2.8	18.4	1.7
mitochondria	3.9	8.0	24.8	7.0
microsomes	3.9	1.1	74.0	7.7
cytoplasm	105	0.2	3.0	7.7

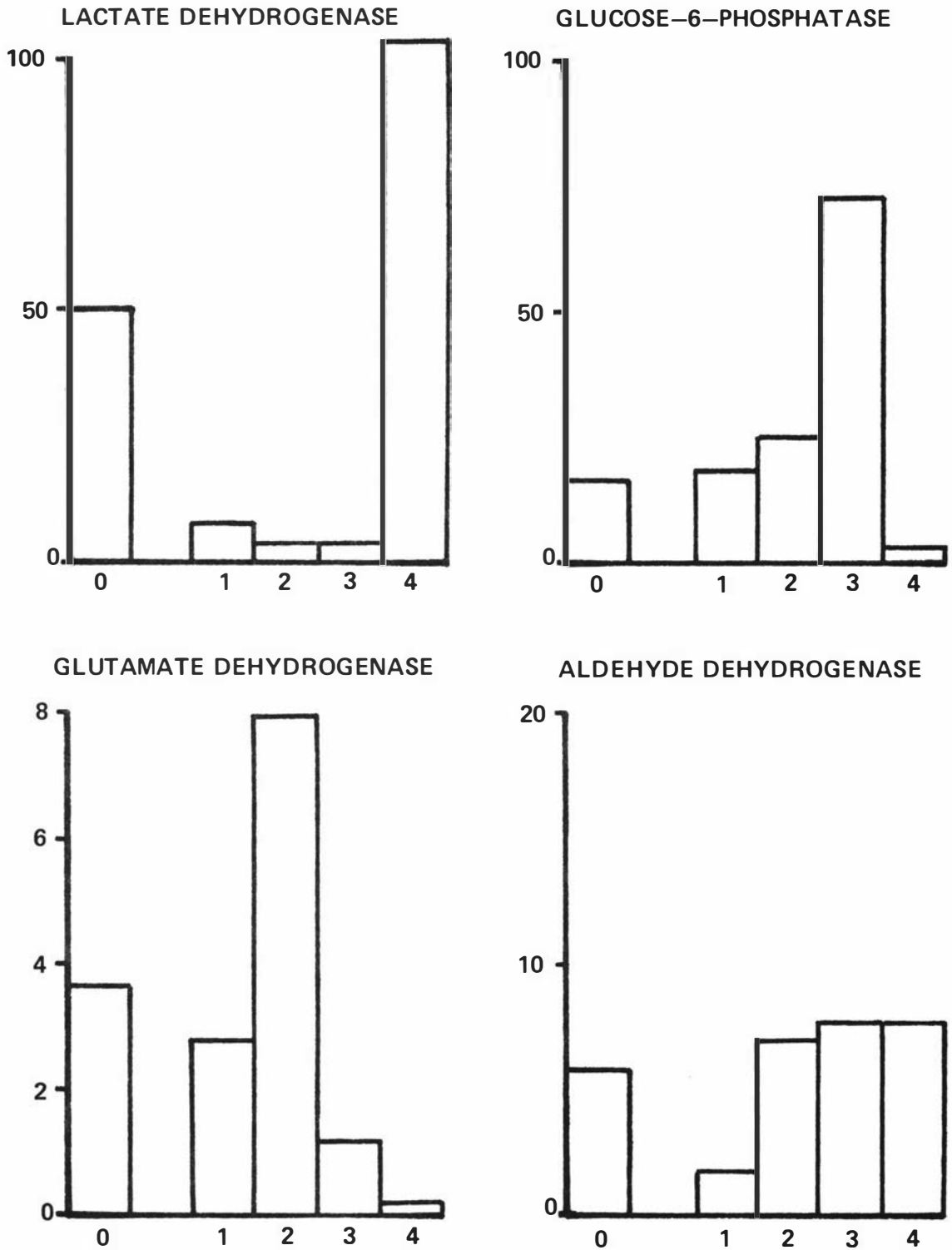
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FIGURE 2.1 DISTRIBUTION OF ACTIVITIES OF MARKER ENZYMES AND ALDEHYDE DEHYDROGENASE IN INTRACELLULAR FRACTIONS



The data that were used to plot the above histograms are given in Table 2.1. The vertical axis of each histogram represents percentage activity. Intracellular fractions are numbered on the horizontal axes as follows: 1 - "500g" precipitate, 2 - mitochondria, 3 - microsomes, 4 - cytoplasm.

FIGURE 2.2 SPECIFIC ACTIVITIES OF MARKER ENZYMES AND ALDEHYDE DEHYDROGENASE IN INTRACELLULAR FRACTIONS



The data that were used to plot the above histograms are given in Table 2.2. The vertical axis of each histogram represents specific activity. Intracellular fractions are numbered on the horizontal axis as follows: 0 – initial homogenate, 1 – 500g precipitate, 2 – mitochondria, 3 – microsomes, 4 – cytoplasm.

dehydrogenase results shown are from one fractionation. A second experiment gave almost identical results with respect to the amount of GDH present in microsomes, the amount of GDH and G-6-Pase present in cytoplasm, and the amount of LDH in mitochondria and microsomes. The amounts of GDH and G-6-Pase in the "500g" precipitate were higher in the second experiment, however, indicating a greater contamination of this fraction with mitochondria and microsomes. This was due to a slightly higher centrifuge speed in the first spin, and does not alter the conclusions which may be reached from the results shown in Tables 2.1 and 2.2. The aldehyde dehydrogenase results for this second experiment are presented in Table 2.4. as no. 6.

The distribution of aldehyde dehydrogenase was checked in several other fractionations using an identical homogenisation technique. Most of these fractionations were carried out primarily to obtain cytoplasm for further purification of cytoplasmic aldehyde dehydrogenase.

Table 2.3 and Figure 2.3 show the average values for 8 such fractionations, as well as for the two in which marker enzymes were also estimated (numbers 6 and 7 in Table 2.4). Maximum and minimum values for the 10 experiments are given.

Table 2.4. gives data for all 10 fractionations, with activity and protein recoveries shown. No. 7 is the fractionation represented in Tables 2.1 and 2.2. No. 11 is a fractionation that was carried out on the liver of a sheep that had been infused with ethanol for several days. There do not appear to be any major differences between results for no. 11 and the other 10 fractionations. The percentage of aldehyde dehydrogenase in the cytoplasm was higher in no. 11 than in any other fractionation, but this cannot be considered a significant difference without other supporting data.

Figures given in Table 2.5 for protein estimation in each fraction are for the same fractionation as the marker enzyme results. Protein recovery in this fractionation was 100%, suggesting that the losses or gains of up to 25% of enzyme activity that occurred were due to activation or deactivation of enzyme, rather than to any loss of protein. Protein recoveries in most other fractionations were also close to 100%, as shown in Table 2.4.

Table 2.6 shows the very large differences in specific activity of aldehyde dehydrogenase which were found, particularly in the cytoplasmic and microsomal fractions, namely 4.8 and 6.5 fold respectively. Mitochondrial specific activity showed only a two fold maximum variation.

TABLE 2.3

AVERAGE INTRACELLULAR DISTRIBUTION OF ALDEHYDE  
DEHYDROGENASE

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Average values for percentage activity, specific activity and percentage recovery of aldehyde dehydrogenase for 10 fractionation runs are shown. Maximum and minimum values are shown in brackets. Units of specific activity: nmoles NADH produced/minute/mg protein.

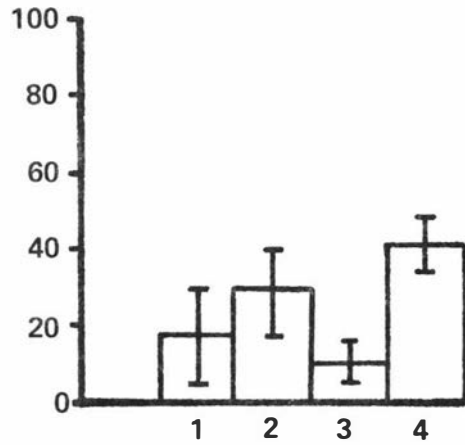
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	Percentage Values	Specific Activity
initial homogenate	-	6.2 (3.2-10.6)
"500g" precipitate	18 (2-29)	3.3 (1.3-8.0)
mitochondria	30 (17-40)	8.0 (5.3-11.6)
microsomes	10 (5-17)	7.5 (2.7-17.4)
cytoplasm	42 (35-49)	7.6 (3.2-15.4)
percentage recovery	90 (70-106)	-

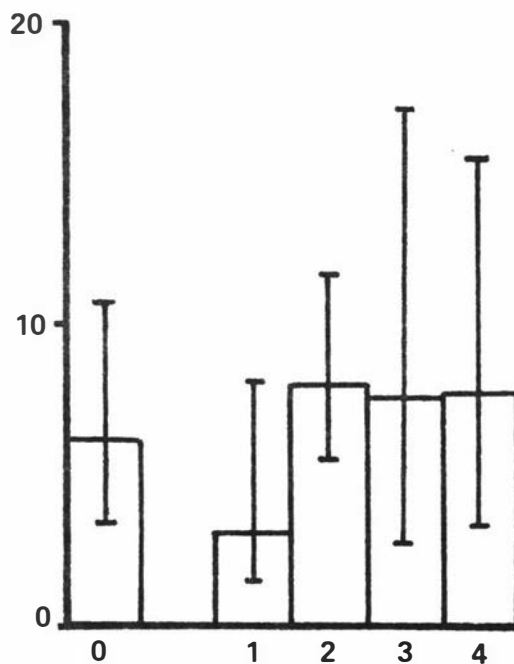
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ALDEHYDE DEHYDROGENASE

a) PERCENTAGE ACTIVITY



b) SPECIFIC ACTIVITY



The data used to plot histograms a and b are given in Table 2.3. The vertical axes represent in a, percentage activity, and in b, specific activity. Fractions are numbered on the horizontal axes as follows: 0 – initial homogenate, 1 – “500g” precipitate, 2 – mitochondria, 3 – microsomes, 4 – cytoplasm. The bars on each histogram represent maximum and minimum values.

TABLE 2.4

DATA FOR FRACTIONATIONS OF ALDEHYDE DEHYDROGENASE

Numbers 1 - 10 represent data obtained from fractionations of sheep livers obtained over a 10 month period. No. 11 is a fractionation carried out on the liver of an animal that had been infused with ethanol in another study. Units of specific activity are nmoles NADH produced /min/mg protein.

<u>No.</u>	1	2	3	4	5	6	7	8	9	10	11
<u>% activity</u>											
"500g" precipitate	22	17	25	2	13	18	9	29	28	16	11
mitochondria	34	33	32	40	32	26	26	17	30	26	19
microsomes	9	5	7	9	9	16	17	10	7	10	11
cytoplasm	35	45	36	49	46	40	48	44	35	48	59
<u>% recovery of enzyme activity</u>											
	73	96	92	70	105	92	96	106	102	73	98
<u>Specific activity</u>											
homogenate	4.5	8.9	10.6	3.2	6.3	7.8	5.9	4.7	3.9	6.0	7.7
"500g" precipitate	2.4	3.9	8.0	1.3	2.1	3.9	1.7	4.3	2.6	2.6	2.9
mitochondria	5.3	11.1	11.6	5.5	9.3	7.8	7.0	7.2	7.6	7.8	7.9
microsomes	2.7	6.0	10.2	3.8	7.0	17.4	7.7	8.9	4.0	7.2	12.8
cytoplasm	4.8	11.6	15.4	3.2	9.4	7.8	7.7	6.4	3.6	6.3	12.1
<u>% recovery of protein</u>											
	70	103	102	90	103	100	100	90	110	76	90

TABLE 2.5

PROTEIN RECOVERY AFTER FRACTIONATION  
OF SHEEP LIVER HOMOGENATE

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The values given in this table as an example of protein recovery from fractionated liver correspond to the same fractionation run as the results given for marker enzyme activities. Protein in each fraction is added, and this total compared with the protein estimation from the original homogenate. Protein recovery was checked for most fractionation runs and was generally close to 100%.

---

Cell Fraction	Total mg Protein
"500g" precipitate	2780
mitochondria	1860
microsomes	1150
cytoplasm	3125
Total	<u>8915</u>
original homogenate	8900
recovery	100%

---

The results overall indicate that aldehyde dehydrogenase activity is present in the mitochondria, microsomes, and cytoplasm. The percentage in mitochondria and cytoplasm is similar, averaging 30% for mitochondria, and 40% for cytoplasm. The percentage in microsomes is much lower, and represents about 10% of the total activity. The specific activity of aldehyde dehydrogenase is not significantly elevated in any one cell fraction.

#### 2.4 DISCUSSION

The results for distribution of marker enzymes shown in Tables 2.1 and 2.2 indicate that there is no more than 5% of microsomal enzyme (G-6-Pase) and no more than 2.5% of mitochondrial enzyme (GDH) in the cytoplasm. Since levels of aldehyde dehydrogenase found in the cytoplasm range from 35 - 49% of the total activity, there is little chance that this could be due solely to contamination of the cytoplasm by mitochondrial enzymes. It is reasonable, therefore, to claim that there is a cytoplasmic aldehyde dehydrogenase in sheep liver. This finding is at variance with a report which appeared while the results given here were being published (Tottmar *et al.*, 1973). In their report, virtually no aldehyde dehydrogenase was found in the cytoplasm of rat liver cells. The finding of a cytoplasmic enzyme in liver agrees, however, with other reports on the intracellular localisation of aldehyde dehydrogenase, namely that of Marjanen (1972) who found 20% of the aldehyde dehydrogenase activity of rat liver cells in the cytoplasm, and Deitrich (1966) who found 86.6% of liver aldehyde oxidising capacity in the cytoplasm, again in rat liver.

Some of the wide variation in cytoplasmic levels in the literature reports quoted may be due to variations in technique. Deitrich (1966) used indole-3-acetaldehyde as a substrate in contrast to other studies (the present investigation; Marjanen, 1972; Tottmar *et al.*, 1973) where acetaldehyde was used. Deitrich also studied total aldehyde oxidising capacity, rather than using a specific aldehyde dehydrogenase assay. These factors may be the cause of the very high level of cytoplasmic activity found by Deitrich. In a later reference where he uses propionaldehyde as a substrate, Deitrich (1971) appears to find a greater percentage of activity in the mitochondria. Also, in the earlier reference (Deitrich, 1966), succinic dehydrogenase was used as a mitochondrial marker. This is a membrane bound enzyme, (Singer and Kearney, 1963),

TABLE 2.6

VARIATION IN SPECIFIC ACTIVITY  
OF ALDEHYDE DEHYDROGENASE

---

From Tables 2.3 and 2.4 and Fig. 2.3 it is apparent that the specific activity of aldehyde dehydrogenase varied widely from liver to liver. The extent of the variation is shown in this table. Variation in specific activity of the "500g" precipitate may be due to variations in experimental technique, but, as discussed later, the variation in microsomal and cytoplasmic levels may be due to differences between the animals used.

Livers used for the ten experiments were from animals of which the sex, age and dietary status were not known. They were obtained over a time range of ten months, from September to June.

---

Cell Fraction	Minimum Specific Activity (nmoles NADH produced/min/mg)	Maximum Specific Activity	Variation
homogenate	3.2	10.6	3.3 fold
"500g" precipitate	1.3	8.0	6.2 fold
mitochondria	5.3	11.6	2.2 fold
microsomes	2.7	17.4	6.4 fold
cytoplasm	3.2	15.4	4.8 fold

---

end is not as readily released into the cytoplasm as are soluble matrix enzymes. Accordingly, Deitrich (1966) may not have detected contamination of the cytoplasm by matrix enzymes. Tottmar et al., (1973) who found no cytoplasmic activity, froze some samples after fractionation of the liver, and before assay. These workers do not show figures demonstrating stability of the cytoplasmic enzyme to freezing, and do not describe in detail which samples were frozen before assay and which were not. This casts some doubt on results which otherwise appear to have been obtained with care.

There appears, from results shown in this section, to be a microsomal aldehyde dehydrogenase. The possibility of this being an artefact due to mitochondrial contamination of the microsomes is unlikely, for two reasons. Firstly, only 5% of a known mitochondrial enzyme (GDH) was found in the microsomal fraction, whereas the levels of aldehyde dehydrogenase in the microsomes averaged 10%. Secondly, as described in Section 3, aldehyde dehydrogenase activity was much more difficult to release from the microsomal fraction than from mitochondria. A microsomal aldehyde dehydrogenase has been reported in rat liver by Deitrich (1966), and by Tottmar et al., (1973) but Marjanen (1972) claimed that his work, also on rat liver, showed no microsomal aldehyde dehydrogenase.

The existence of a mitochondrial aldehyde dehydrogenase in sheep liver is also demonstrated by the figures in Tables 2.1 and 2.2, although the actual percentage of mitochondrial enzyme may be higher than quoted due to an appreciable percentage of mitochondrial activity being in the "500g" precipitate. This is due to heavier mitochondria co-sedimenting with nuclei and whole cells. The same explanation could apply to the actual percentage of microsomal aldehyde dehydrogenase; it may be slightly higher than quoted as some loss occurs both by co-sedimentation of larger microsomes with mitochondria, and by endoplasmic reticulum, which is continuous with the nuclear membrane, sedimenting together with nuclei in the "500g" precipitate. Although mitochondrial and microsomal fractions were not washed, the possibility of aldehyde dehydrogenase activity being due to contamination of these fractions with cytoplasmic enzyme is unlikely, as levels of lactate dehydrogenase were only 2% in the mitochondria, and 1% in the microsomes. This amount would represent cytoplasmic material distributed throughout the precipitate in each case, and would presumably have been removed

by washing the cell fractions. It was considered, however, that washing the fractions would only lower recoveries of enzyme, without making the distribution picture significantly clearer. Levels of lactate dehydrogenase appearing in the "500g" precipitate, which were slightly higher than in the mitochondria and microsomes, were presumably due to the presence of unbroken cells in this fraction.

Levels of mitochondrial aldehyde dehydrogenase have also been reported, in rat liver, by Marjanen (1972) who found 80% of the activity in the mitochondria, Tottmar et al., (1973) who found 70 - 80% of the activity in the mitochondria, and Deitrich (1966).

A recent report (Siew et al., 1974) claims that in rat liver mitochondria there are two distinct aldehyde dehydrogenases, one in the matrix, and another in the intermembrane space. In this work no attempt was made to differentiate between mitochondrial matrix and intermembrane space enzymes. During further purification of the mitochondrial enzyme, however, no evidence has been found for two distinct enzymes in sheep liver mitochondria (Dr T.M.Kitson, personal communication). The enzyme is readily released from mitochondria, indicating that it is probably not membrane bound.

From the difference in levels of mitochondrial, microsomal and cytoplasmic enzyme apparent in recent studies it is obvious that the intracellular distribution of aldehyde dehydrogenase is far from defined. The values obtained for sheep liver lie in an intermediate position in the range of values given in the literature for rat liver. Variability in results in the literature may not only be due to differences in technique. Levels of the enzyme, particularly in the cytoplasm, may be influenced by other factors. Sheep liver cytoplasmic aldehyde dehydrogenase is sensitive to steroid hormones such as progesterone, and the steroid hormone analogue diethylstilbestrol (Crow et al., 1974). This has also been reported for the rabbit liver enzyme (Maxwell and Topper, 1961; Maxwell, 1962). Cytoplasmic aldehyde dehydrogenase is also increased in activity in liver when rats are treated with phenobarbital (Deitrich, 1971). In the work reported here, large variations in specific activity of both microsomal and cytoplasmic aldehyde dehydrogenase were noted (see Table 2.6 in Section 2.3). These variations could be due to hormonal or dietary differences between the animals used. Similar differences could explain some of the wide variation reported for levels of aldehyde dehydrogenase in intracellular fractions from different rat liver preparations.

## SECTION 3

MICROSOMAL ALDEHYDE DEHYDROGENASE3.1 INTRODUCTION

The existence of a microsomal aldehyde dehydrogenase in liver has been reported by three different groups. Deitrich (1966) found aldehyde oxidising capacity in rat liver microsomes which could not be removed by repeated washing in 0.25M sucrose, and was not easily released by sonic disintegration. Tietz et al (1964) reported an aldehyde dehydrogenase capable of oxidising long chain aldehydes in rat liver microsomes. Recently, a microsomal aldehyde dehydrogenase similar to one form of a mitochondrial aldehyde dehydrogenase has been reported, also from rat liver (Tottmar et al, 1973). The latter report appeared following conclusion of the work reported in this section.

In contrast with the three reports above, Marjanen (1972) claimed that there was no aldehyde dehydrogenase activity in rat liver microsomes.

To demonstrate that the activity observed in the microsomes of sheep liver cells was not merely due to contamination of the microsomal fraction with mitochondrial and cytoplasmic enzymes, microsomes were washed and subjected to sonication, freezing and thawing, and detergent treatments, as well as ion exchange chromatography and gel electrophoresis. The results of such treatments are given in this section.

3.2 METHODS

## 3.2.1 Preparation of microsomes.

The microsomal pellet obtained in the final fractionation step (described in Section 2.2) was washed by resuspension in 0.0025M phosphate buffer pH 7.3, containing sucrose (0.25M) and mercaptoethanol, (0.1%), followed by centrifugation at 34,000g for 2.5 hours. The washed microsomes were resuspended in an appropriate buffer for further use.

### 3.2.2 Preparation of mitochondria

Mitochondria were prepared for study by resuspension in sucrose containing buffer, followed by centrifugation at 20,000g for 30 minutes.

### 3.2.3 Sonication

Sonic disruption of particles was carried out using an MSE 100 watt Ultrasonic disintegrator, set at maximum output. Samples were maintained at 0°C during the sonication process.

### 3.2.4 Aldehyde dehydrogenase assay

Aldehyde dehydrogenase was assayed as described in Section 2. In some microsomal samples, endogenous activity appeared on addition of the sample to the buffer/NAD<sup>+</sup> mixture in the absence of acetaldehyde. In such cases the assay mixture was left until the absorbance was stable before acetaldehyde was added. Microsomal assays tended to remain linear for only 2 - 3 minutes, when the rate began to decrease. This effect was less obvious in supernatants from detergent-treated microsomes.

### 3.2.5 Gel electrophoresis.

Gel electrophoresis on slab gels was carried out as described in Section 4.

### 3.2.6 Preparation of DEAE Protion.

DEAE Protion was prepared and equilibrated as described in Appendix 1 (Section 9.2.1).

### 3.2.7. DEAE Protion ion exchange chromatography of microsomal samples.

#### (a) Untreated microsomes.

Washed microsomes, resuspended in 0.0025M phosphate buffer, pH 7.0, were loaded onto a DEAE Protion column equilibrated with the same buffer. Elution with 0.0025M buffer was continued until the absorbance at 280nm dropped to less than 0.05. The column was then washed with 0.05M phosphate buffer, pH 7.0, and all fractions were assayed for aldehyde dehydrogenase activity. Fractions containing activity were recentrifuged at 34,000g for 2.5 hours, and the resultant supernatant and precipitate assayed for aldehyde dehydrogenase activities.

#### (b) Detergent-treated microsomes.

Washed microsomes were resuspended in 0.0025M phosphate buffer pH 7.0,

containing Triton X100 (1.0%). After recentrifugation, the supernatant was applied to and eluted from a DEAE Protion column under the same conditions as with untreated microsomes. Fractions were assayed for aldehyde dehydrogenase activity.

### 3.2.8 Protein estimations.

Protein was estimated in column fractions by reading absorbance at 280nm.

### 3.2.9 Sonication, detergent, and freezing treatment of microsomes.

A freshly obtained microsomal or mitochondrial pellet was washed and resuspended in 0.0025M phosphate buffer, pH 7.3, containing mercaptoethanol (0.1%). A sample of this suspension was retained, and the remainder was divided into 3cm<sup>3</sup> samples. Two 3cm<sup>3</sup> samples were kept as untreated controls, and the rest were subjected to sonication, freezing and thawing, and Triton X100 treatments. Two hours after the initial suspension in dilute buffer, all samples were recentrifuged, and the resultant precipitates were assayed for aldehyde dehydrogenase.

## 3.3 RESULTS

### 3.3.1 DEAE Protion ion exchange chromatography.

#### (a) Untreated microsomes

Two ion exchange column runs were carried out on untreated microsomes. The results of these are shown in Table 3.1. Figure 3.1 illustrates the elution pattern for run 1.

The results in Table 3.1 show that microsomal preparations from two different livers behaved similarly. In both experiments, the aldehyde dehydrogenase activity was unretarded under the conditions used. The enzyme appeared to be still attached to microsomal membranes, as the activity could be spun down during centrifugation. The enzyme appeared to be fairly unstable, as only about 25% of the activity was recovered following this procedure (Table 3.1).

#### (b) Detergent-treated microsomes.

After treatment of microsomes with 1.0% Triton X100 a large percentage of the aldehyde dehydrogenase activity was found in the supernatant following a 2.5 hour, 34,000g centrifugation. The activity of this supernatant also appeared to be unretarded when loaded onto DEAE Protion equilibrated with 0.0025M phosphate buffer pH 7.0. Recovery

TABLE 3.1

DEAE PROTION COLUMN RUNS ON UNTREATED  
MICROSOMES

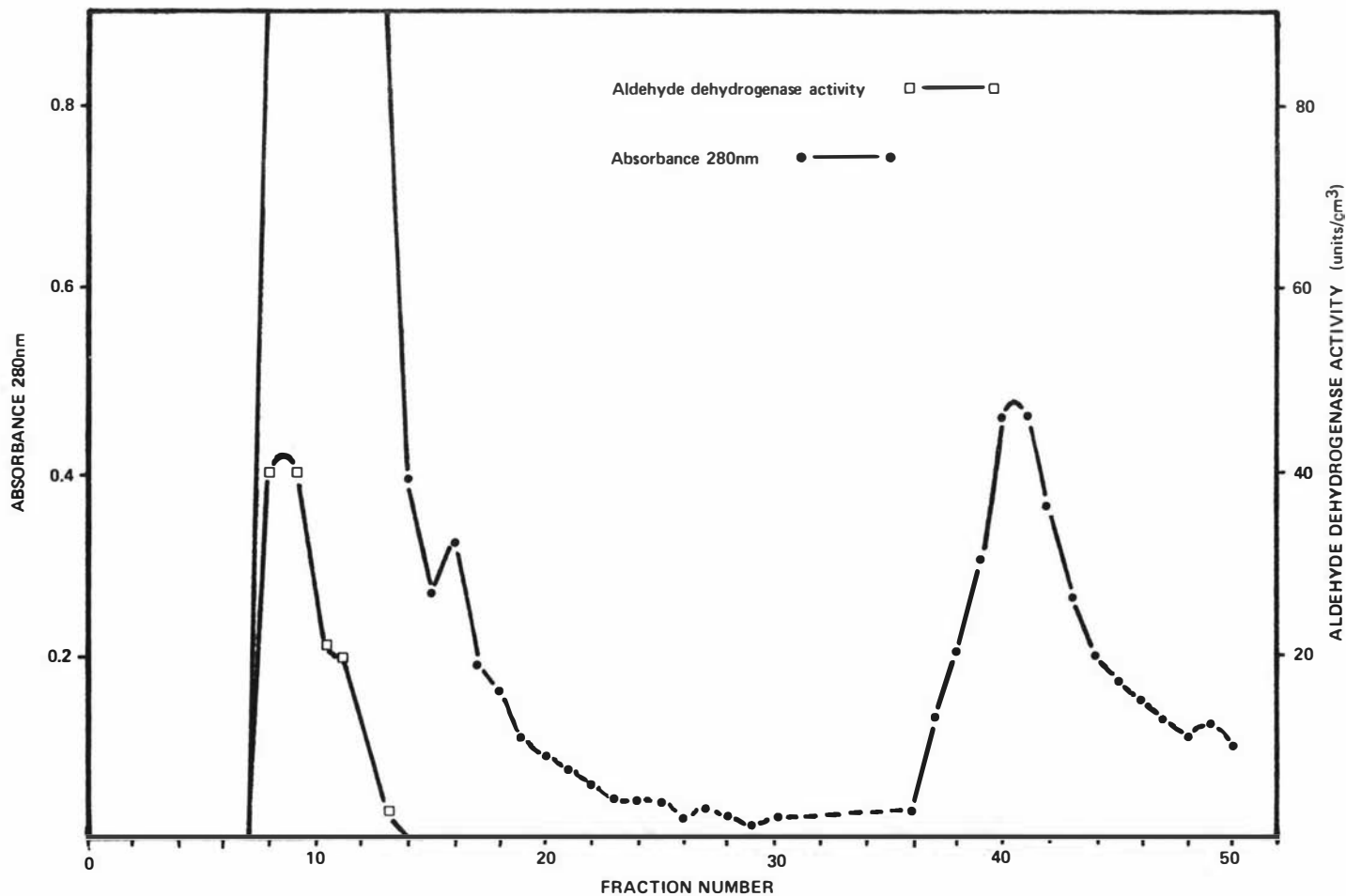
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Results are shown for similar column runs on microsomes from two separate preparations. Untreated microsomes were loaded onto DEAE Protion equilibrated with 0.0025M phosphate buffer, pH 7.0. Elution was continued with 0.0025M buffer until absorbance at 280nm was less than 0.05; then the eluant was changed to 0.05M buffer. Active fractions were bulked and centrifuged for 2.5 hours at 34,000g. Activity is expressed in absorbance units. (1 unit of enzyme activity = 0.001 absorbance units /minute.)

---

	Run 1	Run 2
Activity loaded	960	3,300
Activity recovered in 0.0025M buffer	600	1,400
% recovery	62.5%	42.5%
Activity in 0.05M buffer wash	0	0
Activity in supernatant after centrifuging active fractions	0	0
Activity in precipitate	240	900
Recovery from centrifuging	40%	61%
Overall recovery	25%	27%

FIGURE 3.1 ELUTION PATTERN OF ALDEHYDE DEHYDROGENASE FROM UNTREATED MICROSOMES ON DEAE PROTION



A microsomal pellet obtained from the final step in fractionation was washed by resuspension in 0.0025M phosphate buffer, pH 7.0, containing sucrose, (0.25M) and mercaptoethanol, (0.1%) followed by centrifugation at 34,00g for 2.5 hours. Washed microsomes were resuspended in 0.0025M phosphate buffer, pH 7.0, containing mercaptoethanol, (0.1%), and were applied to a DEAE Protion column equilibrated with the same buffer.

Elution with 0.0025M buffer was continued until the absorbance at 280nm dropped to less than 0.05, then the eluant was changed to 0.05M phosphate buffer, containing mercaptoethanol (0.1%). All fractions were assayed for aldehyde dehydrogenase activity, and absorbance at 280nm was recorded for each fraction. Aldehyde dehydrogenase activity is expressed in absorbance units (1 unit of enzyme activity = 0.001 absorbance units/minute).

of activity was much lower than with untreated microsomes, less than 10% of activity being regained. No further activity was eluted from the column with higher buffer strength, although some protein was removed, indicating that the resin could still bind protein in the presence of Triton X100.

### 3.3.2 Gel electrophoresis of microsomal aldehyde dehydrogenase

Samples of both detergent-treated and untreated microsomes were run on gels, and stained for aldehyde dehydrogenase activity and protein as described in Sections 4 and 5.

Protein from the untreated microsomes remained, as expected, mainly at the top of the gel; the microsomes would be too large to move into the gel pores. A positive aldehyde dehydrogenase activity stain was also obtained in the same position. Detergent-treated microsomes gave a smear of protein extending from the top of the gel to about two thirds of the distance to the dye band. An activity stain extended from the top of the gel down as far as the position of the activity stain usually given by mitochondrial and cytoplasmic aldehyde dehydrogenases. It appeared, therefore, that the enzyme was still bound to membrane fragments, but that the particles involved were of smaller size than the untreated microsomes.

### 3.3.3 Sonication, freezing and thawing, and detergent treatment of microsomes and mitochondria.

Mitochondrial aldehyde dehydrogenase appeared to be readily released by sonication (Dr T.M.Kitson, personal communication). In an initial experiment, microsomal samples were tested over a range of sonication times, from 1 to 3 minutes, and it was found that very little activity was released into the supernatant. Triton X100 treatment released considerably more activity. Results of this first experiment are shown in Table 3.2. Recovery of activity from the control was very low, and this was possibly because microsomes used for this experiment had been left to stand in buffer containing sucrose for several hours before the treatments were started. In the case of sonicated samples, recoveries were slightly higher, with the shortest sonication time of 1 minute giving the best recovery. Only 12 - 18% of activity appeared in the supernatant from sonicated samples. Detergent treated samples showed good recoveries, with 1% Triton X100 giving the greatest release of activity. The higher recovery in the detergent-treated samples probably

TABLE 3.2

PRELIMINARY RESULTS FOR SONICATION AND DETERGENT  
TREATMENT OF MICROSOMES

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Washed microsomes, resuspended in 0.0025M phosphate buffer pH 7.3, were divided into 3cm<sup>3</sup> samples, and treated as listed in the table below. The control suspension was stirred gently for 2 hours, with no other treatment. Sonication was carried out over 30 second intervals, followed by cooling, the number of 30 second intervals for each sample being indicated below. All treated samples, plus the control, were centrifuged for 2.5 hours at 34,000g, then supernatants and precipitates were assayed. The activities recovered in supernatants and precipitates of each centrifuged sample are expressed as a percentage of the total activity recovered from the samples. The total activity recovered from each sample is also shown as a percentage of the total activity found in an uncentrifuged control assayed at the same time. Activity is expressed in units of absorbance, where 1 unit of enzyme activity = 0.001 absorbance units/min.

---

Sample	Total Units		Total activity	% Activity		% recovery
	super-natant	precipitate		super-natant	precipitate	
Uncentrifuged suspension			510			100
Control suspension	0	236	236	0	100	46
Sonicated 2x30	40	282	322	12	88	63
Sonicated 4x30	54	250	304	18	82	60
Sonicated 6x30	54	250	304	18	82	60
Triton X100 .3%	415	150	565	74	26	110
Triton X100 1%	458	44	502	91	9	99
Sonicated 6x30 in Triton X100 1.0%	162	43	205	79	21	40

indicated that the enzyme had been activated by detergent treatment. The sample sonicated in 1% Triton X100 showed a very low recovery. Extensive frothing occurred during sonication, and probably caused denaturation of the enzyme.

On the basis of this preliminary experiment, a further set of freshly prepared microsomal samples was treated in duplicate. The combination of Triton X100 and sonication was not repeated because of the large loss of activity already observed. Freshly prepared mitochondria were similarly treated to give a comparison between the release of mitochondrial and microsomal aldehyde dehydrogenases.

The results of this experiment are given in Table 3.3. Recovery of activity from duplicate microsomal controls was good. Freezing and thawing and sonication both proved ineffective in releasing microsomal aldehyde dehydrogenase. The amount of activity in the supernatant tended to increase with increasing sonication time, but, with the exception of the 4x30 second result, recovery of activity tended to drop. Triton X100 treatment again proved to be most effective in releasing the microsomal aldehyde dehydrogenase. Considerable activation also appeared to occur with the detergent treatment. Triton X100 gave maximum activity in the supernatant and maximum activation at a concentration of 1.0%.

Results for mitochondria contrasted markedly with those for microsomes. Almost all of the mitochondrial aldehyde dehydrogenase appeared in the supernatant when mitochondria were stirred in dilute buffer before centrifuging. In comparable microsomal samples, only 5% of the activity appeared in the supernatant at this stage. Sonication gave no significant increase or decrease in mitochondrial activity, and recovery of activity was close to 100% in all cases.

#### 3.4 DISCUSSION

From the results using DEAE Protion ion exchange chromatography, it appeared that the apparent microsomal aldehyde dehydrogenase activity was not due to cytoplasmic contamination of the microsomes. The enzyme activity from microsomes passed straight through the ion exchange column under conditions in which the cytoplasmic enzyme was bound (see results on purification of cytoplasmic enzyme, in Section 4). In addition, activity was precipitated by centrifugation following the column run, showing that it was still particulate. Detergent-treated microsomal

SONICATION DETERGENT TREATMENT AND FREEZING AND  
THAWING OF MICROSOMES AND MITOCHONDRIA

Key to Table 3.3

Sample	Treatment
Cu .....	Uncentrifuged Control
C .....	Control-original suspension in dilute buffer, stirred for 2 hours, no further treatment
F .....	Frozen and thawed
1x30 .....	Sonicated, 1 30-second burst
2x30 .....	Sonicated, 2 30-second bursts
4x30 .....	Sonicated, 4 30-second bursts
6x30 .....	Sonicated, 6 30-second bursts
0.3 X100 .....	Treated with 0.3% Triton X100
1.0 X100 .....	Treated with 1.0% Triton X100
1.6 X100 .....	Treated with 1.6% Triton X100

Numbers 1 and 2 following sample codes refer to duplicates given identical treatment.

Activity is expressed in units of absorbance (1 unit of enzyme activity = 0.001 absorbance units/min.).

The total activity in the supernatant and precipitate of each sample is expressed as a percentage of the total activity recovered from that sample. The total activity recovered from each sample is expressed as a percentage of the activity in an untreated, uncentrifuged control.

TABLE 3.3

SONICATION, DETERGENT TREATMENT AND FREEZING  
AND THAWING OF MICROSOMES AND MITOCHONDRIA

---

Freshly prepared, washed microsomes and mitochondria were resuspended in 0.0025M phosphate buffer, pH 7.3. Aliquots of the initial suspensions were retained, and the remainder, in 3cm<sup>3</sup> aliquots, treated as shown below.

Sample microsomes	Total Units super- natant	Units precipi- tate	Total activity	% Activity super- natant	% Activity precipi- tate	% recovery
Cu			465			100
C1	24	450	474	5.0	95.0	102.0
C2	24	450	474	5.0	95.0	102.0
F1	30	325	355	8.5	91.5	76.5
F2	24	350	374	6.4	93.6	80.4
1x30 1	30	350	380	7.9	92.1	81.7
1x30 2	60	350	410	14.6	85.4	88.2
2x30 1	45	300	345	13.0	87.0	74.2
2.30 2	69	300	369	18.7	81.3	79.4
4x30	105	380	485	21.5	78.5	104.3
6x30	90	250	340	26.5	73.5	73.0
0.3 X100 1	620	80	700	88.5	11.5	150.5
0.3 X100 2	530	84	614	86.3	13.7	132.0
1.0 X100 1	990	10	1000	99.0	1.0	215.1
1.0 X100 2	715	10	725	98.6	1.4	155.9
1.6 X100 1	595	40	635	93.7	6.3	136.6
1.6 X100 2	595	30	625	95.2	4.8	134.4
<b>mitochondria</b>						
Cu			396			100
C1	390	2.0	392	99.5	0.5	99.0
C2	390	1.6	391.6	99.6	0.4	99.0
1x30	390	0.0	390	100.0	0.0	98.5
2x30	408	2.0	410	99.5	0.5	103.5
4x30	390	1.2	391.2	99.7	0.3	98.8
6x30	426	0.0	426	100.0	0.0	107.6

enzyme, which remained in the supernatant after centrifuging, was not bound by the ion exchange column under conditions in which both the cytoplasmic and mitochondrial enzymes would have been bound. This possibly indicated that the enzyme had different ionic properties from the cytoplasmic and mitochondrial dehydrogenases, although the behaviour of the latter enzymes on DEAE Protion in the presence of Triton X100 was not checked. The lack of binding to the resin could also indicate, however, that the microsomal enzyme was not fully released from membrane particles by the detergent, and so still passed through the column attached to particulate material. Gel electrophoresis results tended to support this possibility. Accordingly, it could not be concluded from the results that the microsomal enzyme definitely had different ionic properties from the mitochondrial and cytoplasmic enzymes.

Attempts to release aldehyde dehydrogenase activity from microsomes showed that dilute buffer, sonication, and freezing and thawing were not very effective. The mitochondrial enzyme, however, was readily released into the supernatant simply by stirring in dilute buffer, indicating that the microsomal activity was probably not due to mitochondrial contamination of microsomes. This agrees with one other result reported in the literature (Deitrich, 1966) where it has been noted that rat liver mitochondrial aldehyde oxidising capacity was nearly all released (97%) by sonication in 0.25M sucrose, whereas only 55% of microsomal aldehyde oxidising capacity was released by similar treatment. Tottmar et al (1973) found that sonication, freezing and thawing, or treatment with detergents all caused higher activity of aldehyde dehydrogenase in rat liver mitochondrial samples, whereas no stimulation of activity was seen after similar treatment of microsomal samples. This contrasts with results reported here, where no increase in mitochondrial activity was found after sonication, and a definite stimulation of microsomal activity occurred after the addition of detergent.

Aldehyde dehydrogenase in rat liver has been reported to occur in both the matrix and intermembrane spaces of mitochondria (Siew et al, 1974). Both enzymes were released into the supernatant simply by sonication, a similar result to the ready release of mitochondrial enzyme noted in this study. The results of Siew et al (1974) contrast with the results of Tottmar et al (1973), where full release of latent mitochondrial activity required detergent treatment, suggesting that at least some of the mitochondrial enzyme was strongly membrane bound.

From results in the literature, there is obviously still doubt as to the exact identity of aldehyde dehydrogenase present in rat liver microsomes and mitochondria. The results for sheep liver, however, appear at this stage to be reasonably clear. There is a readily released mitochondrial enzyme, as well as a distinct, membrane bound, microsomal enzyme. No further purification of the latter enzyme was attempted. The enzyme would probably prove difficult to purify, because of its instability after detergent treatment followed by ion exchange chromatography, and its only partial release from membrane particles by Triton X100.

## SECTION 4

PURIFICATION OF CYTOPLASMIC ALDEHYDE DEHYDROGENASE FROM  
SHEEP LIVER4.1. INTRODUCTION

Mammalian aldehyde dehydrogenase was first purified by Racker (1949). The enzyme was prepared from an acetone powder of bovine liver, using ethanol precipitation, nucleic acid precipitation, and treatment with protamine sulphate. The enzyme was found to be very unstable at cold room temperatures, although it could be stored frozen for several months without complete loss of activity. Deitrich *et al.* (1962), using a modification of the initial procedure described by Racker, obtained preparations of bovine liver aldehyde dehydrogenase of higher specific activity. Maxwell and Topper (1961) isolated an aldehyde dehydrogenase from the soluble fraction of rabbit liver, using ammonium sulphate and acetone treatments, followed by ion exchange chromatography on DEAE and CM cellulose. A modification of the latter procedure, omitting the acetone step, was described subsequently (Maxwell, 1962). This modified method was similar to the later procedures used to isolate liver aldehyde dehydrogenase, for example from supernatant fractions of human liver (Kraemer and Deitrich, 1968; Blair and Bodley, 1969).

Aldehyde dehydrogenase has been isolated from brain as well as from liver. Erwin and Deitrich (1966) isolated the enzyme from bovine brain mitochondria, and from the supernatant of frozen bovine brain, and found that both preparations had similar properties. An aldehyde dehydrogenase has also been purified from pig brain by Duncan and Tipton (1971), who used an acetone powder prepared by the method of Racker (1949). The purified enzyme from pig brain was very unstable, losing half its activity overnight. No activity was found in frozen brain, and the enzyme from isolated, sonicated mitochondria was also unstable.

Shortly after the present purification of sheep liver aldehyde dehydrogenase was undertaken, a report on the purification of rat liver aldehyde dehydrogenase (Shum and Blair, 1972) was published. Although the intracellular distribution and properties of the rat liver enzyme had been described in previous references (Glenn and Vanko, 1959; Deitrich, 1966; Deitrich and Siew, 1971), no purification studies had been carried out. Using the now general techniques of ammonium sulphate precipitation, and DEAE and CM cellulose ion exchange chromatography, Shum and Blair purified two aldehyde dehydrogenases from the supernatant fraction of rat liver which differed in substrate specificity, pH optima, and arsenite inhibition.

Another reference which became available shortly after the work on the sheep liver enzyme commenced described the purification to homogeneity of horse liver aldehyde dehydrogenase (Feldman and Weiner, 1972). This was the first report of a homogeneous preparation of mammalian aldehyde dehydrogenase, but, as with most reports using soluble fractions of tissue homogenates in purifications of aldehyde dehydrogenases, it did not include data to show that mitochondria were not being broken during tissue homogenisation.

Since reports in the literature indicated that there were likely to be both cytoplasmic and mitochondrial aldehyde dehydrogenases in mammalian liver, the present purification was carried out, in association with T.M.Kitson and A.MacGibbon, in an attempt to purify the enzymes from each intracellular location.

## 4.2. METHODS

### 4.2.1. Tissue preparation.

Sheep livers were obtained from the local freezing works within 30 minutes of the death of the animal, and transported in crushed ice. Homogenisation was commenced within 1 hour. Liver extracts were prepared in two ways:

(a) A 50% w/v homogenate of liver tissue was made in a Waring blender, in 0.0025M phosphate buffer pH 7.3, and centrifuged at 27,000 g for 60 minutes in a Sorvall RC-2B centrifuge, at 0°C.

(b) A 20% w/v homogenate of liver was prepared in 0.0025M phosphate buffer, pH 7.3, containing sucrose (0.25M) and mercaptoethanol (0.1%), using a Potter-Elvehjem homogeniser with a teflon head.

This extract was either fractionated as described in Section 2.2.2. or, in the case of large preparations, was centrifuged for 1 hour at 20,000 g followed by 2.5 hours at 34,000 g. During the entire process the homogenate was maintained at 0 - 4°C.

In early preparations, between 50 and 100 g of liver was used, but once a satisfactory method for preparing the enzyme had been established, purifications started with between 500 and 800 g of liver.

The phosphate buffers used throughout aldehyde dehydrogenase preparations were prepared according to Dawson *et al.* (1969, p 490). The concentrations given refer to the initial potassium dihydrogen phosphate and sodium hydroxide solutions, and the actual concentration of phosphate ions in the final buffer is half this value.

#### 4.2.2. Assay of aldehyde dehydrogenase.

Aldehyde dehydrogenase was assayed according to the method given in Section 2.2.3.

#### 4.2.3. Protein determination.

Protein was estimated by the method of Lowry *et al.* (1951), or by measuring absorbance at 280 and 260 nm (Dawson *et al.*, 1969).

#### 4.2.4. Ammonium sulphate fractionation.

Fractionations were carried out by the addition of solid, powdered ammonium sulphate to enzyme solutions which were maintained at 0 - 4°C throughout the addition.

#### 4.2.5. Preparation of resins and gel filtration media.

DEAE Protion was prepared and equilibrated as described in the method in Appendix 1 (Section 9.2.1.). Sephadex G-200, Biogel A 0.5 M, and DEAE cellulose (Whatman DE 32) were prepared according to the instructions supplied with the products.

#### 4.2.6. Column fractionations.

The exact conditions used for fractionations on ion exchange and gel filtration columns are given with the results of individual experiments.

#### 4.2.7. Gel electrophoresis.

Slab gel electrophoresis was carried out using an Ortec 4200 electrophoresis system. A single layer 8% acrylamide gel was normally used. All solutions were made up according to the Ortec manual (Instruction Manual, 4200 Electrophoresis System, Ortec Incorp., 1969), with gels usually being run at pH 9.0. An Ortec 4100 pulsed power supply was used, with settings as follows:

Time (minutes)	Voltage (V)	Pulse rate (/min.)
0 - 5	325	75
5 - 10	325	150
10 - 15	325	225
remainder of run	325	300

Gels were stained for protein using Amido Black, and the aldehyde dehydrogenase activity located by the staining method using phenazine methosulphate and nitroblue tetrazolium described in Section 5.

#### 4.2.8. Assays for contaminant enzymes.

##### (a) Lactate dehydrogenase.

Lactate dehydrogenase was assayed by following the oxidation of NADH at 340 nm, when samples containing enzyme were added to buffer, NADH, and sodium pyruvate (Wroblewski and La Due, 1955).

##### (b) Alcohol dehydrogenase.

Alcohol dehydrogenase activity was determined by following the increase in absorbance at 340 nm when the enzyme was added to an alcohol, buffer and  $\text{NAD}^+$  mixture (Bonnichsen and Brink, 1955).

##### (c) Catalase.

Catalase was assayed by measuring the decrease in absorbance at 240 nm observed when enzyme was added to a solution of hydrogen peroxide (Luck, 1965), or was estimated by measuring absorbance at 400 nm directly.

##### (d) Malate dehydrogenase.

Malate dehydrogenase activity was determined by following the increase in absorbance at 340 nm when sodium malate was added to an enzyme/buffer/ $\text{NAD}^+$  mixture. The assay method was supplied by Boehringer Mannheim.

(e) Aldehyde oxidase.

The dye 2,6-dichlorophenol indophenol was used as an electron acceptor for the assay of aldehyde oxidase. The decrease in absorbance at 600 nm was followed as an indication of the rate of dye reduction, following the addition of acetaldehyde and enzyme to the dye solution (Hendtlass, 1973).

### 4.3. RESULTS

#### 4.3.1. Preliminary results on aldehyde dehydrogenase purification.

A new ion exchange resin, DEAE Protion, was used in initial attempts to purify aldehyde dehydrogenase. This resin eventually proved to be unsatisfactory because of its low capacity, and DEAE cellulose was subsequently used. Appendix 1 describes an improved DEAE Protion, and gives a comparison of this new resin, the original DEAE Protion, and DEAE cellulose used in the separation of aldehyde dehydrogenase.

Before the capacity problem associated with the original preparations of DEAE Protion was fully recognised, a large amount of preliminary work had been carried out using the resin. Some reproducible results were obtained from this work, and those which proved to be relevant to the final purification scheme for aldehyde dehydrogenase, but which are not reported in the appendix, are covered in this section.

Initially, it was hoped that mitochondrial and cytoplasmic aldehyde dehydrogenases would have sufficiently different properties to be separated simply by ion exchange chromatography. With this possibility in mind, a total liver homogenate supernatant, prepared as in method 4.2.1. (a), was used initially. Results from running this supernatant directly on DEAE Protion columns showed two aldehyde dehydrogenase activity peaks, one unretarded in 0.005 M phosphate buffer, pH 7.3, the other retarded and eluted by a gradient or a stepwise change to 0.1 M NaCl. To illustrate that these two activity peaks were not due to overloading the resin, each peak was concentrated using ammonium sulphate, and re-chromatographed under identical conditions. The unretarded activity was still largely unretarded in the second column run (Figure 4.1.a) and the retarded activity was all

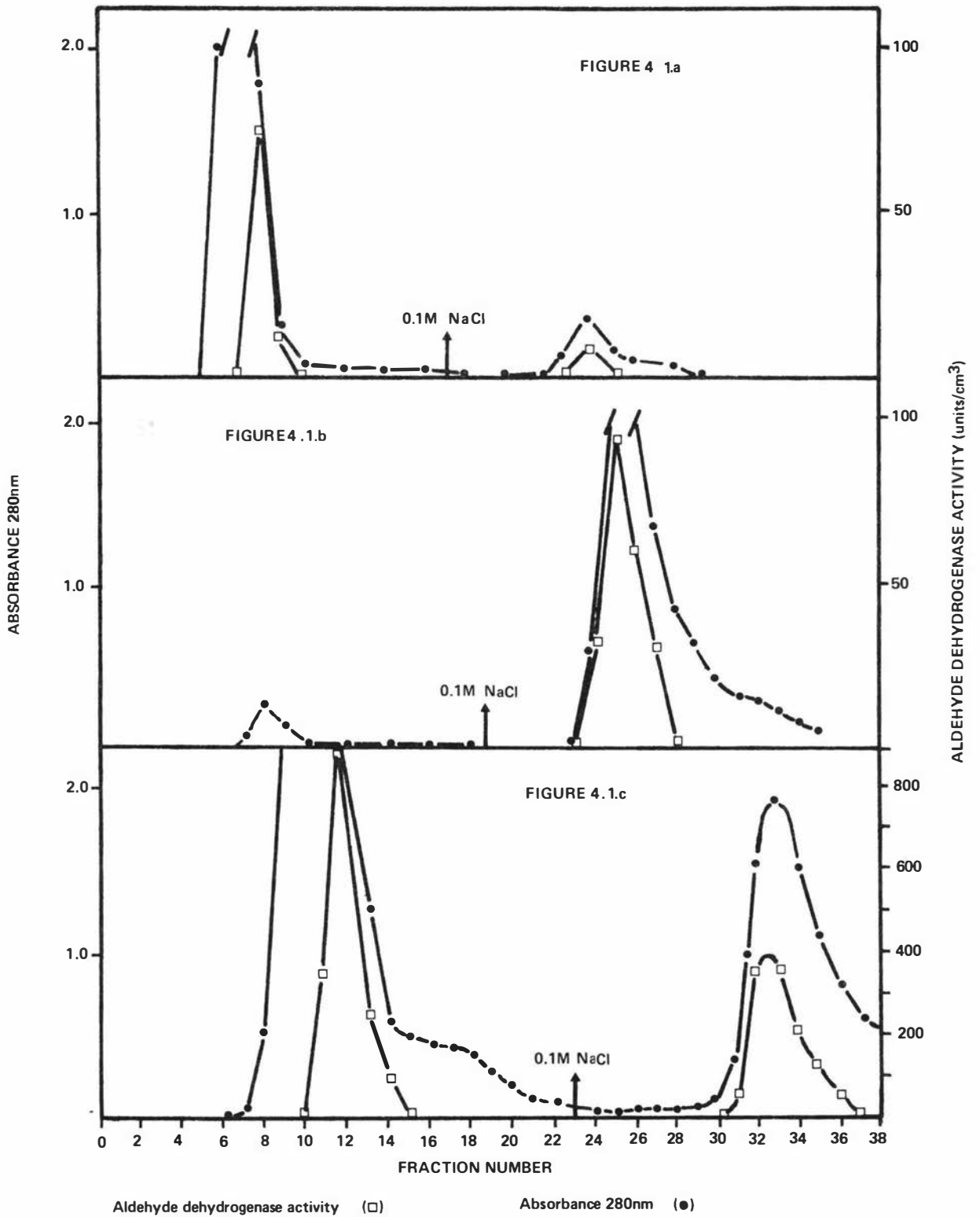
re-bound.(Figure 4.1.b). If the liver supernatant was treated with ammonium sulphate prior to the initial column separation, two activity peaks were still found (Figure 4.1.c). In sodium chloride gradient runs using untreated whole liver supernatant, the retarded enzyme peak tended to be very small. A change to using phosphate gradients appeared to increase the amount of retarded enzyme relative to the amount that was unretarded (Figures 4.2.a and b).

Purification studies carried out simultaneously by Dr T.M.Kitson on aldehyde dehydrogenase from isolated mitochondria showed that a single, bound, peak of activity was found on a column run under similar conditions. It was therefore considered that the unbound activity might be cytoplasmic, and attempts were made to purify this fraction. The unbound activity proved to be extremely unstable, yielding less than 1% recovery after a purification procedure including two DEAE Protion column runs, one CM Protion column run, and two ammonium sulphate fractionations.

The fractionation experiments reported in Section 2 were being carried out concurrently with attempts to purify aldehyde dehydrogenase. These experiments showed that there were possibly three aldehyde dehydrogenases in sheep liver, a microsomal enzyme being present in addition to cytoplasmic and mitochondrial activities. As only two types of activity were distinguished using ion exchange chromatography of whole liver supernatants, and since there now appeared to be enzyme in three intracellular fractions, the most effective way of isolating and identifying these enzymes was considered to be the separation of the cell fractions prior to the purification of the enzyme.

While it was relatively simple to obtain mitochondria free of cytoplasm, by washing mitochondria after an initial centrifugation, the preparation of cytoplasm free of microsomes and the contents of broken mitochondria was more difficult. The method given in Section 4.2.1.(b) for tissue fractionation was used, since this procedure had been shown in Section 2 to give a negligible amount of mitochondrial breakage.

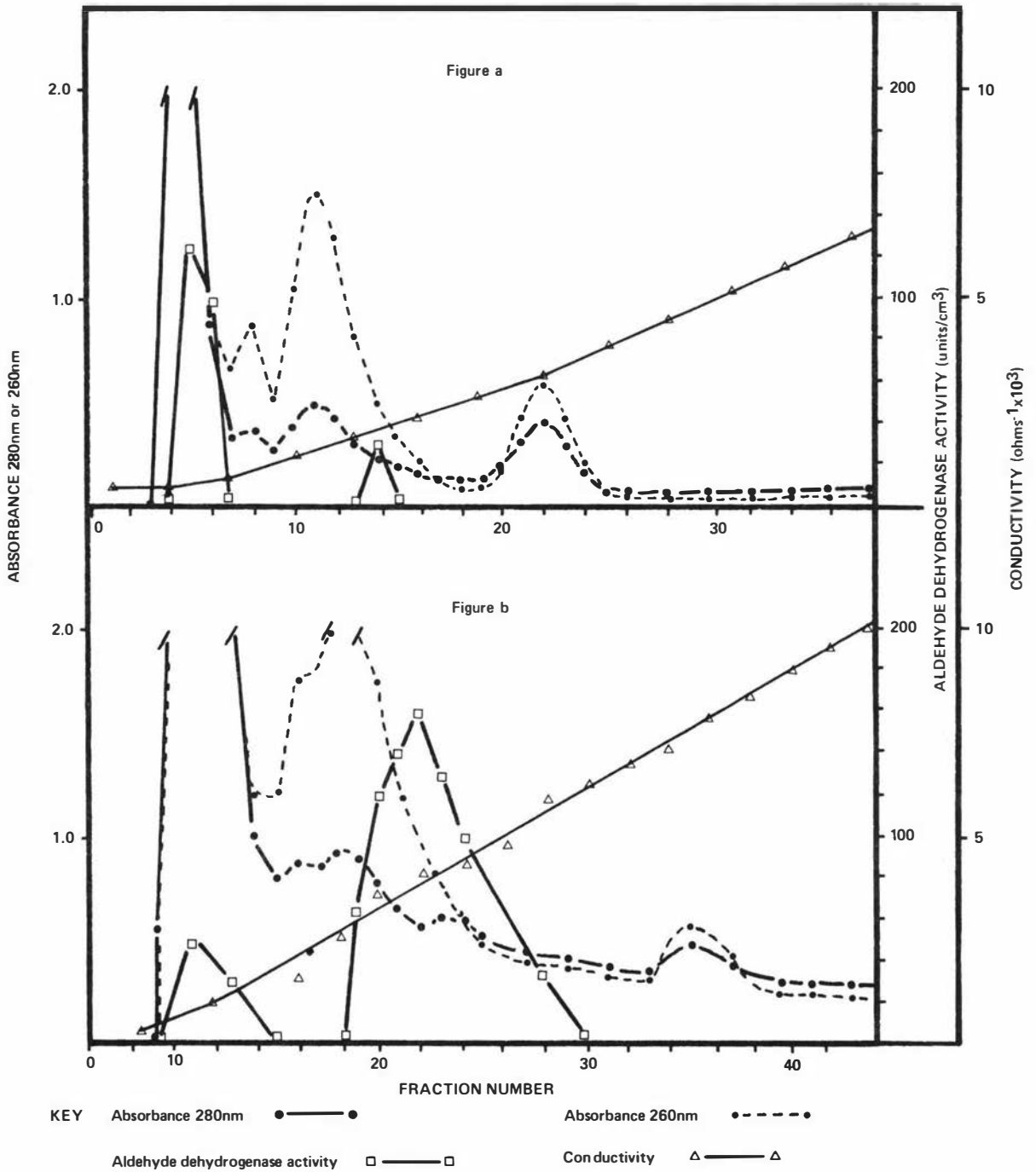
When cytoplasm prepared by method 4.2.1.(b) was run on DEAE Protion under the same conditions as those used for total liver homogenate supernatants, all the aldehyde dehydrogenase activity could be retarded. If an unretarded activity peak did occur in runs on pure cytoplasm, the activity was retarded when concentrated and re-run under the same conditions. When cytoplasm was concentrated using ammonium



Figures 4.1.a and b show the re-elution of aldehyde dehydrogenase from a total liver supernatant bound (b) and unbound (a) in a previous column run, following re-concentration of each fraction by ammonium sulphate precipitation. Figure 4.1.c shows the elution pattern of a sample of total liver supernatant treated with ammonium sulphate prior to a column run. All three DEAE Protion columns (Figures a, b and c) were equilibrated with 0.0025M phosphate buffer, pH 6.8, and eluted with this buffer following sample application, until the absorbance at 280nm dropped to less than 0.01, when the eluant was changed to 0.0025M phosphate buffer containing 0.1 M NaCl. Aldehyde dehydrogenase activity is given in absorbance units (1 unit of enzyme activity = 0.001 absorbance units/minute).

FIGURES 4.2.a and b.

THE ELUTION OF ALDEHYDE DEHYDROGENASE FROM  
TOTAL LIVER SUPERNATANTS: NaCl and PHOSPHATE  
GRADIENTS



The above graphs illustrate the elution of aldehyde dehydrogenase from a total liver supernatant using an NaCl gradient (Figure 4.2.a) and a phosphate gradient (Figure 4.2.b). The NaCl gradient was generated using 200 cm<sup>3</sup> of 0.005M phosphate buffer, pH 7.4, and 200 cm<sup>3</sup> of 0.005M phosphate buffer, pH 7.4, containing 0.1M NaCl. The phosphate gradient was generated using 350 cm<sup>3</sup> of 0.0025M phosphate buffer pH 6.8, and 350 cm<sup>3</sup> of 0.5M phosphate buffer, pH 6.8. Sample volumes were 3 cm<sup>3</sup> in column run (a) and 5 cm<sup>3</sup> in column run (b) of total liver supernatant. Aldehyde dehydrogenase activity is given in absorbance units (1 unit of enzyme activity = 0.001 absorbance units/minute).

sulphate prior to chromatography on DEAE Protion, all the activity could still be retarded.

It was at this point that the low capacity of DEAE Protion compared with that of DEAE cellulose was fully recognised, and a change was made to using DEAE cellulose. By using DEAE cellulose with cytoplasm free of microsomes and mitochondrial contents a single, retarded peak of aldehyde dehydrogenase activity was obtained, and a full purification scheme could finally be developed.

#### 4.3.2. Purification scheme for aldehyde dehydrogenase from cytoplasm.

The final purification scheme for cytoplasmic aldehyde dehydrogenase, an example of which is shown in Table 4.1, follows: -

Cytoplasm, obtained by method 4.2.1.(b), was taken to 45% saturation with ammonium sulphate, centrifuged, and the precipitate discarded. The supernatant was taken to 70% saturation, centrifuged, and the resultant precipitate redissolved in and dialysed against 0.01M phosphate buffer, pH 7.3. Following dialysis, this sample was loaded onto a DEAE cellulose column which had been equilibrated with the same buffer. The column was washed with 0.01 M buffer until the absorbance of the eluate at 280 nm was less than 0.1. The eluant was then changed to 0.05 M phosphate buffer, pH 7.3, and the aldehyde dehydrogenase activity eluting at this concentration was collected. The stepwise elution was developed from gradient runs carried out on DEAE cellulose. An example of a gradient separation of aldehyde dehydrogenase on DEAE cellulose is seen in Appendix 1 (Section 9, Figure 9.3).

Aldehyde dehydrogenase eluted in the stepwise column run was taken to 70% saturation with ammonium sulphate, centrifuged, and the precipitate dialysed against 0.05 M phosphate buffer, pH 7.3. The dialysed sample was loaded onto a Biogel A 0.5M column. Active fractions eluted from this column were suitable for kinetic studies (kinetic characterisation of the enzyme following its purification was carried out by A.MacGibbon), and for use in acetaldehyde assays. Figure 4.3 illustrates a typical column run on Biogel A 0.5M. Sephadex G-200 was used in place of Biogel in some preparations, but proved to be much slower to run, and gave lower recoveries of activity with no greater improvement in purity than that obtained using Biogel.

TABLE 4.1

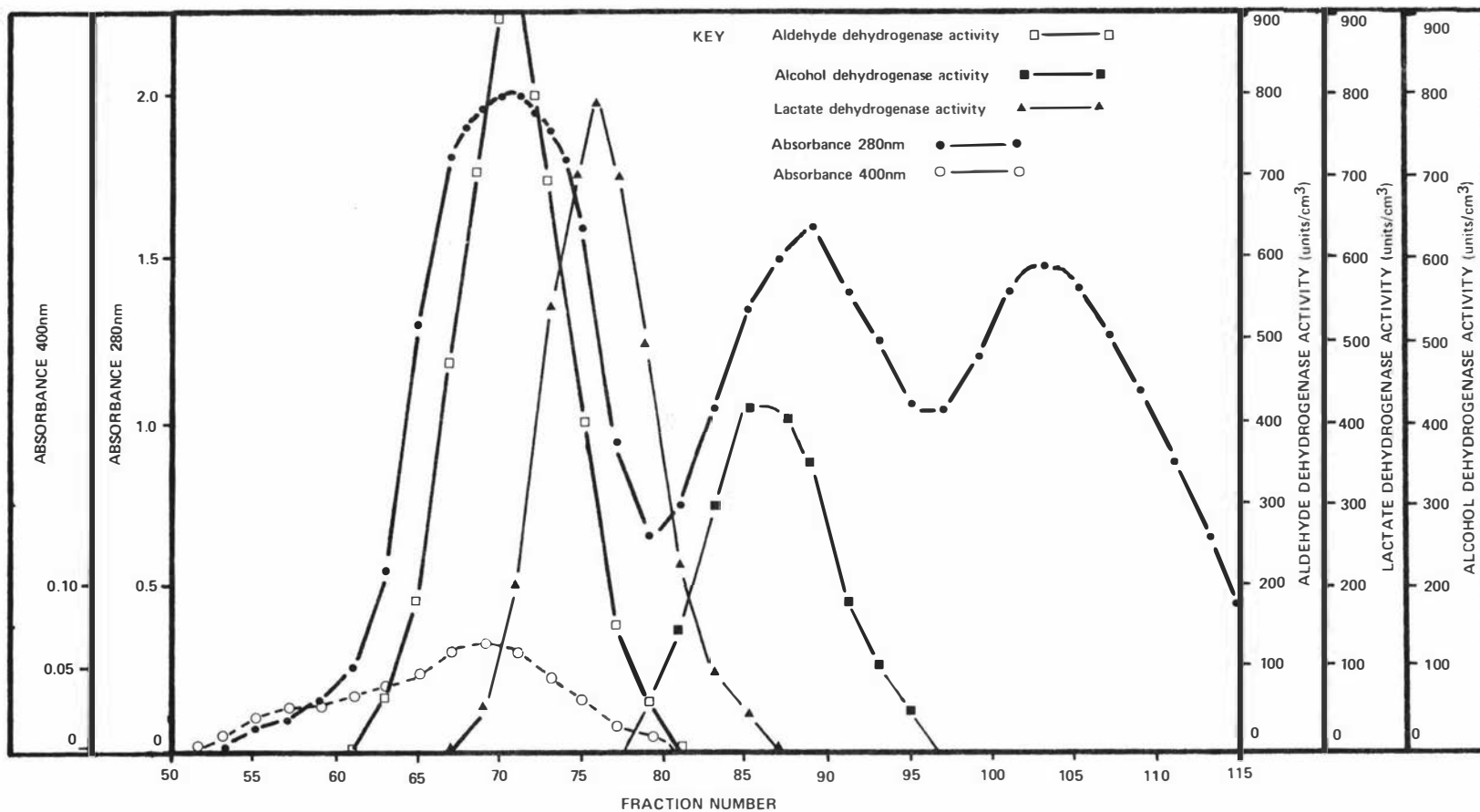
PURIFICATION OF ALDEHYDE DEHYDROGENASE FROM THE  
CYTOPLASM OF SHEEP LIVER

This table gives an example of the final aldehyde dehydrogenase purification scheme. Aldehyde dehydrogenase activity is expressed in absorbance units (1 unit of enzyme activity = 0.001 absorbance units/minute), specific activity in units/mg protein. Post-Biogel results for protein and specific activity are an average over 11 peak fractions from this column run, in which specific activity was fairly constant ( $801 \pm 60$  units/mg).

The preparation shown gave the highest percentage recovery and specific activity achieved, but purity according to gel electrophoresis was similar in all preparations.

Sample	Total Activity (units)	Protein (mg/cm <sup>3</sup> )	Specific Activity (units/mg)	Recovery (%)
Cytoplasm	430,000	11.0	18.2	100
45 - 70% (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> precipitate, dialysed	270,000	35.0	25.7	63
DEAE cellulose effluent	234,000	2.7	333.0	54
0 - 70% (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> precipitate, dialysed	216,000	23.0	391.0	50
Biogel effluent	198,000	4.3	801.0	46

FIGURE 4.3 SEPARATION OF ALDEHYDE DEHYDROGENASE ON BIOGEL



The elution of aldehyde dehydrogenase on Biogel A 0.5M is illustrated. The elution of lactate dehydrogenase and alcohol dehydrogenase is also shown. The activity of each enzyme is given in absorbance units, where 1 unit of enzyme activity = a change of 0.001 absorbance units/minute. Absorbance at 280nm is given to indicate protein concentrations, and absorbance at 400 nm. the presence of catalase. The column dimensions were 70 x 2.5 cm, 6 cm<sup>3</sup> of sample solution was loaded (30 mg/cm<sup>3</sup> protein, 4,500 units/cm<sup>3</sup> aldehyde dehydrogenase activity), and the elution was carried out at a flow rate of 9 cm<sup>3</sup>/hour. The eluting buffer was 0.05M phosphate, pH 7.3, containing mercaptoethanol, 0.1%).

All buffers used throughout the preparation contained 0.1% mercaptoethanol, and were kept degassed where possible. The main factor in maintaining a good yield of aldehyde dehydrogenase activity appeared to be the speed of completion of the purification: The more rapidly a purification could be completed, the higher the percentage recovery of aldehyde dehydrogenase activity that resulted. The minimum elapsed time from whole livers to final product was about 1 week.

Recovery of activity from cytoplasm varied from as low as 5% in a very poor preparation, to as high as 46%. The final specific activities achieved varied from 260 - 800 absorbance units/mg protein (125 - 390 nmoles/min/mg). Table 4.2 gives specific activities and percentage recoveries for 7 preparations. The variation in final specific activity was due mainly to differing retentions of activity; the protein pattern following gel electrophoresis at the final stage did not vary greatly. From 500 g of starting material, the weight of protein finally obtained was between 40 and 300 mg.

#### 4.3.3. Purity of the final preparation determined by gel electrophoresis.

The purity of aldehyde dehydrogenase shown by polyacrylamide gel electrophoresis varied slightly, depending largely on the purification achieved on DEAE cellulose columns. Figure 4.4.a shows a photograph of a slab gel, stained for protein with Amido Black, on which a sample from the final stage of purification was subjected to electrophoresis. The loading on the gel was 22  $\mu$ g of protein. The faint band seen below the aldehyde dehydrogenase band was probably catalase, and, in this case, no other impurities were present in sufficient quantity to show on the gel. This sample was typical of the purity obtained in the final stage of a preparation, where catalase was usually the major contaminant remaining. By measurement of the areas under peaks on a scan of the gel, the amount of contaminating protein present was found to be not more than 5% of the total protein. Identification of the major protein band as aldehyde dehydrogenase was confirmed by activity staining with specific reagents, described in Section 5.

TABLE 4.2

FINAL SPECIFIC ACTIVITY AND PERCENT RECOVERY  
IN ALDEHYDE DEHYDROGENASE PREPARATIONS

These preparations were all carried out with the fully developed purification scheme. The same amount of liver (500g) was used as starting material in all except run 1 (800g). With runs 1 and 2, only a part of the total activity available was put through the final purification step. Recoveries and amounts of protein for these two runs are estimates correcting for the amount of enzyme that was not included in the final step. Run 2 shows extremely low recovery. The sample, in this case, was run through an extra DEAE cellulose column prior to the Biogel column. In runs 1 and 2, NaOH treated DEAE Protion was used rather than DEAE cellulose for the initial column fractionation.

Preparation	Protein (mg)	Specific activity (absorbance units/mg)      (nmoles/ min/mg)		% recovery
1	350	537	260	40
2	40	310	150	5
3	300	353	170	29
4	174	257	125	10
5	216	279	135	13
6	160	320	155	10
7	200	801	390	46

#### 4.3.4. Purity of aldehyde dehydrogenase determined by assay of contaminating enzymes.

Fractions from the Biogel run shown in Figure 4.3 were assayed for the enzymes lactate dehydrogenase, alcohol dehydrogenase, aldehyde oxidase, and malate dehydrogenase. The absorbance at 400 nm was determined as a guide to the presence of catalase. Table 4.3 and Figure 4.3 show the results from these assays. To illustrate the electrophoretic purity of a similar sample, Figure 4.4.b shows a photograph of a gel stained for protein after electrophoresis of the peak aldehyde dehydrogenase fraction from a similar Biogel column from the same preparation. The gel loading was very high (150  $\mu$ g of protein) to check for contaminant bands.

In the example shown in Table 4.3, catalase was present at a maximum level of about 0.04 mg/cm<sup>3</sup>, calculated from  $E_{1\text{cm}}^{1\%} = 17.8$  (Stansell and Deutsch, 1965. Human erythrocyte catalase). Such a level of catalase represented about 2% of the total protein. Removal of catalase depended entirely on the success of the DEAE cellulose column, since, with Biogel, catalase was eluted in almost exactly the same position as aldehyde dehydrogenase. Table 4.4 illustrates the results obtained after assaying fractions from a Biogel column for catalase activity, using hydrogen peroxide. The activity in this assay coincided almost exactly with the aldehyde dehydrogenase peak, as did the 400 nm absorbance peak. Because catalase was eluted from the DEAE cellulose at only a slightly higher ionic strength than aldehyde dehydrogenase, any overloading of this column caused catalase to elute in the 0.05 M buffer wash, instead of remaining bound. Once this had happened, no further separation of the two enzymes could be achieved in the standard purification scheme. Catalase could be removed from contaminated samples by a second DEAE cellulose run, either before or after the Biogel column. This, however, increased the time required for the preparation, and, although a slightly purer product was obtained, yields of activity were lower.

The maximum level of lactate dehydrogenase present in any fraction was 0.38 units/cm<sup>3</sup>, and of alcohol dehydrogenase, 0.2 units/cm<sup>3</sup>, where 1 unit is the standard unit of 1  $\mu$ mole of substrate converted per minute. Lactate dehydrogenase was largely separated from the peak fractions of aldehyde dehydrogenase during the Biogel separation, while alcohol dehydrogenase was completely removed from the region of aldehyde

TABLE 4.3

LEVELS OF CONTAMINATING ENZYMES

Fractions from the Biogel separation shown in Figure 4.3 were assayed for contaminant enzymes as well as aldehyde dehydrogenase. Levels of contaminant enzymes are given in this table both in the fraction containing maximum aldehyde dehydrogenase activity, and in the fraction containing maximum activity for the particular enzyme. Aldehyde dehydrogenase and protein levels are also shown. The Biogel separation used for these assays was from preparation 1 of Table 4.2.

Enzyme	Units	Maximum level	Level at Aldehyde dehydrogenase maximum
Lactate dehydrogenase	$\mu\text{mol. substrate reduced/min/cm}^3$	0.38	0.097
Alcohol dehydrogenase	$\mu\text{mol. substrate oxidised/min/cm}^3$	0.20	no activity
Catalase	$\text{mg/protein/cm}^3$	0.037	0.035
Malate dehydrogenase			no activity
Aldehyde oxidase			no activity
Aldehyde dehydrogenase	$\text{nmoles substrate oxidised/min/cm}^3$	460	460
Protein concentration	$\text{mg/cm}^3$	1.78	1.78

**FIGURE 4.4 GEL ELECTROPHORESIS OF ALDEHYDE DEHYDROGENASE**

**FIGURE 4.4.b**

Figure 4.4.b shows a slab gel to which 150  $\mu\text{g}$  of aldehyde dehydrogenase from preparation 1 of Table 4.2 was applied. The gel was stained for protein using Amido black, following electrophoresis as described in Section 4.2.7.

**FIGURE 4.4.a**

Figure 4.4.a shows a slab gel to which 22  $\mu\text{g}$  of aldehyde dehydrogenase from preparation 7 of Table 4.2 was applied. The gel was stained for protein using Amido black, following electrophoresis as described in Section 4.2.7.

FIGURE  
4 4 a



FIGURE  
4 4 b



TABLE 4.4

CATALASE ACTIVITY, 400nm ABSORBANCE, AND ALDEHYDE  
DEHYDROGENASE ACTIVITY FOLLOWING A BIOGEL RUN

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Fractions from a Biogel column were assayed for catalase activity using hydrogen peroxide in addition to measuring absorbance at 400nm. Catalase and aldehyde dehydrogenase activities are given in absorbance units (catalase, 1 unit of enzyme activity = a change of 1 absorbance unit/minute; aldehyde dehydrogenase, 1 unit of enzyme activity = a change of 0.001 absorbance units/min). This column run was from preparation 4, Table 4.2, and was also assayed for aldehyde oxidase activity, none of which was detected in any fraction. The ratio of 400nm absorbance to catalase activity in the earlier fractions was higher than in subsequent fractions, and since aldehyde oxidase activity was present in the sample loaded onto the Biogel column, (see Table 4.5), the presence of inactive aldehyde oxidase could be contributing to absorbance at this wavelength. The 400nm absorbance peak coincides with both the catalase and aldehyde dehydrogenase activity peaks. This example illustrates the difficulty in separating the two enzymes at this stage.

---

Fraction no.	Catalase activity (units/cm <sup>2</sup> )	400nm absorbance	Aldehyde dehydrogenase act. (units/cm <sup>3</sup> )
31	0.080	0.034	-
33	0.110	0.070	-
35	0.135	0.118	-
37	0.260	0.170	50
39	0.650	0.255	350
41	1.150	0.380	750
43	1.500	0.420	1400
45	1.300	0.315	1200
47	0.700	0.155	800
49	0.275	0.065	400
51	0.090	0.030	150

---

dehydrogenase activity. No malate dehydrogenase was detected in the preparation shown, but trace amounts were found in an earlier Biogel run, the peak activity appearing between that of lactate and alcohol dehydrogenases. The malate dehydrogenase activity did not overlap with the region of aldehyde dehydrogenase activity.

No aldehyde oxidase activity could be detected after the Biogel column shown. As it is a coloured protein, absorbing in the region of 400 nm, the presence of inactive aldehyde oxidase could explain the shoulder observed on the 400 nm absorbance peak (Figure 4.3), as this shoulder, when it occurred, did not show catalase activity. Aldehyde oxidase activity was followed through each step of another aldehyde dehydrogenase preparation. All fractions had to be dialysed before assay since mercaptoethanol rapidly reduced the dye used in this assay. Again, no activity could be detected following the Biogel separation. Results for the assay of aldehyde oxidase are shown in Table 4.5, and demonstrate that even prior to the Biogel column, only 1% of the original aldehyde oxidase activity was retained.

#### 4.4. DISCUSSION

##### 4.4.1. Preliminary results.

In DEAE Protion column runs on supernatant from a total liver homogenate, two enzyme peaks were obtained, one retarded, the other unretarded. The retarded enzyme was more noticeable when phosphate rather than sodium chloride gradients were used. Re-chromatography of unretarded enzyme on a second column showed that the unbound activity was due to a distinct enzyme, and not to an artefact from overloading the column. The unretarded activity was removed after separation of microsomes and mitochondria from cytoplasm, which gave some indication of its source. Activity from mitochondria was retarded under similar conditions (T.M.Kitson, personal communication), but activity from either untreated or detergent-treated microsomes was unretarded (Section 2). The unbound activity was therefore probably of microsomal origin. A further indication of this was the extreme instability noted during attempts to purify the unbound enzyme. Neither the mitochondrial nor the cytoplasmic enzymes showed such instability during purification.

TABLE 4.5

LEVELS OF ALDEHYDE OXIDASE IN AN ALDEHYDE  
DEHYDROGENASE PREPARATION

---

Aldehyde oxidase activity is expressed in absorbance units, where 1 unit of enzyme activity represents a change of 1 absorbance unit/minute at 600nm. Aldehyde dehydrogenase activity is also given in absorbance units (1 unit of enzyme activity = 0.001 absorbance units/minute).

In the preparation given below, the effluent from the DEAE cellulose column was concentrated by two different methods, the usual ammonium sulphate step, and by ultrafiltration. Both methods gave a similar recovery of aldehyde dehydrogenase activity. The preparation shown was 4, in Table 4.2.

---

Fraction	Aldehyde oxidase			Aldehyde dehydrogenase		
	units/cm <sup>3</sup>	total units	% recovery	units/cm <sup>3</sup>	total units	% recovery
cytoplasm	0.050	134	100	200	460,000	100
pre-DEAE-cellulose	0.088	28.1	20	800	256,000	55
bulk post cellulose	0.005	1.3	1.0	450	117,000	25
ammonium sulphate	0.028	0.53		2700	51,300	
ultra-filtration	0.065	0.65	0.9	6500	65,000	25
post-biogel		no activity		-	45,300	10

---

The finding of two aldehyde dehydrogenase activity peaks on DEAE ion exchange resins has been frequently reported. In each case a supernatant fraction has been studied, with varying care in removal of mitochondria and microsomes.

One such report (Sheppard et al., 1970) found almost identical results to those described here for sheep liver. An acetone powder of mouse liver was the starting material, with the initial supernatant being obtained after a low-speed centrifugation. Two peaks of aldehyde dehydrogenase activity were eluted from DEAE cellulose columns, one, a minor unretarded peak, and the other, bound. On re-chromatography under identical conditions, the unretarded peak showed no retarded component. After preparing mitochondria, and finding that the activity from this source was bound, the authors concluded that the bound activity was a combination of both soluble and mitochondrial aldehyde dehydrogenases. No attempt was made to identify the source of the unbound activity. Comparison with the results obtained for sheep liver indicates that the unretarded activity obtained from mouse liver preparations was possibly microsomal in origin.

In a second reference (Blair and Bodley, 1969), a similar situation was noted. Frozen human liver was used, and a supernatant was obtained after a low-speed centrifugation. It seems probable that mitochondria would be broken, and centrifugation would not have been sufficient to remove microsomal material from the supernatant. A third group also used similar methods, with horse liver (Feldman and Weiner, 1972). In both the human and horse liver preparations, two enzyme peaks were found on DEAE cellulose, the minor peak being less strongly bound in each case. No attempt was made by either group to identify the intracellular origin of either activity peak, and both continued purification of the major, bound activity peak. It is possible that the less strongly bound activity observed in these two cases is microsomal in origin.

In a more recent purification of horse liver aldehyde dehydrogenase (Okuda et al., 1973) two activity peaks were found following DEAE Sephadex fractionation. The centrifugation method used was much less likely to have resulted in microsomal contamination of the supernatant in this case, but a smaller, unretarded peak was still found.

This report casts some doubt on a microsomal origin for the unbound activity.

A study by Shum and Blair (1972) of cytoplasmic aldehyde dehydrogenase from rat liver also suggests that microsomal contamination is unlikely to be responsible for a second peak eluted from DEAE cellulose. These authors used sufficient centrifugation to remove microsomal material, and still found two aldehyde dehydrogenases in the supernatant fraction.

Two forms of soluble enzyme, differing in their response to phenobarbital, were reported in rat liver by Deitrich et al., (1972), who also took reasonable care not to break mitochondria, and to exclude microsomes from the supernatant. A third report (Marjanen, 1973) using rat liver, however, gave conflicting results to those above. Only one supernatant aldehyde dehydrogenase was found, and this was unretarded on DEAE cellulose, while the mitochondrial enzyme was retarded. No microsomal aldehyde dehydrogenase was found, and Marjanen suggested that he found only one cytoplasmic enzyme because he used a low acetaldehyde concentration, and failed to detect a high  $K_M$  enzyme.

At this stage there appears to be no clear indication of whether there are one or more cytoplasmic aldehyde dehydrogenases in rat, horse, or human liver, but it can be claimed that some conflicting results are due to the use of varying techniques to obtain "soluble" enzyme. Once cytoplasm, free of microsomes, was prepared there was no evidence of two cytoplasmic enzymes in sheep liver.

Although the unretarded enzyme found in sheep liver was probably microsomal in origin, the results obtained require confirmation from experiments with a conventional ion exchange resin, since DEAE Protion and not DEAE cellulose was used for this work. The main conclusion from these results is that tissue should be adequately fractionated prior to enzyme purification when the enzyme occurs in more than one intracellular fraction, even if intracellular localisation is not of prime interest in the study undertaken.

#### 4.4.2. Final purification of aldehyde dehydrogenase from sheep liver.

Aldehyde dehydrogenase from the cytoplasmic fraction of sheep liver was purified 45 fold. Table 4.6 compares previous purification attempts, and shows that few attempts to purify properly separated

TABLE 4.6

## SUMMARY OF ALDEHYDE DEHYDROGENASE PURIFICATIONS

Tissue	Fraction	Assay Method	Temp. (°C)	pH	Substrate	Final S.A. (units/mg)	Units	Purification (fold)	Reference
Bovine liver	acetone powder	spectrophotometric	-	9.3	acetaldehyde	700	absorbance	23 from aqueous extract of powder	Racker, 1949
Rabbit liver	total supernatant	spectrophotometric	25	9.5	acetaldehyde	2700	absorbance	30 from initial extract	Maxwell and Topper, 1961
Bovine liver	acetone powder	spectrophotometric	23	9.6	acetaldehyde	1300	absorbance	-	Deitrich <u>et al.</u> , 1962
Bovine brain	frozen supt mitochondria	fluorimetric	30	-	indole-3-acetaldehyde	23.0	nmoles/min	9 from mitochondrial supernatant	Erwin and Deitrich, 1966
Human liver	total supt	spectrophotometric	-	7.4	indole-3-acetaldehyde	106	nmoles/min	23 from initial extract	Kraemer and Deitrich, 1968
Human liver	total supt	spectrophotometric	22	9.6	acetaldehyde	680	nmoles/min	20 from initial extract	Blair and Bodley, 1969
Mouse liver, 3 strains	acetone powder	spectrophotometric	25	9.6	acetaldehyde	972 387 568	nmoles/min	9 - 10 from post cellulose	Sheppard <u>et al</u> 1970
Pig brain	acetone powder	spectrophotometric	30	7.2 9.6	heptaldehyde	185 740	nmoles/min	92 from extract of acetone powder	Duncan and Tipton, 1971
Rat liver	cytoplasm	spectrophotometric	22	8.0	acetaldehyde	390	nmoles/min	48 from cytoplasm	Shum and Blair 1972

TABLE 4.6 (continued)

Tissue	Fraction	Assay Method	Temp. (°C)	pH	Substrate	Final S.A. (units/mg)	Units	Purification	Reference
Horse liver	total supt	fluorimetric	25	9.0	propion-aldehyde	1800	nmoles/min	250 from ammonium sulphate cut	Feldman and Weiner, 1972
Horse liver	total supt	spectrophotometric	-	9.3	acetaldehyde	75	nmoles/min	75 from initial supernatant	Okuda <i>et al.</i> , 1973
Rat liver	mitochondria	gas chromatographic and spectrophotometric	25	7.4	acetaldehyde	21	nmoles/min	9 from initial supernatant	Marjanen, 1973
	cytoplasm						7 from initial supernatant		
Sheep liver	mitochondria	spectrophotometric	25	9.3	acetaldehyde	705	absorbance	16 from first ammonium sulphate cut	Dr T.M. Kitson, pers. comm. This study
	cytoplasm					340	absorbance	45 from cytoplasm	
						800	nmoles/min	390	

The table compares some of the reported purifications of mammalian aldehyde dehydrogenase. Where properly separated intracellular fractions have been used, the initial fraction is described as "cytoplasm" or "mitochondria". If separation of intracellular fractions has not been shown, the starting material is described as a "total supernatant (supt)" or "acetone powder" as appropriate. The results for sheep liver aldehyde dehydrogenase are from this study on the cytoplasmic enzyme, and from the work of Dr T.M. Kitson on the mitochondrial enzyme. The purity of the final sheep liver enzyme preparations has been improved considerably since the results of similar purifications were initially published (Crow *et al.*, 1974).

mitochondrial and cytoplasmic aldehyde dehydrogenases have been made. The purification of sheep liver cytoplasmic enzyme, combined with the results of Dr T.M.Kitson for the sheep liver mitochondrial enzyme, is the most extensive study that has been completed. Some of the properties of the two enzymes purified from sheep liver will be discussed in Section 5. The only other study comparing properly separated enzymes (Marjanen, 1973) was published while the present work was in progress. In his paper, Marjanen reported a 9 fold purification of rat liver mitochondrial aldehyde dehydrogenase, and a 7 fold purification of the cytoplasmic enzyme.

The only mammalian aldehyde dehydrogenase purified to homogeneity (horse liver, Feldman and Weiner, 1972) was from a total liver supernatant so that its intracellular origin is not defined. A specific activity of 1800 nmoles/minute/mg protein was the best achieved by the authors, with values of 700 - 1000 nmoles/minute/mg being normal. These values were obtained for enzyme which had been purified by isoelectric focusing, and values prior to this step, where a similar yield of protein to that in the final step of sheep liver preparations was obtained, were considerably lower. As an example, a specific activity of 120 nmoles/minute/mg was obtained after ammonium sulphate, CM cellulose, and two DEAE cellulose treatments. The sheep liver enzyme, following a similar but more simple purification, had a specific activity of up to 390 nmoles/minute/mg. Isoelectric focusing was tried in an attempt to purify further the sheep liver enzyme, but the slowness of the technique meant that it was not a worthwhile purification step in view of the quantity of enzyme needed for further studies.

The final cytoplasmic aldehyde dehydrogenase product was free of alcohol dehydrogenase, malate dehydrogenase, and aldehyde oxidase activities. Contamination by lactate dehydrogenase was slight, and could be completely avoided by using only the first fractions from Biogel columns. Catalase ususally represented less than 2% of the total protein. From these results, and the results of gel electrophoresis, the enzyme was judged to be about 95% pure after the purification scheme described. It was suitable for use in kinetic studies, comparative and characterisation work, and for use in the assay of acetaldehyde.

## SECTION 5

CHARACTERISATION OF ALDEHYDE DEHYDROGENASE5.1 INTRODUCTION.

When this work commenced, mammalian aldehyde dehydrogenase had not been purified to homogeneity, although several estimates of the molecular weight of the enzyme had been reported in the literature, and other characteristics had been described using semi-purified preparations. The molecular weight estimates for the enzyme varied considerably. Two values for the human liver enzyme had been reported, one of 90,000 (Kraemer and Deitrich, 1968) and the other, 200,000 (Blair and Bodley, 1969). The aldehyde dehydrogenase from pig brain was reported to have a molecular weight of 130,000 (Duncan and Tipton, 1971) and two enzyme forms from rat liver were both estimated to have molecular weights of near 180,000 (Shum and Blair, 1972).

Among other characteristics reported was the ability of the enzyme to produce a positive staining reaction with phenazine methosulphate-nitroblue tetrazolium (Robbins, 1966). Robbins used a crude total extract of beef liver, and found only one band of aldehyde dehydrogenase activity after polyacrylamide gel electrophoresis. Semi-purified aldehyde dehydrogenase from pig brain (Duncan and Tipton, 1971), however, did not give the same activity stain.

The sensitivity of the enzyme to some steroidal hormones was reported for rabbit liver aldehyde dehydrogenase in 1961 (Maxwell and Topper). Other kinetic, inhibitor, and pH dependence data had been reported by 1972, but since these aspects of enzyme characterisation have been studied by other research workers in the group, these results will only be discussed briefly at the end of this section.

During the course of this study, the first report of a homogeneous preparation of mammalian aldehyde dehydrogenase was published by Feldman and Weiner (1972), and two more recent reports have further described the aldehyde dehydrogenases from rat liver (Marjanen, 1973; Siew et al., 1974).

## 5.2 METHODS

### 5.2.1. Gel electrophoresis

Gel electrophoresis was carried out as described in Section 4.2.7.

### 5.2.2. Activity staining for aldehyde dehydrogenase on polyacrylamide gels.

Aldehyde dehydrogenase was detected after slab gel electrophoresis by formation of formazan bands on the gel in the presence of phenazine methosulphate (PMS) and nitroblue tetrazolium (NBT), with suitable substrates. The method followed that described by Robbins (1966) except that (a) larger volumes of solution were used so that the slab could be fully immersed, and (b) pyrophosphate buffer was used at pH 9.3 instead of pH 8.8. Lactate dehydrogenase was stained as described in the same reference.

### 5.2.3. Isoelectric focusing.

#### (a) Polyacrylamide gels.

Polyacrylamide gel isoelectric focusing was carried out on slab gels, in a cold room at 4°C, using an Ortec method for high resolution electrophoresis. (Ortec Application Note AN 32, Techniques for high resolution electrophoresis). Samples were dialysed prior to loading to remove mercaptoethanol; the presence of this substance prevented polymerisation of the acrylamide. pH values following electrofocusing were determined by cutting a strip from the gels into approximately 0.5 cm pieces, which were then soaked in distilled water overnight. The pH of each gel portion was then taken as the pH of the solution in which it had been soaking.

#### (b) Glass column.

Isoelectric focusing using a glass column was carried out as described in the LKB 8100 instruction manual (LKB 8100 Ampholine Electrofocusing Equipment Instruction Manual, LKB-Produkter, Sweden). A glass column of 110 cm<sup>3</sup> volume, and teflon fittings, made in the departmental workshops, copied descriptions in this manual. Sucrose gradients were generated manually, using the table of volumes with the LKB instructions. Enzyme samples were dialysed against water prior to electrofocusing runs, and fractions (2cm<sup>3</sup>) were collected by hand from

the column after each run, at a flow rate of 0.5 cm<sup>3</sup>/minute. The fractions were stored in stoppered test tubes, and the pH measured using a 2 cm<sup>3</sup> water-jacketed container to retain the temperature of 18°C used in the focusing run. Aldehyde dehydrogenase assays were carried out by the standard method. Fractions required for gel electrophoresis were run through a Sephadex G-25 column to remove ampholytes and sucrose.

A narrow range pH gradient was obtained by the method described in the LKB instruction manual, using method B, i.e. a pre-run without sample.

#### 5.2.4. Molecular weight by gel filtration.

Molecular weight estimations were carried out using a 70 x 2.5 cm Pharmacia column packed with Biogel A 0.5M, and calibrated with cytochrome C, myoglobin, malate dehydrogenase, ovalbumin, lactate dehydrogenase, yeast and liver alcohol dehydrogenases, catalase, aldolase, urease, and glutamate dehydrogenase. Most standards were run in two separate runs, either with several other proteins, or alone. The proteins used were detected as follows:

cytochrome C	.....	absorbance at 400 nm
myoglobin	.....	absorbance at 400 nm
malate dehydrogenase	...	assayed by following the oxidation of NADH in the presence of oxaloacetate (assay method: Boehringer Mannheim).
lactate dehydrogenase	..	assay: Section 4.2.8.a (Wroblewski and La Due, 1955).
liver alcohol dehydrogenase	.....	assay: Section 4.2.8.b (Bonnichsen and Brink, 1955).
yeast alcohol dehydrogenase	.....	assayed as for liver alcohol dehydrogenase.
aldolase	.....	absorbance at 280 nm.
catalase	.....	absorbance at 400 nm.
ovalbumin	.....	absorbance at 280 nm.
glutamate dehydrogenase	.....	assayed by following NAD reduction in the presence of glutamate (Hogeboom and Schneider, 1953).

### 5.2.5. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

Sodium dodecyl sulphate (SDS) gels were run by two different methods. The first (Weber and Osborn, 1969) used 0.1 M sodium phosphate buffer, pH 7.2, containing SDS, 0.1%. Samples were prepared according to the reference, except that 5% mercaptoethanol was used rather than 1%. Samples were incubated in a boiling water bath for 5 minutes and were not dialysed prior to loading onto the gel. The latter variations were described in a subsequent reference by the same authors (Weber *et al.*, 1972). Gels were prepared according to the reference, except that slab gels rather than disc gels were used. These were run on the Ortec electrophoresis apparatus described in Section 4.2.7. SDS gels run by this method took up to  $5\frac{1}{2}$  hours, at a final setting of 325 volts, 115 ma.

The second method for running SDS gels (Cashmore, personal communication) used tris-glycine buffer, pH 8.9, containing 0.1% SDS. The gel mixture was as follows:

acrylamide (30%) + bisacrylamide (0.17%)	15 cm <sup>3</sup>
tris-glycine buffer ( 1 M )	3 cm <sup>3</sup>
H <sub>2</sub> O	11 cm <sup>3</sup>
SDS ( 10% )	0.3 cm <sup>3</sup>
tetramethylethylenediamine (10% in ethanol)	0.15 cm <sup>3</sup>
ammonium persulphate ( 10% )	0.15 cm <sup>3</sup>

Samples prepared as described for the previous method were dialysed against 0.1 M tris-glycine buffer, pH 8.9, containing SDS, 1%, and mercaptoethanol, 5%, before use in this method. Slab gels were again used, and could be run in 45 minutes, at final settings of 325 volts, 80 ma.

The same standard proteins were used in each procedure:

standard protein	final concentration in treated solution ( mg / cm <sup>3</sup> )
liver alcohol dehydrogenase	0.60
bovine serum albumin	0.54
ovalbumin	0.50
cytochrome C	0.52
lactate dehydrogenase	0.50

standard protein	final concentration in treated solution ( mg / cm <sup>3</sup> )
pyruvate kinase	0.50
catalase	0.50
aldolase	0.50
carboxypeptidase A	0.70

The final volumes of samples loaded are given with the results.

Both mitochondrial and cytoplasmic aldehyde dehydrogenase preparations were examined, and samples from the final stage of purification were treated similarly to the standard proteins. Final concentrations of the two enzymes in treated solutions were as follows:

mitochondrial (a)	0.17 mg/cm <sup>3</sup>
(b)	0.34 mg/cm <sup>3</sup>
cytoplasmic (a)	0.52 mg/cm <sup>3</sup>
(b)	0.26 mg/cm <sup>3</sup>

Samples containing two levels of protein were used as a check that reaction with SDS was complete at the higher protein concentrations. Amounts of protein finally loaded onto the gels are given in the results section.

Following electrophoresis, gels were stained for protein using Coomassie blue, and destained in methanol, acetic acid, and water as described by Weber and Osborn (1969). Protein band mobilities were calculated as described in the above reference, with measurements taken from the leading edge of the protein band in each case.

#### 5.2.6. Stability of aldehyde dehydrogenase to freezing.

To investigate the possibility of freezing as a means of storing aldehyde dehydrogenase, samples taken after the DEAE cellulose and Biogel purification steps were frozen under different conditions. Samples of enzyme solution (5 cm<sup>3</sup>) with additions of acetaldehyde, glycerol, ammonium sulphate, zinc sulphate, and NAD<sup>+</sup> were frozen at -20°C. A control sample was kept refrigerated at 4°C. All samples were assayed for residual aldehyde dehydrogenase activity after 4 weeks.

### 5.3. RESULTS

#### 5.3.1. Isoelectric focusing of cytoplasmic aldehyde dehydrogenase.

##### (a) Polyacrylamide gels

Isoelectric focusing of aldehyde dehydrogenase on polyacrylamide gels was carried out to give an indication of the isoelectric point of the enzyme before attempting to use column isoelectric focusing as a preparative step.

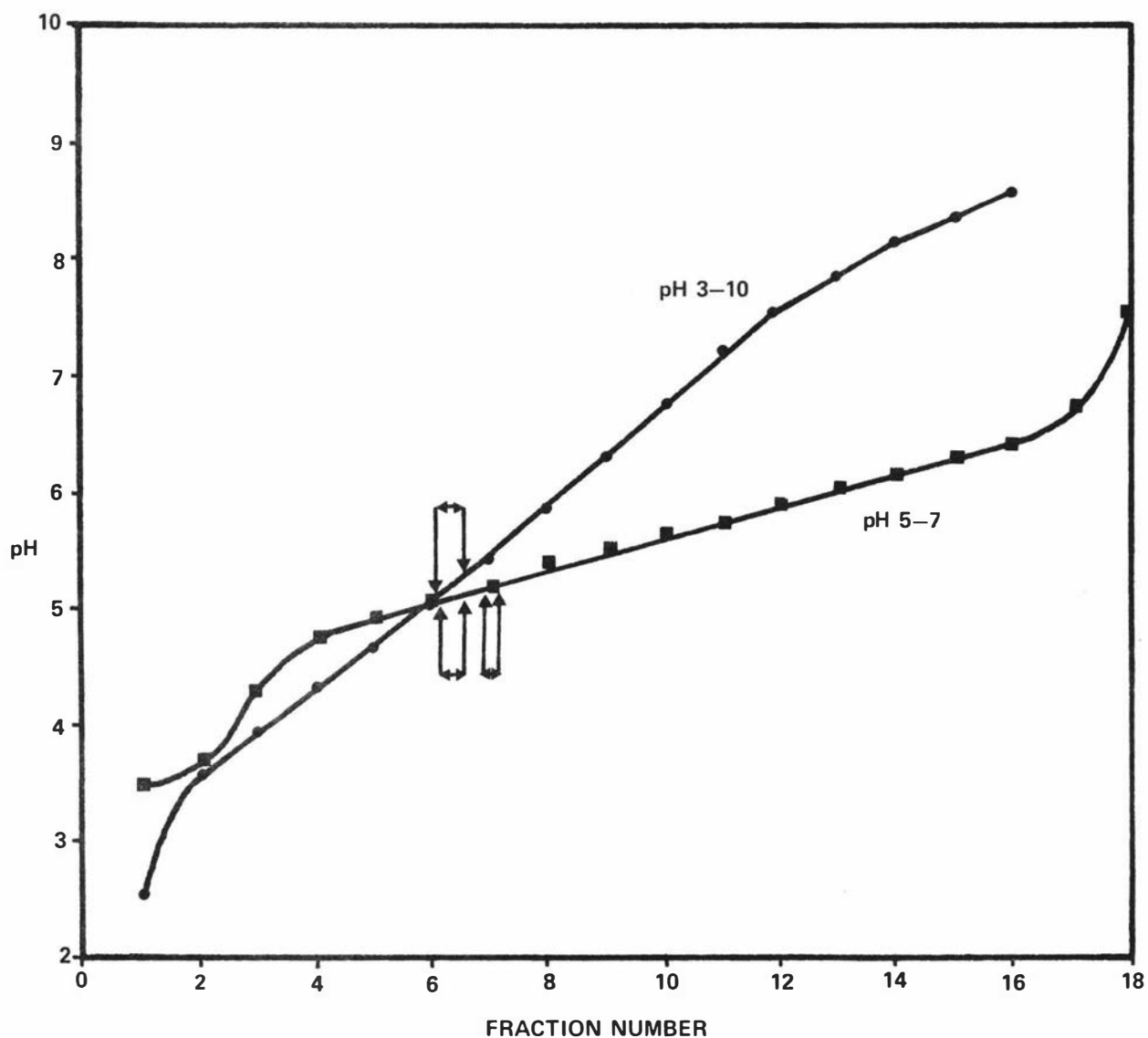
A pH 3 - 10 gradient was used initially, and duplicate enzyme samples gave isoelectric points at pH 5.05 and 5.10 calculated from the position of the activity stains, and at pH 5.15 and pH 5.25 calculated from protein stains. These values gave an average isoelectric point of  $\text{pH } 5.14 \pm 0.10$ . A narrower gradient, between pH 5 and 7 was also used, and this gave two bands of aldehyde dehydrogenase activity with isoelectric points at pH 5.35 and 5.58, after averaging duplicate results from protein and activity stains. In this case, non-linearity of the gradient and broadness of the bands indicated that focusing was not complete. In a second pH 5 - 7 run, two separate bands could only just be resolved in two out of four activity stains. In the remaining two activity stains, separate bands could not be resolved. Average results from four protein stains gave isoelectric points of  $5.070 \pm 0.005$  and  $5.16 \pm 0.01$ , and from two activity stains,  $5.11 \pm 0.01$  and  $5.18 \pm 0.02$  for the two enzyme bands. The pH 3 - 10 gradient and the second pH 5 - 7 gradient are shown in Figure 5.1, with the range in isoelectric points calculated from the mid-points of aldehyde dehydrogenase bands being shown in each case.

##### (b) Glass column.

Following the above preliminary tests on gels, glass column isoelectric focusing was carried out, as it was possible that the use of this technique on a preparative scale could provide an alternative to Sephadex G-200 column separations, which were being used at this stage, and which, as described in Section 4.3.2, had not proved satisfactory.

Two initial separations using a pH 3 - 10 gradient were carried out to establish the stability of the enzyme to focusing at  $18^{\circ}\text{C}$ . In the first separation, 10 hours focusing at 300 V gave a non-linear gradient, and an isoelectric point for the enzyme far removed from that

FIGURE 5.1 pH GRADIENTS OBTAINED IN ISOELECTRIC FOCUSING ON POLYACRYLAMIDE GELS



Two pH gradients covering ranges pH 3 - 10 and 5 - 7, obtained from separate focusing runs on polyacrylamide gels, are shown. Arrows indicate the range of isoelectric point for aldehyde dehydrogenase in each run, as determined from the mid points of protein and activity stain bands. The linearity of the gradients indicates complete focusing in each case.

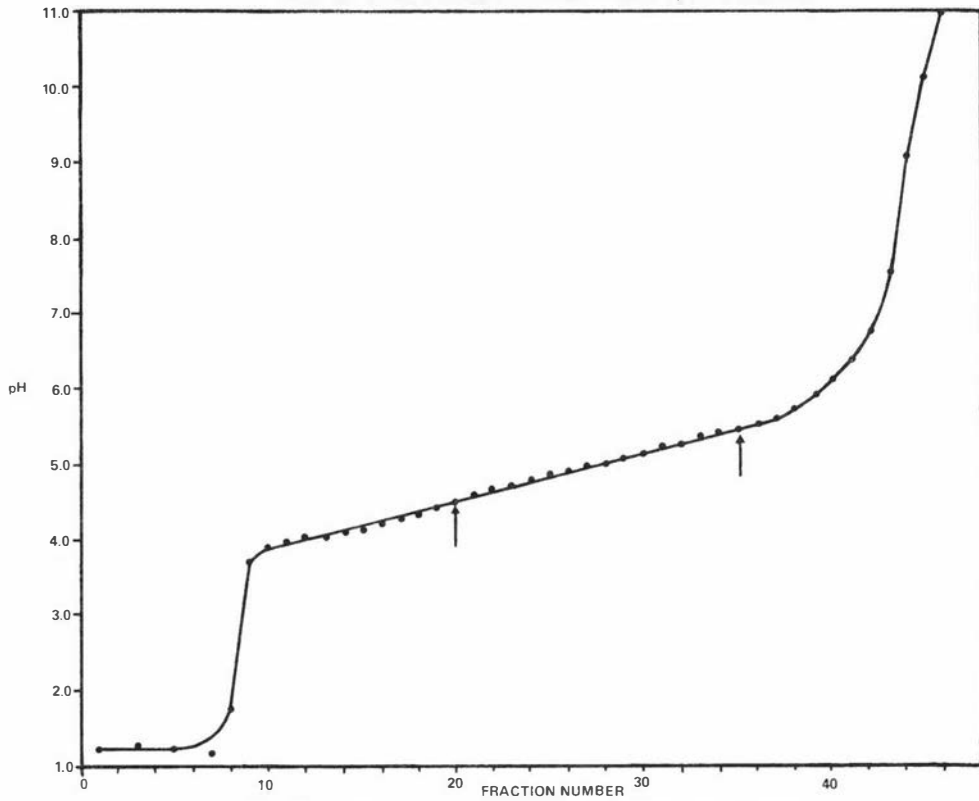
found on gels. In the second pH 3 - 10 run, focused for 27 hours at 300 V, a nearly linear gradient was formed, and an isoelectric point at pH 4.8 was observed. About 90% of the initial activity was recovered in this separation, compared with 100% in the first.

Enzyme samples used for these separations were pre-gel filtration samples, with the possibility that gel filtration might replace isoelectric focusing as a final purification step. However, pH 3 - 10 gradients gave very little separation of contaminant proteins from aldehyde dehydrogenase. A pH 3 - 6 gradient focused for 31 hours at 450 - 475 V gave a better separation, but only 50% of activity was recovered. A completely linear gradient was obtained, and the isoelectric point was pH 5.05. Because of the loss of activity, and the demonstration at this stage that Biogel resin was suitable for a final purification step, the use of isoelectric focusing for preparative work was not pursued.

A final electrofocusing experiment was carried out in an attempt to separate the two forms of enzyme seen on gels. In this run, a pH 4 - 6 gradient was generated without enzyme, and the fractions in the pH range 4.5 - 5.5 were refocused in a subsequent experiment with enzyme present. The initial gradient required 68 hours to focus, at 240 V, and was linear over the range of pH 4.0 - 5.6 (Figure 5.2). The second gradient required 72 hours to focus, at 225 V and was linear in the pH range 4.5 - 5.5 (Figure 5.3). The enzyme used in this run was a post-gel filtration sample, with a specific activity of 174 units/mg. A total of 34.5 mg of protein was loaded onto the column, representing 6,200 units of activity. Following the separation, a total of 8,000 units of activity was recovered. This high recovery of activity was surprising, when compared with the 50% recovery in an earlier experiment. The extra activity noted after the run probably reflects errors introduced by summing the activities of the separate fractions eluted from a column.

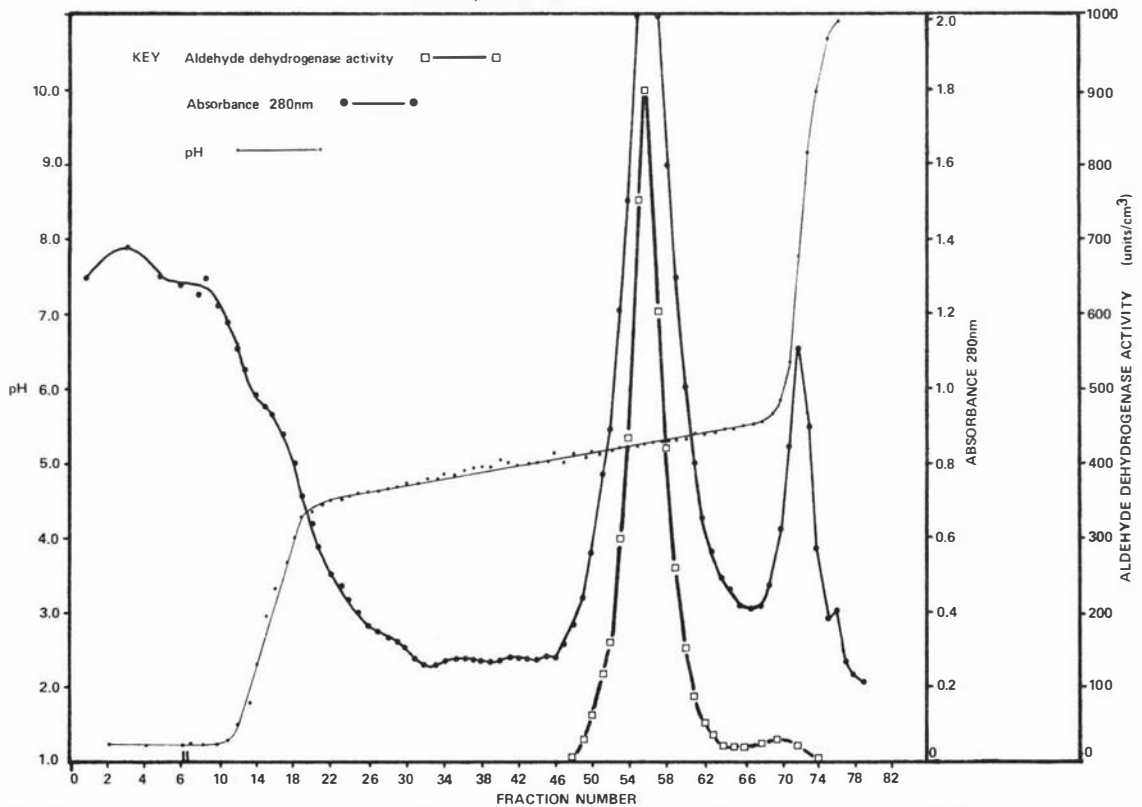
Protein levels were not accurately estimated in the fractions from the column, since ampholytes interfere with standard protein estimation techniques (Wrigley, 1971). However, an approximate value from 280 nm readings showed the specific activity to have been increased about two fold. The main enzyme peak, detected by assaying for aldehyde dehydrogenase activity was well separated from any obvious contaminant peaks, and was symmetrical, with no evidence of two enzyme

FIGURE 5.2 DENSITY GRADIENT ELECTROFOCUSING : pH 4 - 6 GRADIENT



The pH 4 - 6 gradient shown was generated by electrofocusing ampholytes of pI 4 - 6 in a sucrose gradient without any added sample. Fractions in the pH range 4.5 - 5.5 were re-focused in a subsequent experiment, with enzyme present, to give the gradient shown in Figure 5.3

FIGURE 5.3 SEPARATION OF ALDEHYDE DEHYDROGENASE ON A NARROW RANGE pH GRADIENT



A narrow range (pH 4.5 - 5.5) pH gradient was generated with an aldehyde dehydrogenase sample present, using appropriate fractions from the run shown in Figure 5.2. Following the focusing run, fractions (2 cm<sup>3</sup>) were collected, and assayed for aldehyde dehydro-

genase activity. Absorbance at 280 nm was also recorded for each fraction, and the pH measured using a water-jacketed cell. Aldehyde dehydrogenase activity is expressed in absorbance units (1 unit of enzyme activity = 0.001 absorbance units/min).

species (Figure 5.3). The isoelectric point obtained from the centre of the peak was pH 5.25. This was a little higher than the upper limit of values obtained from isoelectric focusing on gels, but the range of experimental error would be expected to be greater when comparing two different methods. A very slight tail of activity following the enzyme peak was probably due either to slight mixing during emptying the column, or to the separation of a much less active form of the enzyme from the major peak. The amount was not sufficient to account for the second band of enzyme observed on gels, where the two bands obtained were of similar intensity with respect to both activity and protein stains. As the sample used on gels was from a similar stage in the purification scheme, and the gradient used in the final column run should have been sufficiently narrow to separate two such forms of the enzyme had they occurred, it was concluded that the appearance of two forms of the enzyme in gel isoelectric focusing was probably an artefact caused by the gel environment. Apparent heterogeneity of proteins can arise in gel electrofocusing through (a) chemical gel polymerisation (Wrigley, 1971, p322), which was used in this case, and (b) the occurrence of polymeric forms of the protein being focused (Wrigley, 1971, p 325). These artefacts could not occur in density gradient electrofocusing as (a) depends on the use of persulphate to polymerise gels and (b) is due to the rate of movement of proteins being limited by the gel medium. Such effects could explain the appearance of two forms of aldehyde dehydrogenase in gel electrofocusing that could not be detected in fractions from the column.

If narrow gradient isoelectric focusing had separated two forms of aldehyde dehydrogenase, it would have been essential as a final purification step. However, although recovery of activity was good in the final electrofocusing run, the fact that only a single form of the enzyme was found, and the time involved in the run (a total of nearly 6 days) showed that the method was not of real value for preparative work.

To compare the purity of samples before and after electrofocusing, ampholytes were removed from the column fractions by passage through Sephadex G-25, and the samples were run in the usual gel electrophoresis system. Results from these experiments showed a single protein band in fractions containing aldehyde dehydrogenase after the narrow gradient electrofocusing run. Protein and activity bands appeared in the same

relative position following electrophoresis on gels for sample of the enzyme from before and after electrofocusing.(Figure 5.4). Protein bands from the contaminant peak in the electrofocusing run (Figure 5.3) were close to the position of the enzyme in electrophoresis (Figure 5.4). Scans of the gels showed that one band in the electrofocusing contaminant peak corresponded to a shoulder seen in the enzyme peak prior to electrofocusing. A second band from the contaminant peak appeared to correspond in position to the aldehyde dehydrogenase protein stain, but no activity stain was obtained. This second band could have been an inactivated form of aldehyde dehydrogenase.

### 5.3.2. Molecular weights of cytoplasmic and mitochondrial aldehyde dehydrogenases determined by gel filtration.

Figure 5.5 shows the molecular weight calibration curve obtained for a Biogel A 0.5M column, prepared as described in Section 5.2.4. The average position for cytoplasmic aldehyde dehydrogenase from 6 column runs on three different enzyme preparations is marked on the graph. The average molecular weight of cytoplasmic aldehyde dehydrogenase obtained from the calibration curve was 212,000, with minimum and maximum values for the six runs at 205,000 and 220,000. The position of mitochondrial aldehyde dehydrogenase determined in one run is also marked on the graph, and the molecular weight was calculated at 205,000.

Several of the standards used did not fall very close to a straight line. The reason for this is not known, but duplicate runs which were carried out on a majority of the standards gave very little variation in elution volumes, indicating that the non-linearity was a consistent property of the system. Because the proteins exhibiting non-linear behaviour fell close to the elution volume of aldehyde dehydrogenase, as many standards as possible were used in this region, and an effective calibration curve was prepared in spite of the apparently anomalous behaviour of four of the ten standards. Molecular weights used for the standard proteins are shown in Table 5.1.

#### FIGURE 5.4

#### GEL ELECTROPHORESIS OF ALDEHYDE DEHYDROGENASE FOLLOWING NARROW GRADIENT ISOELECTRIC FOCUSING

The photograph illustrates samples of aldehyde dehydrogenase following narrow gradient isoelectric focusing, separated on a slab gel, with, as comparison, similar samples prior to electrofocusing. Fractions from the electrofocusing column were passed through Sephadex G-25 to remove ampholytes before gel electrophoresis. Samples and volumes applied to the gel from left to right were as follows:

wells 1 and 2	- fraction 16, 80 $\mu$ l
well 3	- fraction 51, 50 $\mu$ l
well 4	- fraction 56, 30 $\mu$ l
well 5	- fraction 62, 50 $\mu$ l
well 6	- fraction 72, 80 $\mu$ l
wells 7 and 8	- sample prior to electrofocusing

Fraction numbers refer to the electrofocusing run shown in Figure 5.3. Fraction 16 shows no protein stain following electrophoresis, indicating that the 280 nm absorbance observed in this region following the column run was not due to contaminating protein in the aldehyde dehydrogenase sample. Fractions 51, 56 (peak aldehyde dehydrogenase activity) and 62, which contained aldehyde dehydrogenase activity, show a single protein band in a similar position to that seen in samples prior to electrofocusing.

Fraction 72, with only very slight aldehyde dehydrogenase activity, shows two definite protein bands, indicating that the 280 nm absorbance seen in the region of this fraction following the electrofocusing run was due to contaminating protein in the enzyme sample. The dark bands at the base of the gel in wells 3, 4, 5 and 6 are due to traces of ampholytes still remaining after the Sephadex G-25 column treatment.

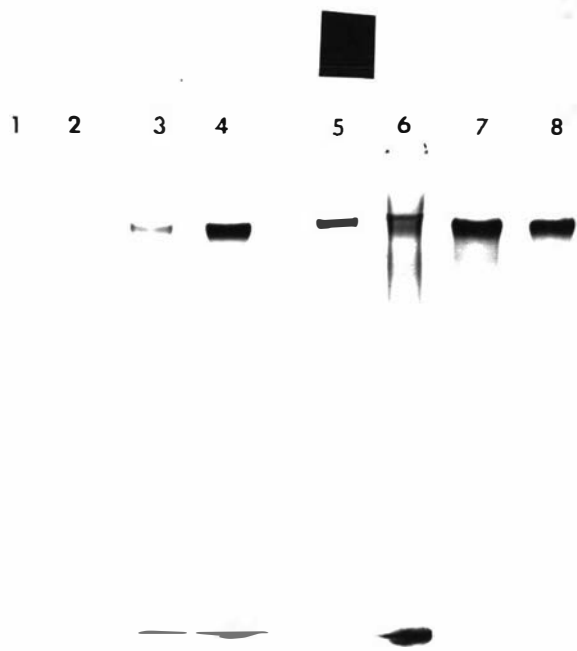
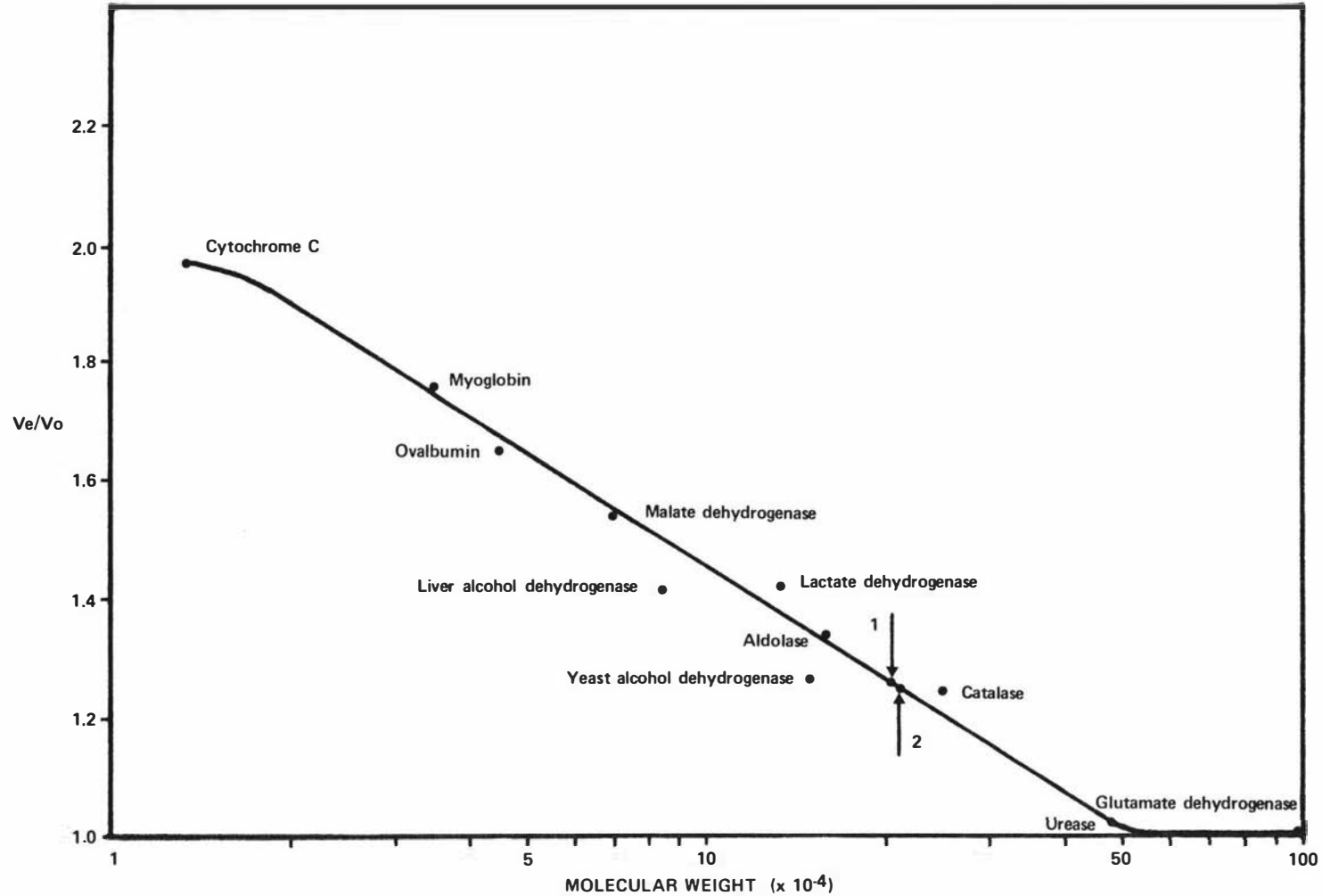


FIGURE 5.5 MOLECULAR WEIGHT CALIBRATION OF BIOGEL A 0.5M



Biogel A 0.5M was calibrated with standard proteins as described in Section 5.2.4. The molecular weights used for the proteins are shown in Table 5.1. The column used was a 70 x 2.5 cm Pharmacia column, run at a flow rate of 9 cm<sup>3</sup>/hour. The void volume was 154 cm<sup>3</sup>, and the total volume, 307 cm<sup>3</sup>. The elution buffer used for all runs was 0.05M phosphate, pH 7.3, containing 0.1% mercaptoethanol. The positions of the two aldehyde dehydrogenases are shown on the calibration curve marked (1) mitochondrial, and (2) cytoplasmic. The cytoplasmic enzyme position is a result of averaging 6 separate elution volumes, but the mitochondrial enzyme position was determined in only a single experiment.

TABLE 5.1

## MOLECULAR WEIGHTS OF STANDARD PROTEINS

## A. Calibration of Biogel - native molecular weights

Standard protein	Type	Source	Molecular weight	Reference
cytochrome C	horse heart	Sigma	11,700	1
myoglobin (dimer)	horse heart	Sigma	17,200 (34,400)	1
ovalbumin	egg white	Sigma	43,000	1
malate dehydrogenase	pig heart	Sigma	70,000	2
alcohol dehydrogenase	horse liver	Sigma	84,000	3
lactate dehydrogenase	rabbit muscle	Sigma	132,000	4
alcohol dehydrogenase	yeast	Sigma	151,000	3
aldolase	rabbit muscle	Sigma	156,500	5
catalase	bovine liver	Sigma	248,000	3
urease	soy bean	Sigma	480,000	3
glutamate dehydrogenase	bovine liver	Sigma	1,000,000	3

## B. Calibration of SDS gels - subunit molecular weights

Standard protein	Type	Source	Molecular weight	Reference
cytochrome C	horse heart	Sigma	11,700	1
carboxypeptidase A	bovine pancreas	Sigma	34,600	1
lactate dehydrogenase	rabbit muscle	Sigma	36,000	6
aldolase	rabbit muscle	Sigma	40,000	1
alcohol dehydrogenase	horse liver	Sigma	41,000	1
ovalbumin	egg white	Sigma	43,000	1
pyruvate kinase	rabbit muscle	Sigma	57,000	1
catalase	bovine liver	Sigma	60,000	1
serum albumin (dimer)	bovine	Fluka	68,000 (136,000)	1

## References:

- 1 - Weber and Osborn, 1969;      2 - Thorne and Kaplan, 1963;  
 3 - Dixon and Webb, 1964, pp 453 - 455;      4 - Fromm, 1963  
 5 - Castellino and Barker, 1968;      6 - Weber et al., 1972

5.3.3. Molecular weights of cytoplasmic and mitochondrial aldehyde dehydrogenases determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

Sodium dodecyl sulphate (SDS) gels were carried out using two different buffer systems, as described in Section 5.2.5. Determinations of subunit molecular weights of both cytoplasmic and mitochondrial aldehyde dehydrogenases were obtained simultaneously.

Figure 5.6.a shows a calibration curve obtained using the first buffer system. The graph appears to be slightly curved, particularly in the higher molecular weight region. The authors of the paper who described this method (Weber and Osborn, 1969) obtained a hyperbolic calibration curve when standards were used in the region of 50,000 to 100,000 molecular weight. Figure 5.7.a shows a photograph of an SDS gel obtained by this method. The subunit molecular weights used for standard proteins are shown in Table 5.1. Volumes of aldehyde dehydrogenase samples loaded onto the gel follow:

mitochondrial solutions	(a) 60 $\mu$ l
	(b) 30 $\mu$ l
cytoplasmic solutions	(a) 20 $\mu$ l
	(b) 40 $\mu$ l

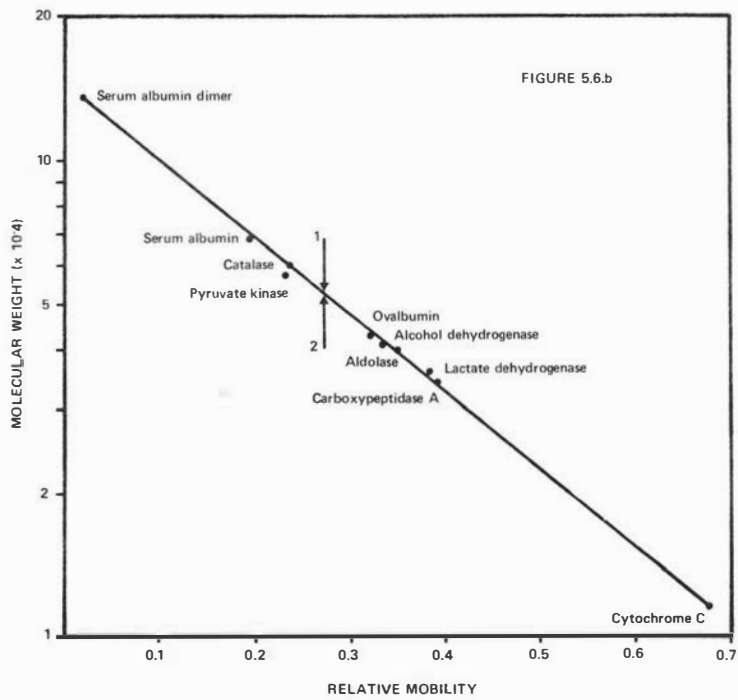
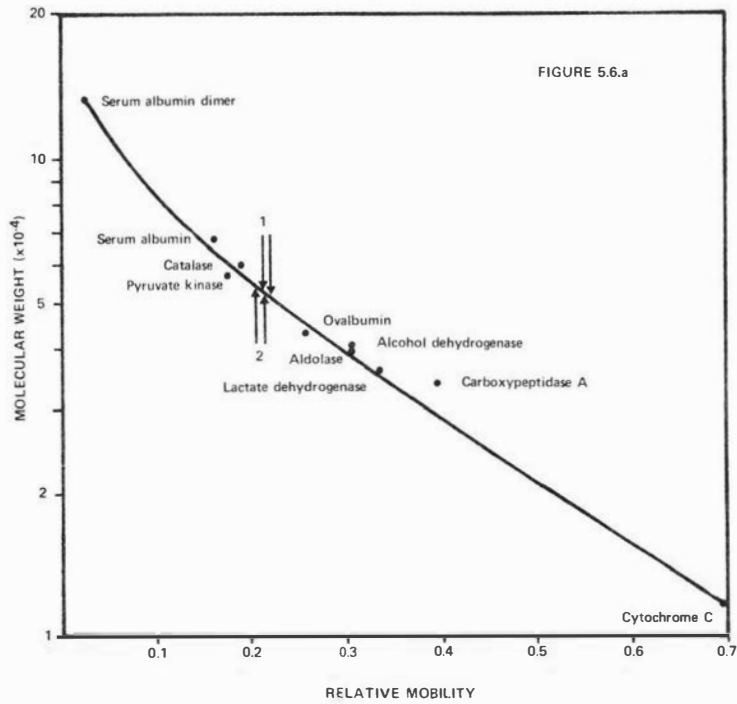
These volumes represented a total of 10.2  $\mu$ g of protein for each mitochondrial sample, and 10.4  $\mu$ g for the cytoplasmic samples. Single protein bands were observed on the gel for both mitochondrial and cytoplasmic aldehyde dehydrogenases (Figure 5.7.a).

The subunit molecular weights for the two enzymes calculated from the calibration curve were:

mitochondrial	(a) 52,000
	(b) 53,000
cytoplasmic	(a) 53,000
	(b) 55,000

Figure 5.6.b shows a calibration curve obtained by the second method used for running SDS gels. In this case the graph is linear even in the higher molecular weight region. Two runs were carried out by this method, with the calibration curve shown for the second run (Figure 5.6.b).

FIGURES 5.6a and b. CALIBRATION CURVES FOR SODIUM DODECYL SULPHATE - POLYACRYLAMIDE GEL ELECTROPHORESIS.



Calibration curves were prepared by running standard proteins as described in Section 5.2.5. Figure 5.6a shows the curve obtained using the first buffer system, and Figure 5.6.b that obtained for the second buffer system. The volumes of the standard protein solutions used are given with Figures 5.7.a and b. The positions of mitochondrial (1) and cytoplasmic (2) aldehyde dehydrogenases are marked by arrows, numbered appropriately, and the amounts of these enzymes applied to the gels are given in Section 5.3.3. The molecular weights of standard proteins are given in Table 5.1

In the first run samples were loaded as follows:

mitochondrial	(a) 60 $\mu$ l (10.2 $\mu$ g)
	(b) 60 $\mu$ l (20.4 $\mu$ g)
cytoplasmic	(a) 50 $\mu$ l (26.0 $\mu$ g)
	(b) 50 $\mu$ l (13.0 $\mu$ g)

Subunit molecular weights calculated from this run were:

mitochondrial	(a) 54,000
	(b) 55,000
cytoplasmic	(a) 51,500
	(b) 53,000

In the second run using this method, only one sample of each enzyme was applied to the gel, the amounts being 60  $\mu$ l (20.4  $\mu$ g) of mitochondrial sample b, and 60  $\mu$ l (31.2  $\mu$ g) of cytoplasmic sample a. The subunit molecular weights calculated from this gel were 52,000 for each enzyme. Figure 5.7.b shows a photograph of the gel. The amount of protein loaded was double in the case of the mitochondrial enzyme, and three times for the cytoplasmic enzyme, compared with those for the gel shown in Figure 5.7.a. With this increase in the amount of protein, several minor contaminants appeared. In the case of cytoplasmic enzyme there was one main contaminant band, and the subunit molecular weight for this band was calculated as 62,000, which corresponded closely to that of the standard, catalase. As described in Section 4, catalase was usually the major contaminant at the final stage of cytoplasmic enzyme preparations.

The average subunit molecular weights obtained from each method, the overall average, and the total molecular weight calculated assuming 4 subunits, are shown in Table 5.2.

Averaging the results from both methods gave a subunit molecular weight of  $53,100 \pm 2,000$  for both the mitochondrial and cytoplasmic enzymes. Apparent differences occurring between the two enzymes in some runs appeared to be due to random error, as the average results for each enzyme were identical. The molecular weight, calculated assuming there to be 4 subunits, agrees closely with that obtained for the native enzymes using gel filtration, particularly in the case of the cytoplasmic enzyme. Figure 4.7.c shows the electrophoretic pattern of the native protein for both cytoplasmic and mitochondrial samples corresponding to those used on SDS gels.

FIGURES 5.7.a, b and o

SODIUM DODECYL SULPHATE - POLYACRYLAMIDE GELS

Figures 5.7.a and b show SDS gels on which both standard proteins and aldehyde dehydrogenase samples were separated. Figure 5.7.o shows a native gel run on the same aldehyde dehydrogenase samples. The gels in Figures 5.7.a and b gave the calibration curves shown in Figures 5.6.a and b respectively. The samples and standards were loaded onto the gels as follows:

Figure 5.6.a

- well 1 - bovine serum albumin, lactate dehydrogenase, cytochrome C, 20  $\mu$ l.
- well 2 - mitochondrial sample (a), 60  $\mu$ l
- well 3 - cytoplasmic sample (a), 20  $\mu$ l
- well 4 - pyruvate kinase, aldolase, 20  $\mu$ l, liver alcohol dehydrogenase, 30  $\mu$ l.
- well 5 - mitochondrial sample (b), 30  $\mu$ l
- well 6 - cytoplasmic sample (b), 40  $\mu$ l
- well 7 - blank
- well 8 - catalase, 20  $\mu$ l, ovalbumin, carboxypeptidase, 30  $\mu$ l

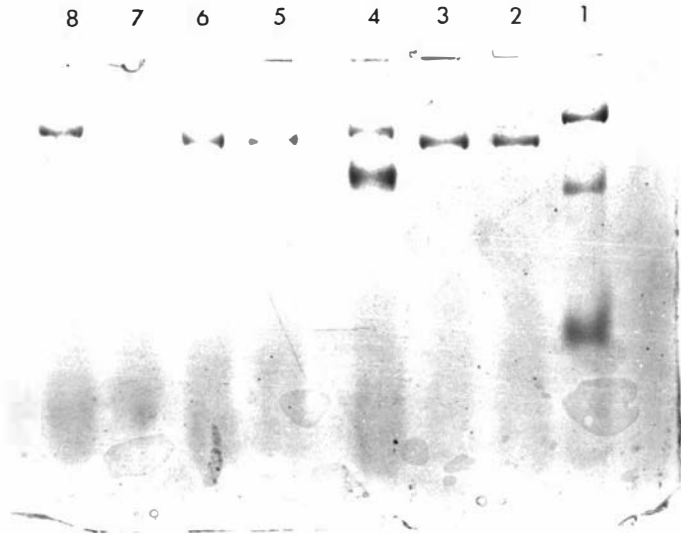
Figure 5.6.b

- well 1 - carboxypeptidase, 60  $\mu$ l
- well 2 - ovalbumin, 60  $\mu$ l
- well 3 - liver alcohol dehydrogenase, 60  $\mu$ l
- well 4 - pyruvate kinase, aldolase, 30  $\mu$ l
- well 5 - bovine serum albumin, cytochrome C, 30  $\mu$ l
- well 6 - mitochondrial sample (b), 60  $\mu$ l
- well 7 - cytoplasmic sample (a), 60  $\mu$ l
- well 8 - lactate dehydrogenase, catalase, 30  $\mu$ l

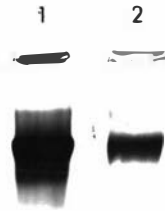
Figure 5.6.o

- well 1 - cytoplasmic enzyme, 200  $\mu$ g
- well 2 - mitochondrial enzyme, 68  $\mu$ g

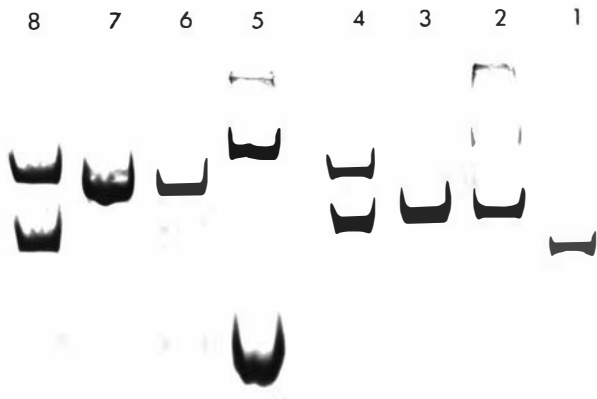
**a**



**c**



**b**



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TABLE 5.2

NATIVE AND SUBUNIT MOLECULAR WEIGHTS FOR  
CYTOPLASMIC AND MITOCHONDRIAL ALDEHYDE DEHYDROGENASES

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Results are shown for subunit molecular weights determined from SDS gels run in two different buffer systems (First and Second methods), and using two separately treated samples ( (a) and (b) ) of each enzyme. The variation shown is that between the maximum and minimum of the five values calculated for each enzyme. The native molecular weights for each enzyme determined by gel filtration are also shown.

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	Cytoplasmic enzyme	Mitochondrial enzyme
Subunit molecular weights		
First method	(a) 53,000	(a) 52,000
	(b) 55,000	(b) 53,000
Second method, run 1	(a) 51,500	(a) 54,000
	(b) 53,000	(b) 55,000
Second method, run 2	(a) 52,000	(b) 52,000
Average result, First method	54,000	52,500
Average result, Second method	52,200	53,700
Average result, Both methods	53,100	53,100
Variation	±2,000	±2,000
Molecular weight, calcu- lated assuming 4 subunits	212,400	212,400
Native molecular weight	212,000 (205-220,000)	205,000

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#### 5.3.4. Gel electrophoresis and activity staining.

##### (a) Gel electrophoresis of mitochondrial and cytoplasmic enzymes

As described in Section 4, gel electrophoresis of cytoplasmic aldehyde dehydrogenase at the final stage of purification gave a single major protein band, and the activity stain for aldehyde dehydrogenase coincided with this band.

Figure 5.8 shows typical activity stains and corresponding protein stains for both cytoplasmic and mitochondrial aldehyde dehydrogenase preparations.  $R_f$  values for protein and activity stains are shown in Table 5.3, as "Gel 1". While the total units of each enzyme applied to the gel in Figure 5.8 were similar, a very much lighter activity stain reaction was obtained for the mitochondrial enzyme. The  $R_f$  values for the two enzymes in the run represented in Figure 5.8 were similar but not identical (Table 5.3, Gel 1). A second gel (Table 5.3, Gel 2) using the same samples showed closer agreement in the  $R_f$  values. A larger volume of mitochondrial enzyme was loaded in the "Gel 1" experiment to obtain similar total units for each enzyme. This probably was responsible for the mitochondrial enzyme not running as far as the cytoplasmic enzyme.

No reason was found for the reproducible difference in the degree of colour yielded by the two enzymes. Both gave similar recovery of activity when diluted 10 fold in electrophoresis buffer, left 2 hours, then assayed. The mitochondrial enzyme retained 85% of its activity under these conditions, and the cytoplasmic enzyme, 70%. The use of tris-borate buffer did not therefore explain the difference in degree of staining. Attempts to extract the enzymes from gels after electrophoresis were unsuccessful. Neither cytoplasmic nor mitochondrial activity could be detected either in supernatants from gels homogenised in buffer, or in buffers in which gels were soaked overnight. If the activity stain was carried out in solution, both enzymes reacted equally well, indicating that the mitochondrial enzyme could undergo the reaction as fully as the cytoplasmic enzyme when not in a gel

(b) Aldehyde dehydrogenase colour reaction in the absence of added substrate.

If either  $\text{NAD}^+$  or acetaldehyde, or both these substrates, were omitted from the mixture used for staining gels for aldehyde dehydrogenase activity, a coloured band was still observed corresponding to the

FIGURE 5.8

ACTIVITY STAIN FOR ALDEHYDE DEHYDROGENASE

The gel shown in Figure 5.8 illustrates two features of the aldehyde dehydrogenase activity stain - the lesser ability of the mitochondrial enzyme to react, and the production of a colour reaction in the absence of added substrate.

The gel was divided into four sections, which numbered from the left show:

1 - Mitochondrial (a) and cytoplasmic (b) enzymes reacted with the complete activity stain mixture.

2 - Mitochondrial (a) and cytoplasmic (b) enzymes, with  $\text{NAD}^+$  omitted from the activity stain mixture.

3 - Mitochondrial (a) and cytoplasmic (b) enzymes, with acetaldehyde omitted from the activity stain mixture.

4 - Mitochondrial (a) and cytoplasmic (b) enzymes, stained with Amido black to show the corresponding protein bands.

The amounts of each enzyme applied to the gel are given in Table 5.3, where this gel is described as "Gel 1".

ACTIVITY STAIN FOR ALDEHYDE DEHYDROGENASE

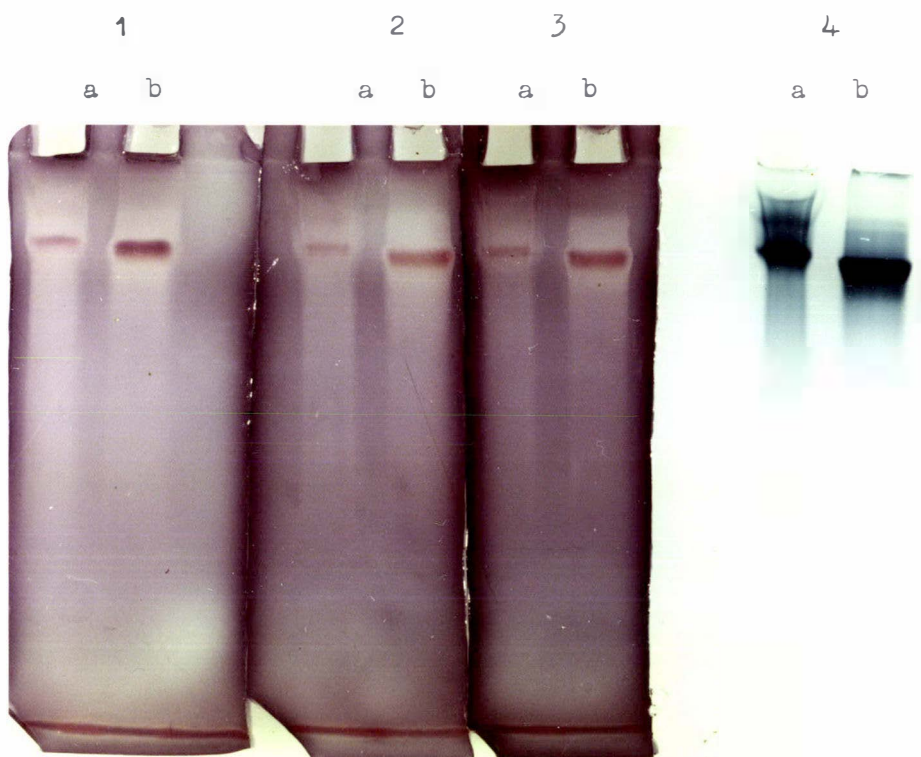


TABLE 5.3

GEL ELECTROPHORESIS AND ACTIVITY STAINING OF  
MITOCHONDRIAL AND CYTOPLASMIC ALDEHYDE DEHYDROGENASES

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The Table shows levels of protein and activity loaded on two gels. Gel 1 is shown in Figure 5.8. Gel 2 was run using the same samples as for gel 1, but with a smaller loading of mitochondrial enzyme.  $R_f$  values were calculated using the distance moved by a dye front and the distance moved by the sample, as measured from the bottom of the sample well to the centre of the protein or activity band. The activity is given in absorbance units.

---

Sample	Activity (units/cm <sup>3</sup> )	Protein (mg/cm <sup>3</sup> )	Activity loaded units	Protein loaded μg	$R_f$	
					Activity	Protein
<u>Gel 1</u>						
cytoplasmic enzyme	1500	5.2	60.0	200	0.16	0.17
mitochondrial enzyme	425	1.7	42.0	170	0.14	0.15
<u>Gel 2</u>						
cytoplasmic enzyme	1500	5.2	60.0	200	0.15	0.15
mitochondrial enzyme	425	1.7	17.0	68	0.15	0.15

---

major protein band. This is illustrated by Figure 5.8. Some further study was carried out in an attempt to explain the reactions involved. It was finally concluded that the colour occurring in gels in the absence of substrate was probably due to a reagent-enzyme complex formed between the dye phenazine methosulphate (PMS) and sulphhydryl groups on the enzyme. The observations that led to this conclusion are outlined below.

(1) Colour of the aldehyde dehydrogenase activity stain.

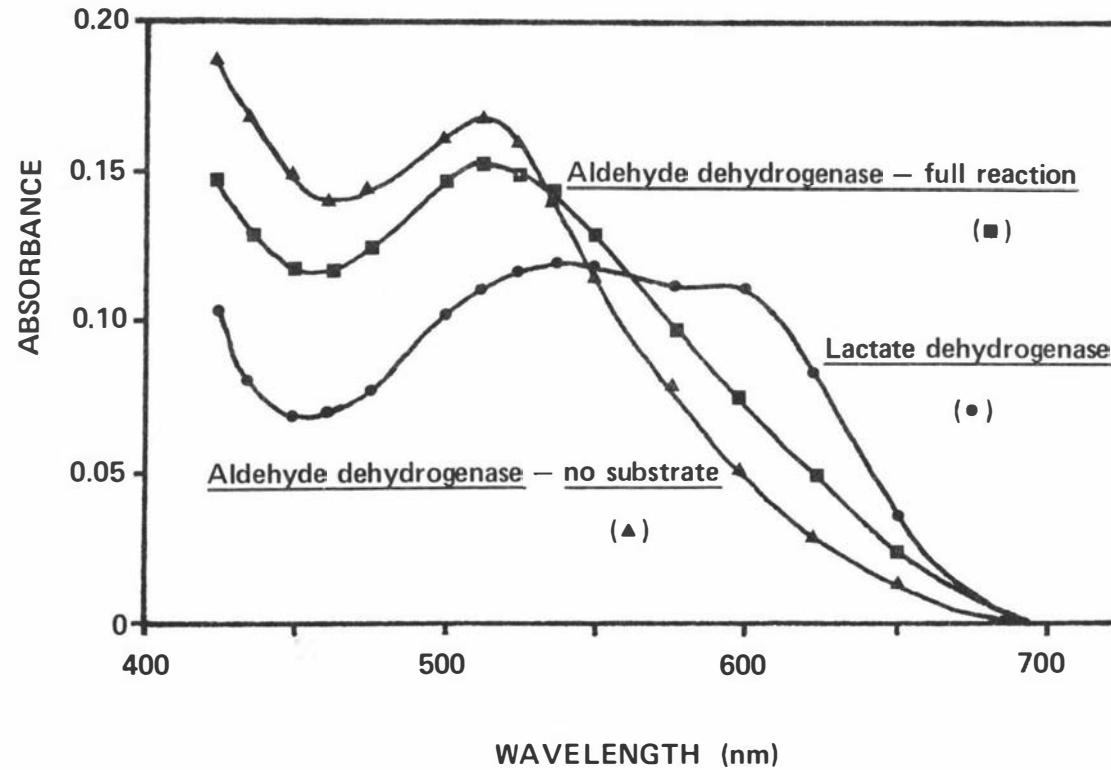
It was noted that the band formed in the absence of either or both substrates was a different colour from that seen when all reagents were present, being red, rather than purple. The red band tended to intensify in colour when gels were placed in 10% acetic acid for storage and also seemed to be of equal intensity throughout the entire gel thickness, whereas the darker purple full activity stain remained at the surface of the gel.

Aldehyde dehydrogenase gave a different colour with this activity stain from that given by lactate dehydrogenase. The red colour appeared to form even in the presence of all substrates, and contributed to the final colour of the activity stain. In Figure 5.8, the background colour of the gel is the same as that given by the lactate dehydrogenase activity stain. The difference in colour between the two activity stains is illustrated also by Figure 5.9 where scans of each stain are shown. The lactate dehydrogenase activity stain has a broad absorption peak from 520 - 560 nm, and a shoulder in the 600 nm region, the aldehyde dehydrogenase full reaction had a peak at 515 nm, and a very slight shoulder at about 550 - 600 nm, and the aldehyde dehydrogenase reaction without substrate showed a single peak at 510 nm.

(2) Reactions of cytoplasmic and mitochondrial aldehyde dehydrogenases with dye reagents in solution.

Reactions with both cytoplasmic and mitochondrial aldehyde dehydrogenases were carried out in solution as well as in gels. The results listed below were the same for both enzymes. Solutions of all reagents were made up as for normal activity stains, and the volumes used follow:

FIGURE 5.9 ABSORPTION SPECTRA OF ACTIVITY STAINS ON GELS



Slab gels were cut and fitted into a gel scanner on a Beckman ACTA-3 spectrophotometer. The gel was positioned at the point of maximum absorbance at a fixed wavelength between 500 and 600 nm, to ensure that the activity stain was directly in the light path, then was scanned over the range 400 - 700 nm.

phenazine methosulphate	0.2 cm <sup>3</sup>
nitroblue tetrazolium	0.2 cm <sup>3</sup>
NAD <sup>+</sup>	0.2 cm <sup>3</sup>
buffer	0.4 cm <sup>3</sup>
acetaldehyde	0.4 cm <sup>3</sup>
enzyme	0.1 cm <sup>3</sup>

The results for reactions in solution showed that:

(a) Mercaptoethanol present in the enzyme sample was sufficient to reduce rapidly the activity stain reagents. For all further work in solution, mercaptoethanol was removed from the enzyme sample by dialysis. Reactions on gels were checked using dialysed enzyme, and it was found that the removal of mercaptoethanol did not alter the results.

(b) With all reagents present, both mitochondrial and cytoplasmic enzymes gave an equally rapid reaction from a clear yellow to a dark purple solution.

(c) With either or both the substrates NAD<sup>+</sup> and acetaldehyde omitted a slow reaction to give a purple colour occurred over several hours.

(d) With PMS omitted, the solution remained clear yellow, and with NBT omitted, a clear green colour.

(e) When reaction mixtures giving a purple colour were centrifuged all the coloured compound was precipitated. The precipitate was insoluble in 10% acetic acid, and partially soluble in ethanol. The ethanol soluble component was reddish-purple in colour, and the ethanol-insoluble component, blue-black. There was more of the ethanol-soluble component present in reactions occurring in the absence of one or both substrates than in the full reaction. A scan of the ethanol soluble component showed an absorption peak at 520 nm, and a shoulder at 560 nm.

### (3) Reaction of dye reagents with reducing agents.

NBT and PMS were reacted with the reducing reagents sodium borohydride and mercaptoethanol, in the absence of enzyme. Results were as follows:

(a) NBT, reduced by either concentrated mercaptoethanol (1 M) or sodium borohydride, and extracted into ethanol, gave an absorption spectrum with a peak at 520 nm and a shoulder at 560 nm. The same spectrum was obtained from NBT and PMS, left in buffer for several hours and then extracted into ethanol.

(b) PMS reduced by sodium borohydride gave a colourless, cloudy precipitate, which changed to a clear blue colour over several hours. Reaction of PMS with mercaptoethanol (1 M) gave a cloudy precipitate, which changed over several hours to give an orange-red colour. Centrifugation of the mixture produced a precipitate, which was soluble in 10% acetic acid, and the resultant red solution showed a single absorption maximum at 510 nm.

(4) Correlation of results from gels and reactions in solution.

From the results listed above, which are summarised in Table 5.4, it appeared that the closest correlation of the absorption spectrum of the reaction on gels was given by the absorption spectrum of the product of PMS reacting with mercaptoethanol. This led to the conclusion stated earlier, that the red band seen on gels was a product of PMS reacting not with mercaptoethanol, but directly with the enzyme, the common factor in the two reactions being the presence of sulphhydryl groups. The presence of sulphhydryl groups in aldehyde dehydrogenases is well documented (Jakoby, 1963).

Further attempts to produce the 510 nm absorbing compound by direct reaction between PMS and aldehyde dehydrogenase in solution were not successful - probably a sufficiently high ratio of enzyme to PMS was not achieved with the quantities of enzyme available. Demonstration of a direct reaction between PMS and enzyme would be essential to provide confirmation of the explanation proposed for the appearance of a coloured band on gels in the absence of substrate. However, since finding suitable reaction conditions to produce the enzyme-PMS complex in solution could have required considerable time and enzyme, the problem was not pursued further at this stage.

The ethanol-soluble compound which was formed in greater quantity in reactions without substrate (2e) was initially considered as a possible explanation for the band appearing on gels in the absence of substrate. This explanation appeared unlikely, however, when it was found that the absorption spectrum of the ethanol-soluble compound did not correspond to that of the red band on gels. A possible explanation for the increased amounts of the ethanol-soluble compound appearing in reactions without substrate is suggested in Section 6, below.

TABLE 5.4

ABSORPTION MAXIMA OF COMPOUNDS FORMED FROM PMS AND NBT  
WITH AND WITHOUT ALDEHYDE DEHYDROGENASE AND SUBSTRATES

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The table summarises results obtained after scanning the products formed on reaction of PMS and NBT with various combinations of reagents in solution and on gels. "Reaction with substrates" refers to the complete reaction mixture containing  $\text{NAD}^+$  and acetaldehyde, and "reaction without substrates" refers to mixtures not containing  $\text{NAD}^+$  or acetaldehyde. When reactions were carried out in solution, the mixtures were centrifuged after several hours, and the precipitates dispersed in 10% acetic acid. Material insoluble in acetic acid was removed by centrifugation, and dissolved in ethanol. Ethanol-insoluble material was removed by a third centrifugation. Supernatants from each centrifugation were scanned using a Perkin Elmer model 124 spectrophotometer. Gels were stored in 10% acetic acid once reaction with the activity stain reagents was complete.

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Sample	Absorption Maxima (nm)	
	10% acetic acid	absolute ethanol
<u>reactions in solution</u>		
reaction with substrates	-	520, shoulder 560
reaction without substrates	-	520, shoulder 560
NBT + $\text{NaBH}_4$ or mercapto ethanol	-	520, shoulder 560
NBT + PMS, in buffer	-	520, shoulder 560
PMS + mercaptoethanol	510	-
PMS + $\text{NaBH}_4$	-	-
<u>reactions on gels</u>		
lactate dehydrogenase	520-560 (broad peak) shoulder, 600 region	
aldehyde dehydrogenase reaction with substrates	515, slight shoulder, 550-600 region	
aldehyde dehydrogenase reaction without substrates	510	

---

(5) Source of the ethanol soluble component of reduced NBT.

The appearance of an ethanol-soluble, red-purple compound in enzyme reactions in solution, and on reduction of NBT, was probably explained by the presence of a monotetrazolium contaminant in the ditetrazolium salt, NBT. Contamination of ditetrazolium salts by their monotetrazolium equivalent commonly occurs (Karmarkar *et al.*, 1959). In the case of NBT, the monoformazan resulting from reduction of the monotetrazolium salt is red, and has an absorption maximum at 520 nm (Tsou *et al.*, 1956). The diformazan produced on reduction of the ditetrazolium salt is blue-black in colour, and has an absorption maximum at approximately 550 nm (Dawson *et al.*, 1969). It is less soluble than the monoformazan (Tsou *et al.*, 1956). The ethanol soluble compound seen in this work was probably due predominantly to monoformazan, which contributed the 520 absorption maximum, with some diformazan present to contribute the shoulder at 560 nm.

(6) Possible reason for increased amounts of ethanol soluble compound appearing in reactions without substrate.

It is possible that the reaction occurring without substrate, in solution, was due to the high pH of the buffer used, rather than to the reagent-enzyme complex which appeared to cause the red band on gels. The use of pH 7.0 buffer, rather than a more alkaline pH, prevents darkening of gels stained by this method (Feinstein and Cameron, 1972). The monotetrazolium salt may be more easily reduced by this means than the ditetrazolium salt, whereas both were probably reduced with equal ease in the full reaction mixture. This could have given rise to a greater proportion of the ethanol-soluble monoformazan in reactions catalysed by the high pH, than in reactions catalysed by aldehyde dehydrogenase in the presence of both substrates.

#### 5.3.5. Stability of cytoplasmic aldehyde dehydrogenase to freezing.

Results of tests on the stability of aldehyde dehydrogenase to freezing are shown in Table 5.5. Sample A, which was an enzyme preparation prior to gel filtration, was more stable when frozen with  $\text{NAD}^+$  or ammonium sulphate than enzyme at the final stage of purification. Both samples were completely stabilised by freezing in glycerol, and completely inactivated when frozen untreated or with acetaldehyde.

TABLE 5.5

STABILITY OF ALDEHYDE DEHYDROGENASE TO FREEZING

Samples of enzyme (5cm<sup>3</sup>) from the final and pre-gel filtration steps of purification were treated as described, and frozen at -20°C for 4 weeks. All samples were then thawed and assayed, together with a sample stored at 4°C. Units of activity are absorbance units, and recoveries are calculated as the percentage of the total activity in the initial sample still present in the treated samples.

Sample	Treatment	Total Units	Percent Recovery
<b>Initial samples</b>			
A	Pre-Biogel	2,550	100
B	Post-Biogel	1,200	100
<b>Frozen samples - pre-biogel</b>			
A1	Untreated	0	0
A2	17% (v/v) glycerol	2,600	102
A3	9mM NAD <sup>+</sup>	950	37
A4	4mM acetaldehyde	0	0
A5	50% satd. ammonium sulphate	2,100	82
<b>Frozen samples - post-biogel</b>			
B1	Untreated	0	0
B2	17% (v/v) glycerol	1,200	100
B3	28% (v/v) glycerol	1,250	104
B4	9mM NAD <sup>+</sup>	50	4
B5	50% satd. ammonium sulphate	600	50
B6	4mM acetaldehyde	0	0
B7	1mM zinc sulphate	0	0
<b>Refrigerated sample - post-biogel</b>			
B8	Untreated	1,100	92

When samples A2 and B3 were dialysed free of glycerol and re-assayed, considerable loss of activity occurred. After 2 days dialysis against 0.05 M phosphate buffer, pH 7.3, containing 0.1% mercaptoethanol, 80% of the activity of A2 remained, and only 60% of the activity of B3. Sample B2 was refrozen for a further 8 weeks, and after this time, only 37% of the activity remained.

Cytoplasmic aldehyde dehydrogenase could be frozen in 0.05 M phosphate buffer and re-thawed immediately with only a small loss in activity. A total of 92% of the initial activity was retained in a sample from the pre-Biogel stage of the preparation following this treatment. With subsequent freeze-drying, however, loss of activity increased. After freeze-drying overnight, and redissolving in buffer containing mercaptoethanol (0.1%), the same sample retained only about 10% of its activity. If the enzyme was redissolved in buffer without mercaptoethanol, complete deactivation occurred. With freeze-drying for 2 hours, until the sample was just dry, and redissolving in buffer containing mercaptoethanol, 70% of the original activity could be retained. Long-term tests on the stability of freeze-dried enzyme to storage were not carried out as the initial losses in activity indicated that this method of storing the enzyme would not give a high retention of activity.

The most suitable means of storage of the enzyme appeared from the above results to be storage in 0.05 M phosphate buffer, pH 7.3, containing 0.1% mercaptoethanol, at 4°C. Freezing in glycerol gave good initial recoveries, but losses during dialysis to remove the glycerol decreased the yield. Initial losses in activity on freeze-drying prevented use of this technique, so enzyme required for acet-aldehyde assays and kinetic work was prepared as fresh as possible, and stored at 4°C until required. Samples could be kept for up to three months in this way, with an overall loss of about 50% of their initial activity, but the usual time of storage was for up to one month, when only small losses in activity occurred.

## 5.4 DISCUSSION

### 5.4.1. Native and subunit molecular weights for mitochondrial and cytoplasmic aldehyde dehydrogenases.

Mitochondrial and cytoplasmic aldehyde dehydrogenases from sheep liver appear to have nearly identical molecular weights as determined by gel filtration. The two enzymes also have identical subunit molecular weights determined from experiments with SDS-polyacrylamide gel electrophoresis. Each enzyme gave a single major band on SDS gels, and the subunit molecular weights calculated were  $53,100 \pm 2,000$  in each case. Results from gel filtration showed a molecular weight of  $212,000 \pm 8,000$  for the cytoplasmic and  $205,000$  for the mitochondrial enzyme. The results thus indicated that each enzyme probably consists of four subunits of equal molecular weight. A urea gel showed a single band for cytoplasmic aldehyde dehydrogenase, indicating that this enzyme probably consisted of four subunits identical in charge as well as in molecular weight (T.Braggins, personal communication). Urea gels have not as yet been run for mitochondrial enzyme, but studies of the behaviour of the two enzymes on these gels are continuing.

The molecular weights found in this work were similar to those found for horse liver aldehyde dehydrogenase (Feldman and Weiner, 1972),  $245,000 \pm 20,000$  for the native enzyme, and a subunit molecular weight of  $57,000 \pm 6,000$ . The isoelectric point for sheep liver cytoplasmic aldehyde dehydrogenase (5.05 - 5.25) was also similar to that from horse liver (5.05). It is not clear whether the horse liver enzyme is cytoplasmic or mitochondrial in origin. The only other direct comparison of molecular weights of mitochondrial and cytoplasmic aldehyde dehydrogenases (Marjanen, 1973) showed that in rat liver these two enzymes had different molecular weights, 180,000 and 110,000 respectively. Another recent, brief report on the rat liver mitochondrial enzyme, however, claimed that there are two mitochondrial aldehyde dehydrogenases, with molecular weights of 62,500 and 275,000 (Siew et al., 1974).

#### 5.4.2. Activity staining of aldehyde dehydrogenase.

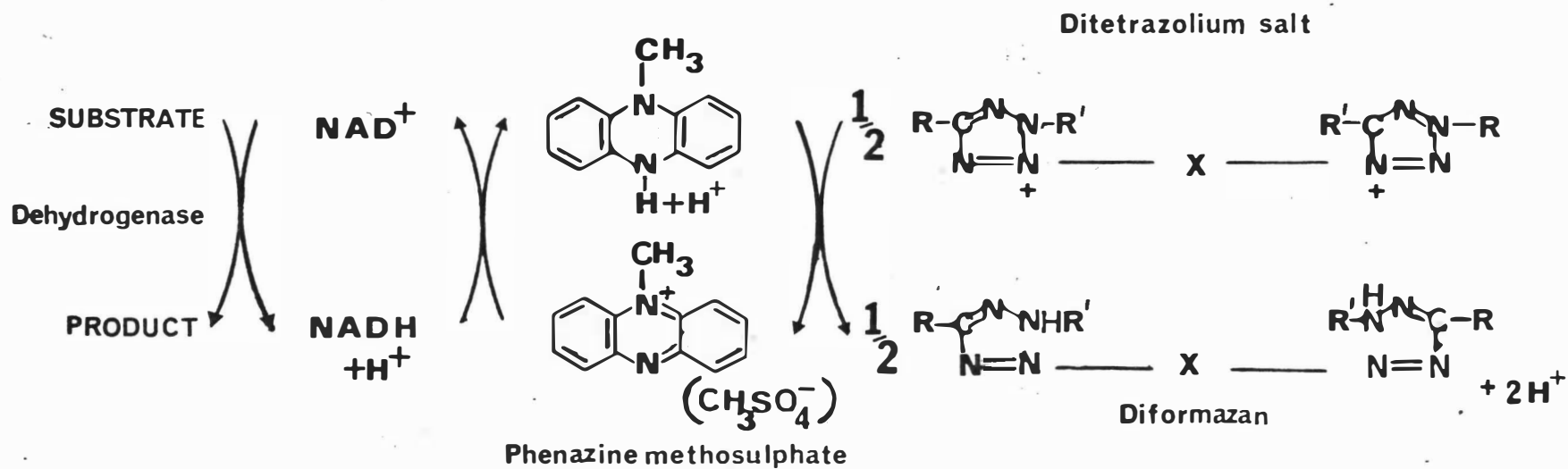
There have been varied reports on the activity staining of aldehyde dehydrogenase by the PMS-NBT technique. Robbins (1966), who first used this technique to stain for aldehyde dehydrogenase, successfully stained the beef liver enzyme, and Marjanen (1973) obtained activity stains for both mitochondrial and cytoplasmic enzymes from rat liver. Feinstein and Cameron (1972) also obtained an aldehyde dehydrogenase activity stain for the rat liver enzyme, obtaining a single band of activity from homogenates of normal rat liver. However, Duncan and Tipton (1971) failed to produce an activity stain using pig brain aldehyde dehydrogenase. In the work reported here, aldehyde dehydrogenase from sheep liver has been successfully stained by this technique, although two anomalies were observed. Firstly, the mitochondrial enzyme showed a weak response to the stain, and secondly, the colour of the activity band varied from that given by other dehydrogenases.

The reason for the poor response of mitochondrial enzyme to the activity stain was not determined. The enzyme could be successfully reacted with the dye reagents in a test tube, and was not deactivated by the buffer used in gel electrophoresis. It appeared, therefore, that the polyacrylamide gel itself in some way inhibited the mitochondrial enzyme. Marjanen, who successfully stained both mitochondrial and cytoplasmic enzymes from rat liver, used starch rather than polyacrylamide gels. The results of Duncan and Tipton (1971) who failed to find an activity stain for pig brain aldehyde dehydrogenase may have been due to their enzyme being mitochondrial in origin. The majority of activity from bovine brain appears to be mitochondrial (Erwin and Deitrich, 1966), so that the same may apply to pig brain.

The sequence of reactions involved in the PMS-NBT staining technique is shown in Figure 5.10. It is apparent that the final product should be the same regardless of the initial source of NADH. The different colour given by aldehyde dehydrogenase was probably explained by the contribution to the colour of the final activity stain of a reagent-enzyme complex forming between aldehyde dehydrogenase and phenazine methosulphate.

Phenazine methosulphate reaction with sulphhydryl groups, including sulphhydryl enzymes, has been mentioned previously (Singer and Kearney, 1957), but no evidence for the reaction was quoted, and no mechanism postulated.

FIGURE 5.10 REACTION SEQUENCE FOR PHENAZINE METHOSULPHATE-NITROBLUE TETRAZOLIUM ACTIVITY STAIN



X - 3,3<sup>1</sup>(CH<sub>3</sub>O)<sub>2</sub> - 4,4<sup>1</sup>- biphenylene

R - C<sub>6</sub>H<sub>5</sub>

R' - C<sub>6</sub>H<sub>4</sub>NO<sub>2</sub><sup>-P</sup>

Nitroblue tetrazolium

References - reduction of phenazine methosulphate, King, 1963;  
ditetrazolium salt, Tsou et al., 1956

The possibility of the reaction on gels being due to aldehyde oxidase, rather than to aldehyde dehydrogenase, as suggested by Feinstein and Lindahl (1973) was discounted because reaction of enzyme on gels in the presence of acetaldehyde, FMS and NBT showed less colour than with all the reagents present, or than with  $\text{NAD}^+$ , FMS, and NBT.

Activity occurring in the absence of substrate has been reported in various electrophoretic media for other dehydrogenases, for example for lactate dehydrogenase, by Robbins (1966), Barnett (1964), Graymore, (1965) and Shaw and Koen (1965), and for alcohol dehydrogenase by Shaw and Koen in the previous reference. Lactate dehydrogenase activity in the absence of added lactate was also observed during staining of aldehyde dehydrogenase samples in this work. The colour developed for lactate dehydrogenase activity in the absence of added substrate was the normal colour for the activity stain, and other references have made no mention of different colours being developed in reactions occurring without substrate. The explanation for the above cases of lactate dehydrogenase activity occurring in the absence of known added substrate appeared to be the presence of lactate in the enzyme samples, as the reactions appeared to be dependant on  $\text{NAD}^+$  (Barnett, 1964; Graymore, 1965). Some mention was made in an earlier paper by Wieme (1963) of Barnett having found two types of "nothing reaction" with lactate dehydrogenase. One such type was said to be independent of  $\text{NAD}^+$ , and was thought to be due to direct reduction of tetrazolium salts by sulphhydryl groups on the enzyme, but this explanation appeared to be disregarded by Barnett in a later reference (Barnett, 1964). Such reduction of NBT by sulphhydryl groups would still yield the same final colour in the activity stain, and was not considered to be a likely explanation for the aldehyde dehydrogenase activity in the absence of substrate.

The reason for a FMS-protein reaction occurring with aldehyde dehydrogenase, but not apparently with other dehydrogenases, is not known, but might be due to the large amounts of protein that were used to obtain activity stains for aldehyde dehydrogenase. A positive activity stain for lactate dehydrogenase often occurred in the presence of barely detectable amounts of protein. If production of formazan due to endogenous substrate, which occurs even when using crystalline lactate dehydrogenase (Robbins, 1966) could be prevented, and if a

large amount of lactate dehydrogenase was loaded onto gels, then possibly a red sulphdryl-IMS product might be seen.

The production of a different coloured activity stain for aldehyde dehydrogenase has not previously been reported, but Robbins (1966), who first used the stain, and Feinstein and Cameron (1972), used only crude preparations of aldehyde dehydrogenase, in which the colour difference might not have been as marked, and Marjanen (1973) who did use purified enzyme, studied this on starch gels rather than polyacrylamide gels, so that any colour difference might not have been observed. All these workers also used a lower pH than was used in this work, particularly Feinstein and Cameron (1972) who stained their gels at a pH as low as 7.0, and this could have prevented the formation of the proposed dye-enzyme complex.

#### 5.4.3. Comparison of mitochondrial and cytoplasmic aldehyde dehydrogenases.

From the results reported in this section, the only significant difference noted between mitochondrial and cytoplasmic aldehyde dehydrogenases was the lesser ability of the mitochondrial enzyme to produce an activity stain on polyacrylamide gels. Other differences have been found between the two enzymes, however, by T.M. Kitson and A. MacGibbon, who are studying the kinetic of the enzymes. Some of these differences were reported in a recent publication (Crow *et al.*, 1974) while further results are being submitted for publication. Briefly, the differences noted between mitochondrial and cytoplasmic aldehyde dehydrogenases are:

- (a) The two enzymes have different  $K_M$  values for  $NAD^+$
- (b) The specificities of the two enzymes are significantly different with respect to benzaldehyde, glyceraldehyde, and glycolaldehyde.
- (c) The sensitivity of the two enzymes to steroid hormones differs.
- (d) Phosphate causes a different degree of activation with each enzyme.
- (e) The sensitivity of the enzymes to disulfiram (tetraethylthiuram disulphide - used as a deterrent to drinking in the treatment of alcoholics) differs.

It would appear that the differences between the two enzymes are sufficient to affect activity towards some substrates and inhibitors, but not to affect the overall charge or molecular weight of the enzymes. The only other comparative results available in the literature for mitochondrial and cytoplasmic aldehyde dehydrogenases are for rat liver. Kinetic and specificity differences were noted for the rat liver enzymes, as well as the molecular weight differences already quoted (Marjanen, 1973). Sensitivity of the enzymes to steroid hormones was not studied by Marjanen, but another paper, specifically on mitochondrial aldehyde dehydrogenase from rat liver, noted that it was insensitive to steroid hormones (Grunnet, 1973). Sensitivity to steroid hormones has only been reported for one aldehyde dehydrogenase apart from the sheep liver enzyme. Maxwell and Topper (1961) reported that the enzyme from rabbit liver was sensitive to, among other related compounds, progesterone, and the steroid hormone analogue, diethylstilbestrol. The intracellular source of the rabbit liver enzyme was not determined, but the results found were very similar to those for cytoplasmic aldehyde dehydrogenase from sheep liver.

A brief report noted following completion of this section of the thesis gives close agreement with results for characterisation of mitochondrial and cytoplasmic aldehyde dehydrogenases. Eckfeldt *et al.* (1975) reported two aldehyde dehydrogenase isozymes from horse liver, both with native molecular weights of 200 - 250,000 and subunit molecular weights between 52,000 and 53,000, but with differences in their  $K_M$  values for  $NAD^+$  and acetaldehyde and in their sensitivity to disulfiram. Preliminary localisation experiments by these workers indicated that one enzyme was primarily cytosolic, the other mitochondrial.

## SECTION 6

THE DEVELOPMENT OF AN ENZYMIC ASSAY FOR ACETALDEHYDE USING  
SHEEP LIVER ALDEHYDE DEHYDROGENASE6.1 INTRODUCTION

Reported methods for the assay of acetaldehyde in blood and tissue samples include chemical, enzymic and gas chromatographic procedures. Chemical methods were the first to be developed, and the two most widely used have been those of Stotz (1943) and Burbridge et al., (1950). The method developed by Stotz was based on the reaction of acetaldehyde with p-hydroxybiphenyl in concentrated sulphuric acid solution, and used a special distillation apparatus which made the method inconvenient for routine use. The chemical method developed by Burbridge et al. depended on the diffusion of acetaldehyde into a semicarbazide solution using Conway diffusion units. This method gave positive results with acetoacetate (Lundquist, 1958) which, under the conditions of the assay, yielded acetone by decarboxylation, forming an acetone semicarbazone.

Several enzymic methods for the determination of acetaldehyde have been described. One, which used yeast alcohol dehydrogenase, was initially described by Holzer et al. (1955) and also by Racker (1957) and Bergmeyer (1965). This method was of limited use when large amounts of alcohol were present, since the equilibrium of the enzyme favours formation of ethanol. In addition, the method was inaccurate at low levels of acetaldehyde, because it depended on the measurement of a small decrease in a large absorbance from NADH (Lundquist, 1958). A method using yeast aldehyde dehydrogenase was developed by Lundquist (1958) and although this appeared to be a satisfactory method, the author noted that the ox liver enzyme used by Racker (1957) would be preferred because its low  $K_M$  for acetaldehyde would simplify the assay of this compound. Despite this stated preference, Lundquist did not apply the mammalian enzyme successfully for assays, primarily because preparations of the enzyme contained lactate and malate dehydrogenases, which interfered in the assays.

Duritz and Truitt (1964) published the first method for the simultaneous determination of acetaldehyde and ethanol in blood, using gas chromatography. Because of its rapidity, specificity, and apparent simplicity, this became the most commonly used method during the following 10 years. The original technique, which involved analysis of head space gas over a deproteinised blood sample, was varied considerably. One modification used a blood sample placed on a paper disc before equilibration with head space gas (Boiteau and Mousson, 1968). Other variations included the direct injection of deproteinised supernatants into the gas chromatograph (Roach and Creaven, 1968), and the direct injection of whole blood (Baker *et al.*, 1969). In 1970, Truitt published a further report which showed that his original method required modification to avoid artificially high levels of acetaldehyde due to its production from blood precipitates in the presence of ethanol.

When the gas chromatographic estimation of acetaldehyde was introduced in this laboratory by K. Couchman numerous problems were encountered, and these are summarised as follows:

(a) One of the most commonly used gas chromatographic column packings, Porapak Q, was found to bind acetaldehyde, and acetaldehyde peaks could be eluted from a column previously used for acetaldehyde estimations following subsequent injections of distilled water.

(b) Another column coating, Carbowax 1500, also commonly used for such estimations, was found to give acetaldehyde as a decomposition product.

(c) The injection of whole blood onto the column resulted in acetaldehyde binding to charred deposits remaining in the injector port from previous injections.

(d) The use of samples on filter paper to equilibrate with head space gas failed to give reproducible results as the amount of acetaldehyde finally injected was very small.

(e) The direct injection of supernatants of deproteinised blood samples gave results which were not reproducible to the degree required for accurate studies, again because of the extremely low levels of acetaldehyde.

(f) Both acetaldehyde production and disappearance were observed in blood samples under different conditions.

Because of such problems, it was considered essential to develop an alternative assay which would complement results obtained using gas chromatographic methods for acetaldehyde assays. Since sheep liver aldehyde dehydrogenase had been isolated (Section 4), it was decided to attempt to develop an assay for acetaldehyde using this enzyme. Lundquist (1958) had found ox liver aldehyde dehydrogenase unsuitable for the assay of acetaldehyde, as already noted. Although sheep liver aldehyde dehydrogenase was free of malate dehydrogenase, lactate dehydrogenase was known to contaminate enzyme preparations (Section 4), and it was therefore decided that a diffusion assay should be developed to avoid lactate from blood samples coming into contact with the assay reaction mixture.

## 6.2 METHODS

### 6.2.1. Preparation of sheep liver aldehyde dehydrogenase.

Cytoplasmic sheep liver aldehyde dehydrogenase was prepared by the procedures described in Section 4. Post-gel filtration enzyme samples were used for all assays.

### 6.2.2. Acetaldehyde solutions.

Acetaldehyde was re-distilled and the refractive index of the resultant product was found to correspond exactly to that of pure acetaldehyde as reported in the literature. Dilute solutions of acetaldehyde were prepared by pipetting the required amount of acetaldehyde, kept at 4°C, into cold, deionised water and diluting by the correct amount.

### 6.2.3. Approximate $K_M$ for acetaldehyde.

The approximate  $K_M$  of aldehyde dehydrogenase for acetaldehyde was determined using the spectrophotometric assay described in Section 2, with the concentration of acetaldehyde in the reaction mixture varied from 0.83 to 83 M. In addition to the measurement of initial reaction rates, each assay was allowed to continue to completion and the final absorbance change was noted.

#### 6.2.4. Fluorometry.

All measurements were carried out using a Turner model 430 spectrofluorometer. For NADH determination the excitation wavelength was set at 350 nm and the emission at 460 nm.

#### 6.2.5. Assay reagents.

Pyrophosphate buffer and  $\text{NAD}^+$  were used at the same concentrations used in the aldehyde dehydrogenase assays (Section 2.2.3). Solutions were made up using deionised water. It was necessary to filter the buffer through a sintered glass filter to remove dust particles before the solution was used for fluorometric assays.

#### 6.2.6. Assay of alcohol dehydrogenase.

Alcohol dehydrogenase was assayed as described in Section 4.2.8. For the determination of activity using mercaptoethanol as a substrate, appropriate concentrations were substituted for ethanol.

### 6.3 RESULTS

#### 6.3.1. Development of the assay.

##### 6.3.1.1. Approximate $K_M$ for acetaldehyde.

The  $K_M$  of sheep liver aldehyde dehydrogenase for acetaldehyde was in the range of 1 - 10  $\mu\text{M}$ , determined using spectrophotometric assays. More accurate measurements of the  $K_M$  were difficult to obtain because of the extremely low values. However, results showed that the enzyme, due to its high affinity for acetaldehyde, would be suitable for use in the enzymic determination of this compound. The final absorbance change measured in each assay indicated that almost all of the acetaldehyde could be accounted for as NADH produced, which supported the view that the enzyme would be most suitable for acetaldehyde estimations.

##### 6.3.1.2. Use of fluorometry.

It was assumed from the levels of blood acetaldehyde reported in the literature that measurement of levels lower than 0.1 mg% (0.1 mg/100 cm<sup>3</sup>) might be routinely required. This was less than the minimum level that could be detected by the spectrophotometer using a

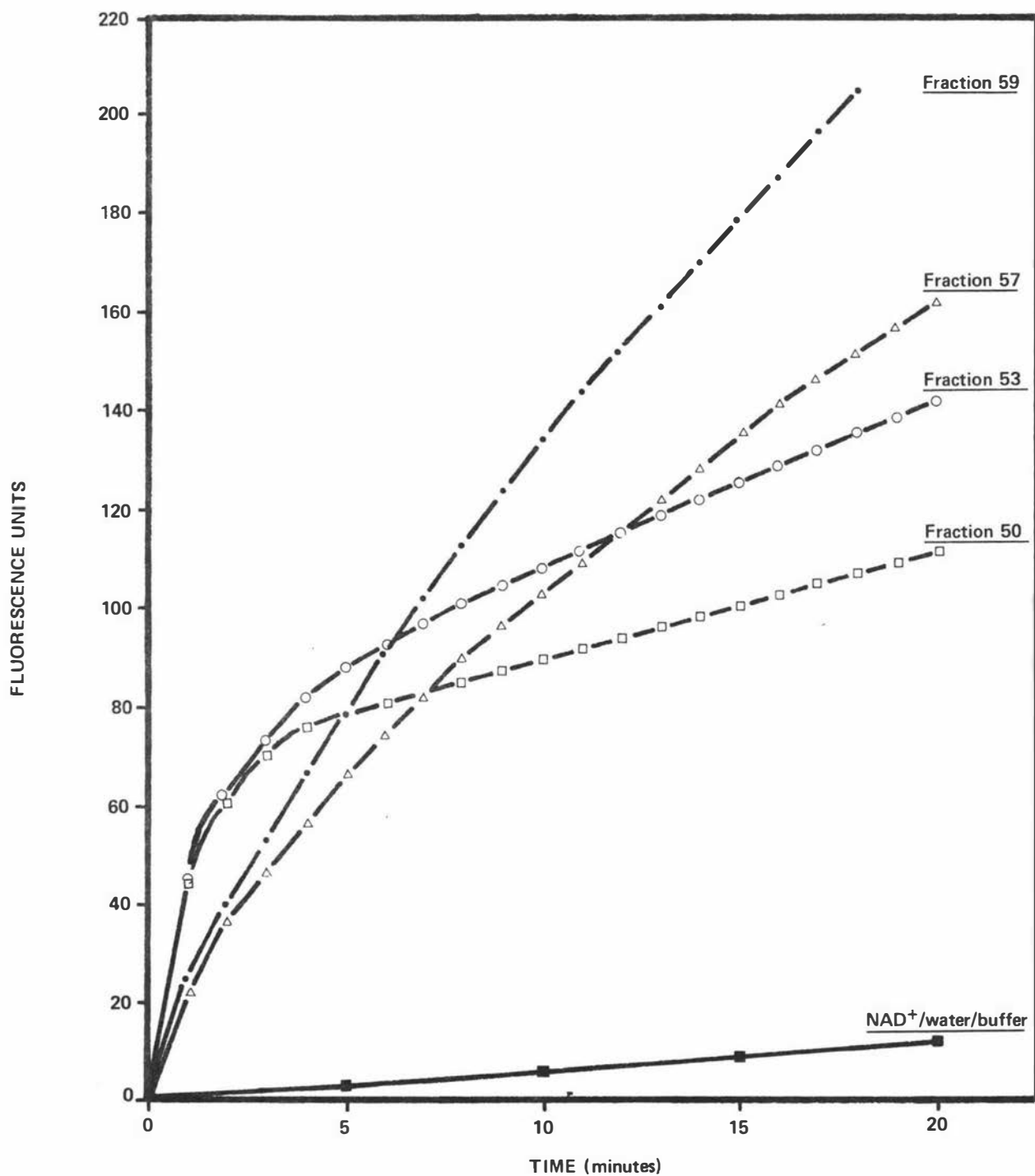
0.1 cm<sup>3</sup> sample, so that all further work on developing the assay was carried out with fluorometry, which could provide up to a 100 fold increase in sensitivity.

#### 6.3.1.3. Endogenous activity in aldehyde dehydrogenase samples.

Although the use of the fluorometer gave increased sensitivity, it accentuated a problem which only caused minor interference in spectrophotometric analyses. Some preparations of aldehyde dehydrogenase had considerable endogenous activity, where NADH production appeared to take place in the assay mixture without added acetaldehyde. It was possible to inhibit this endogenous activity by addition of p-chloromercuribenzoate (PCMB), but since this also inhibited aldehyde dehydrogenase, it was necessary to add it at the end of any reaction with acetaldehyde. While this method of stopping the endogenous activity worked reasonably satisfactorily for low sensitivity readings, at high sensitivities too much error was introduced by the endogenous activity. It was therefore essential to either remove the endogenous activity, or find a specific inhibitor which could be used prior to the acetaldehyde assay.

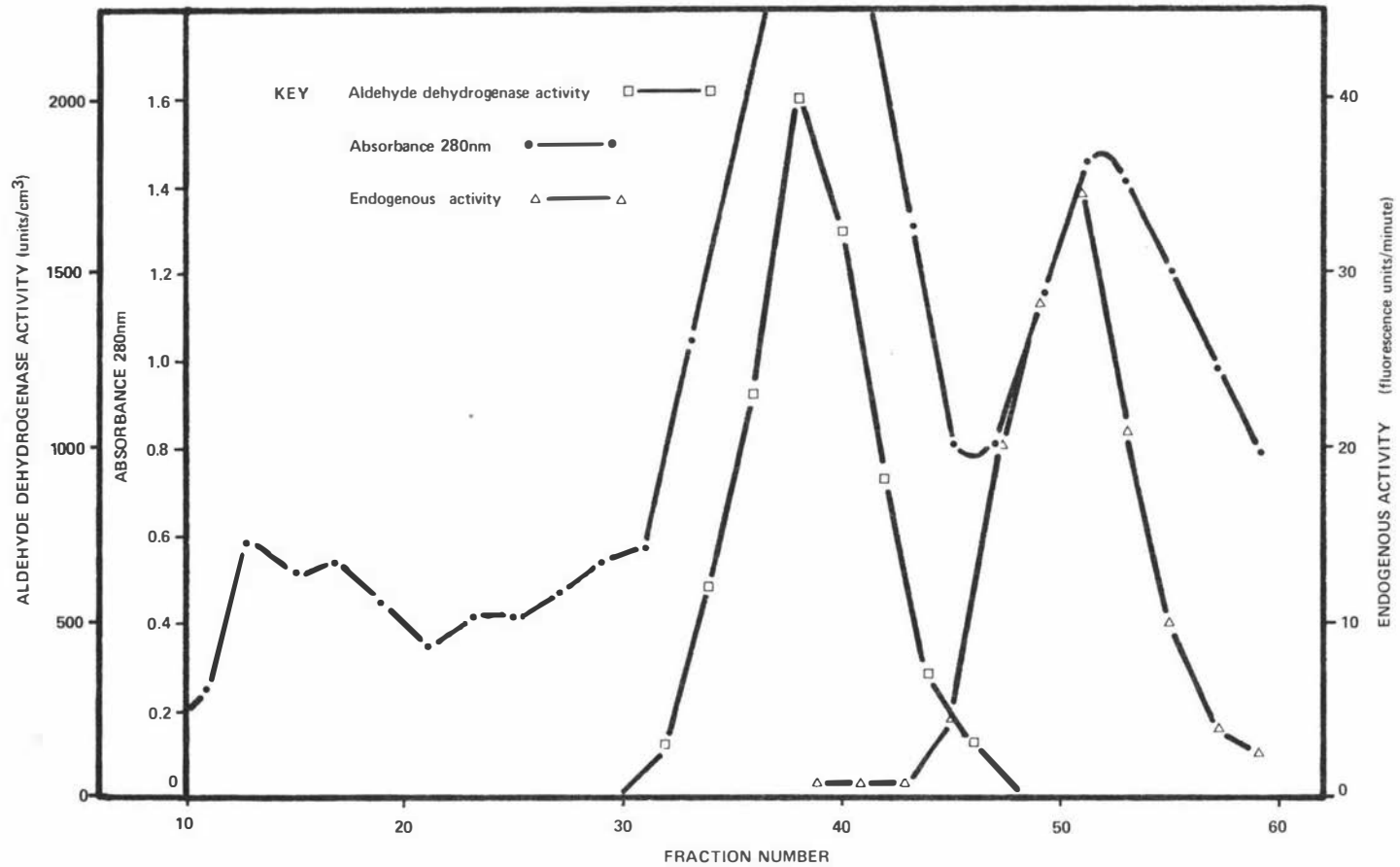
Subsequent results showed that there were in fact two types of endogenous activity, separable by gel filtration, and Figure 6.1 shows assays on fractions from a Sephadex G-200 column which exhibited both types of activity. Samples at peak aldehyde dehydrogenase activity, (fraction 50), exhibited a very high initial rate of endogenous NADH production which decreased within 5 minutes to a much lower constant rate, but subsequent fractions, with decreasing aldehyde dehydrogenase activity, showed a higher constant rate of NADH production. Endogenous activity of the type shown by fraction 50 did not interfere with the assay procedure and could be ascribed to contamination of the assay reagents with aldehydes. The more persistent endogenous activity of later fractions in gel filtration runs required a different explanation. The peak of this activity was well separated from the aldehyde dehydrogenase activity peak (Figure 6.2) indicating that it was probably due to a contaminating enzyme. The reaction was found to be dependent on NAD<sup>+</sup> with a  $K_M$  of 0.05 mM, and the product corresponded to NADH in absorption, excitation, and emission spectra, (Figures 6.3.a, b and c). Extensive dialysis of the enzyme sample against the usual buffer of the final enzyme preparation step (0.05 M phosphate, pH 7.3, containing

FIGURE 6.1 ENDOGENOUS ACTIVITY IN FRACTIONS FROM SEPHADEX G-200



The above figure illustrates the increase in fluorescence that was obtained when aldehyde dehydrogenase samples (fractions 50, 53, 57, and 59 from a Sephadex G-200 column run) were added to buffer/NAD<sup>+</sup> solution. Following mixing of the enzyme with the buffer/NAD<sup>+</sup> solution, the increase in fluorescence was recorded at 1 minute intervals for 15-20 minutes.

FIGURE 6.2 ENDOGENOUS ACTIVITY IN FRACTIONS ELUTED FROM BIOGEL A 0.5M



"Endogenous activity" in fractions from a Biogel column was estimated by adding samples to a buffer/NAD mixture, and recording the steady rate of fluorescence production that resulted after 1.5 minutes. The rate of increase is expressed in fluorescence units/minute. Aldehyde dehydrogenase activities for fractions from the column are also shown, expressed in absorbance units (1 unit of enzyme activity=0.001 absorbance units/minute).

FIGURE 6.3.a ABSORBANCE SCANS 300-370 nm

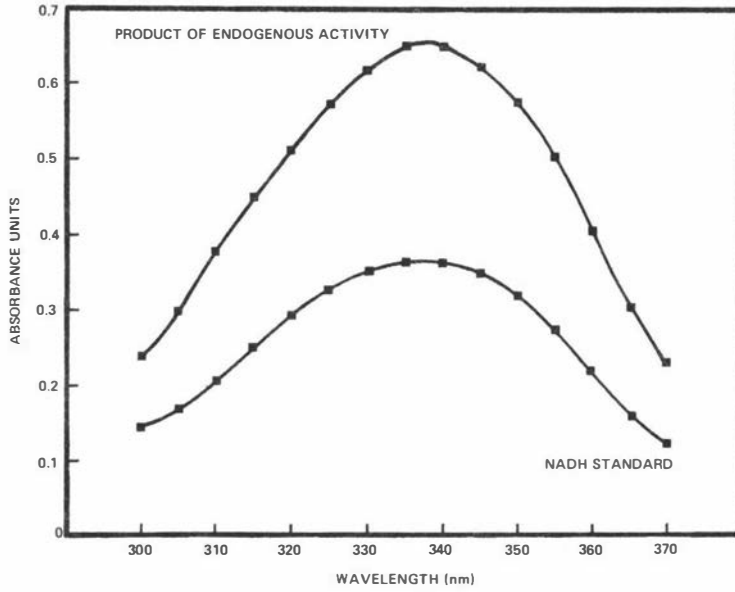


FIGURE 6.3.b EXCITATION SCANS 300-600 nm

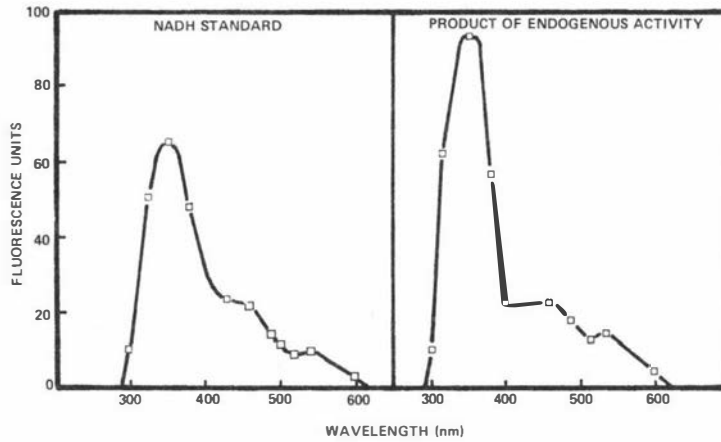
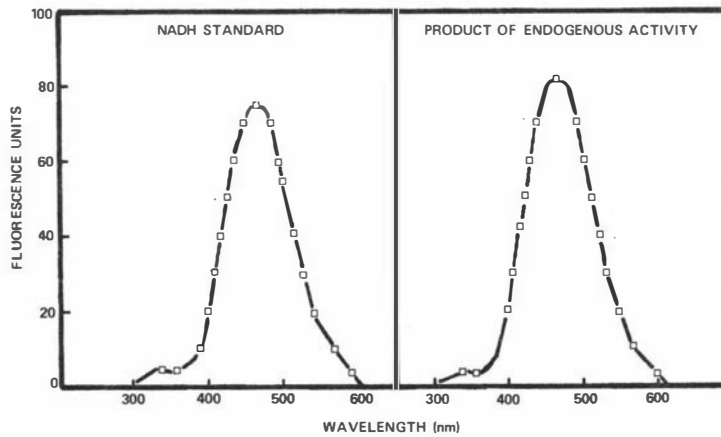


FIGURE 6.3.c EMISSION SCANS 200-600 nm



Figures 6.3 a, b, and c show absorbance, excitation and emission scans for the product of the endogenous reaction observed with aldehyde dehydrogenase. Scans of a standard NADH solution are also shown.

0.1% mercaptoethanol) did not remove the contaminating enzyme activity, but dialysis against the same buffer without mercaptoethanol did so. Similarly, running the enzyme through Sephadex G-25, with 0.05 M phosphate buffer and no mercaptoethanol removed the contaminating enzyme activity. After both dialysis and G-25 treatment in the absence of mercaptoethanol, the contaminating enzyme activity could be restored by the addition of mercaptoethanol. At this stage it seemed likely that the more persistent endogenous activity was due to alcohol dehydrogenase acting on mercaptoethanol as a substrate. Following are further results which indicated that the assumption was probably correct:

(a) The addition of ethanol to samples containing contaminating endogenous activity caused an increase in the rate of NADH production.

(b) Alcohol dehydrogenase was found to elute from Biogel A 0.5 M in the same position as the contaminating enzyme activity peak.

(c) Pyrazole, which did not inhibit the endogenous NADH production coinciding with aldehyde dehydrogenase activity was found to inhibit the second type of endogenous activity. Pyrazole is a potent inhibitor of alcohol dehydrogenase.

(d) Mercaptoethanol was found to be a substrate for purified horse liver alcohol dehydrogenase, reacting at about 3% the maximum rate for ethanol as a substrate.

The simplest procedure to avoid the interference caused by the second type of endogenous activity was to discard any fractions containing aldehyde dehydrogenase from gel filtration separations, which also contained alcohol dehydrogenase. This was preferable to the removal of mercaptoethanol by dialysis, since aldehyde dehydrogenase was less stable to storage without mercaptoethanol and in addition, samples to be assayed for acetaldehyde would often contain ethanol which would cause interference if alcohol dehydrogenase was present. The importance of removing alcohol dehydrogenase from samples used in acetaldehyde assays is further illustrated in Section 7.3.9.

The complete removal of alcohol dehydrogenase from aldehyde dehydrogenase preparations left only a very slight endogenous activity which gave a rate of less than 1 fluorescence unit/minute at the most sensitive setting used. This did not interfere in the assay as long as suitable blanks were used.

#### 6.3.1.4. $K_M$ of alcohol dehydrogenase for mercaptoethanol.

Mercaptoethanol has been reported as an inhibitor of alcohol dehydrogenase (Drum *et al.*, 1969), and it was also confirmed in the present work that mercaptoethanol inhibited the activity of alcohol dehydrogenase with ethanol as a substrate. No report has been found, however, of mercaptoethanol acting as a substrate for alcohol dehydrogenase. To obtain further information on the reaction of alcohol dehydrogenase on mercaptoethanol, a  $K_M$  was calculated using purified horse liver alcohol dehydrogenase. A  $K_M$  of 0.5 mM was found, at pH 7.9, and and  $NAD^+$  concentration of 0.4 mM.

The  $K_M$  for mercaptoethanol was similar to the value of 0.55 mM at pH 7.15 reported for ethanol oxidation by horse liver alcohol dehydrogenase (Wratten and Cleland, 1963). Further results using mercaptoethanol as a substrate for alcohol dehydrogenase have been noted by Blackwell and Hardman (1975).

#### 6.3.1.5. Standard curve for acetaldehyde, 2.5 - 12.5 nmoles.

Literature values for blood acetaldehyde indicated that measurement of levels at least as low as 0.1 mg% might be required, and so a standard curve of acetaldehyde concentration against fluorescence was initially determined in the range 2.5 - 12.5 nmoles, which represented an amount equivalent to that present in 0.1 cm<sup>3</sup> of a 0.1 - 0.5 mg% solution.

The standard curve was constructed by the following method: 1.2 cm<sup>3</sup> of deionised water, 1.0 cm<sup>3</sup> of pyrophosphate buffer, 0.5 cm<sup>3</sup> of  $NAD^+$  and 0.1 cm<sup>3</sup> of aldehyde dehydrogenase were added to each of six fluorometer tubes. To five of the tubes, 0.01 - 0.05 cm<sup>3</sup> of 0.25 mM acetaldehyde solution was added. After 10 - 15 minutes, the fluorescence of each was read against that of the sixth tube, to which no acetaldehyde had been added. An example of a standard curve obtained is shown in Figure 6.4.a.

#### 6.3.1.6. Standard curve for acetaldehyde: Conway diffusion procedure.

A similar standard curve to that obtained by the direct addition of acetaldehyde to the assay mixture could be obtained by placing the assay system in the outer ring of a Conway diffusion unit, and acetaldehyde in the centre well. A standard curve from this procedure is shown in Figure 6.4.b. The Conway units were kept ice cold until

FIGURE 6.4.a

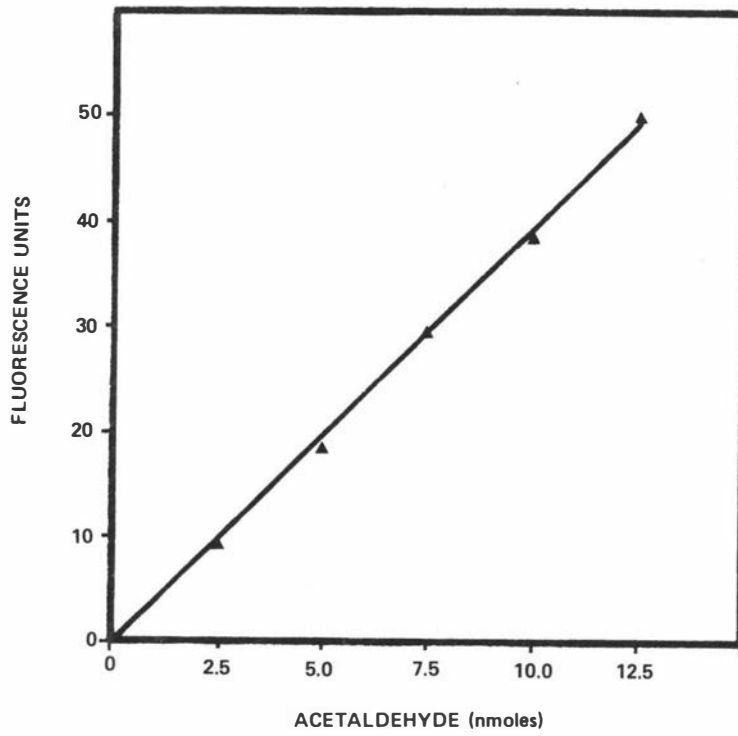
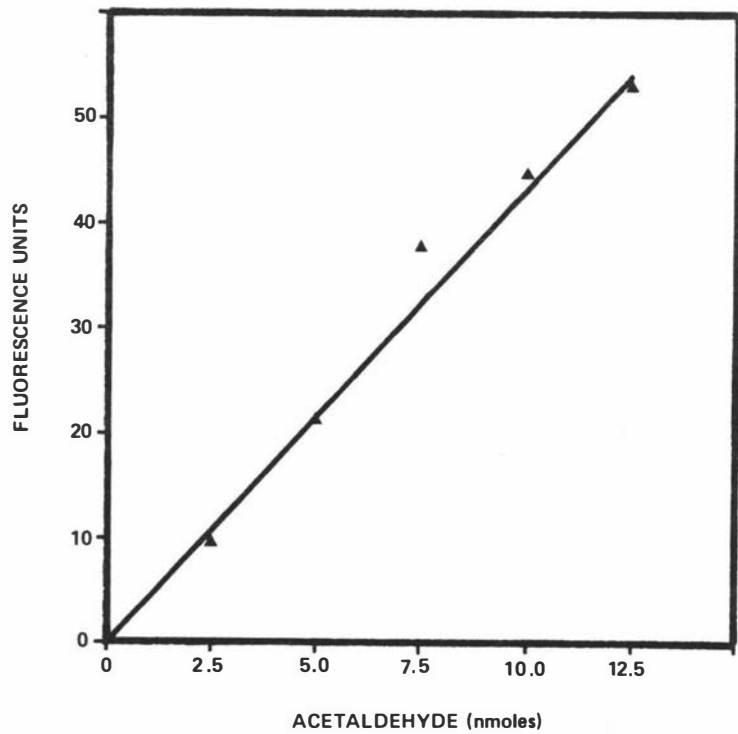


FIGURE 6.4.b



The standard curve shown in Figure 6.4.a was constructed by adding 0.0 – 0.05 cm<sup>3</sup> of 0.25M acetaldehyde to a standard assay mix containing pyrophosphate buffer (1.0cm<sup>3</sup>), NAD (0.5cm<sup>3</sup>), water (1.2cm<sup>3</sup>) and aldehyde dehydrogenase, (0.1cm<sup>3</sup>). After 10 - 15 minutes at room temperature, tubes containing acetaldehyde were read against the blank tube, the fluorescence values recorded, and plotted against the amount of acetaldehyde added initially. The curve shown in Figure 6.4.b was obtained using the same reagent volumes, but acetaldehyde was diffused into the mixture using Conway units, instead of being added directly.

acetaldehyde had been added and the unit sealed. They were then incubated at a temperature of 30 - 32°C above a water bath for 30 minutes. The assay mixture was removed from the outer well, transferred to a fluorometer tube and the fluorescence read against a blank (without acetaldehyde) which had been treated similarly. The same volumes of assay reagents and of standard acetaldehyde solution were used as described for the standard curve obtained by the direct addition of acetaldehyde to the assay system.

A direct comparison of standards determined by diffusion and direct addition, using the same enzyme samples and standard acetaldehyde solution, is shown in Figure 6.5.a. Figure 6.5.b shows that increasing the volume of acetaldehyde solution appeared to delay diffusion, but between 0.05 and 0.5 cm<sup>3</sup> of solution, most of the acetaldehyde reacted.

#### 6.3.1.7. Modification of the diffusion method.

The Conway diffusion method of acetaldehyde determination was capable of measuring down to 2.5 nmoles of acetaldehyde, or the equivalent of the acetaldehyde in an 0.1 cm<sup>3</sup> sample of a 0.1 mg% solution. The volume of sample used in the diffusion method could possibly have been increased to measure acetaldehyde in a 0.5 cm<sup>3</sup> sample which would have permitted the determination of levels lower than 0.1 mg%. The main limiting factor in the Conway diffusion assay was, however, the error introduced by transferring the assay mixture, after diffusion of acetaldehyde, from Conway units to fluorometer tubes. Frothing of the enzyme-containing mixture, which was difficult to avoid, caused an increase in fluorescence and this could be as great as that due to the acetaldehyde measured. An alternative method of diffusing acetaldehyde from samples into the assay system was developed which avoided the transfer step, and involved placing the sample containing acetaldehyde onto glass fibre paper, which was then suspended above the assay mixture in a sealed fluorometer tube. Glass fibre paper was selected for the purpose because it absorbed the samples readily, and had no apparent acetaldehyde contamination. In addition to avoiding the transfer step, this procedure allowed all assay mixtures to be checked for fluorescence before starting the assay and made it possible to continue readings during the diffusion stages.

## FIGURES 6.5 a and b ASSAY OF ACETALDEHYDE BY DIFFUSION IN CONWAY UNITS

FIGURE 6.5.a

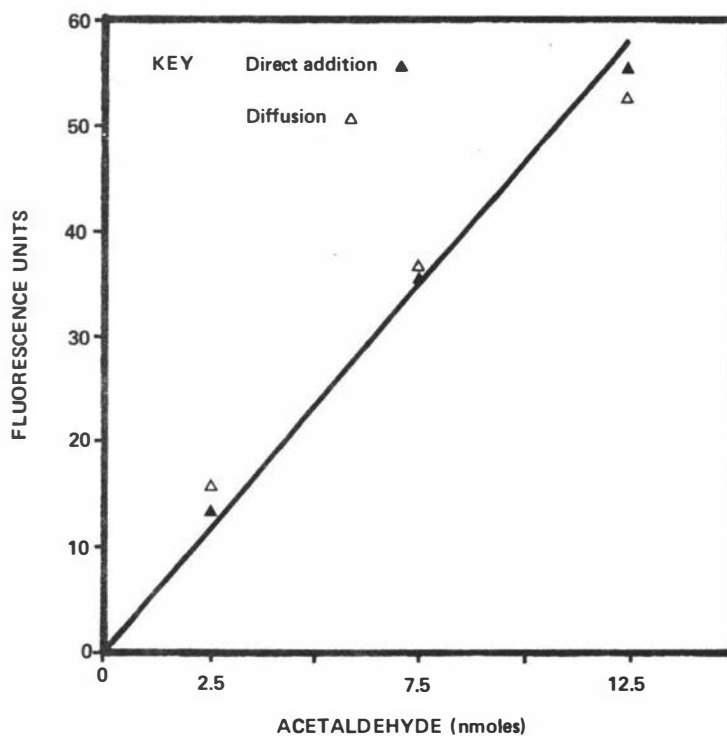


FIGURE 6.5.b

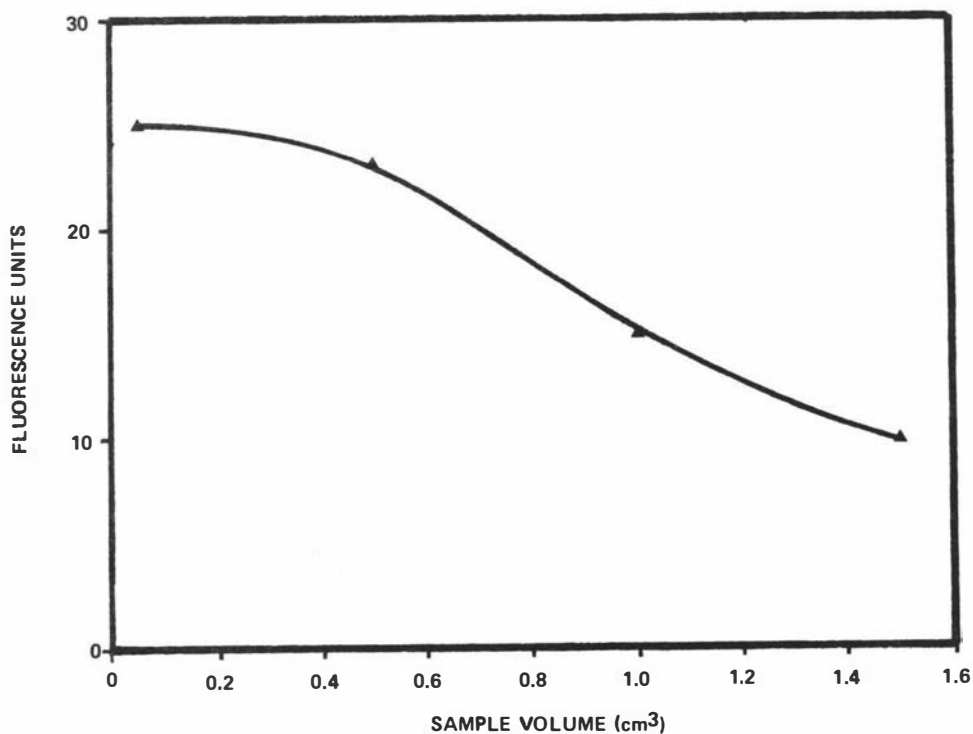


Figure 6.5 a shows standard amounts of acetaldehyde determined by diffusion into and direct addition to the assay mix, using the same enzyme samples and standard acetaldehyde solution. Figure 6.5.b shows a standard amount of acetaldehyde (10 n moles) diffused into the assay from a total volume of 0.05, 0.5, 1.0, and 1.5cm<sup>3</sup>. The fluorescence units used are arbitrary as the fluorimeter was not standardised for these experiments.

For the detection of acetaldehyde levels down to 0.25 nmoles, checking of all tubes prior to starting the assay was found to be essential. Initially, large variations in blank levels of fluorescence were found, but these were reduced by thoroughly pre-mixing the assay reagents, boiling all tubes in 50% nitric acid-water, and, after final washing, heating the tubes and all pipettes in an oven. It was also necessary to store concentrated acetaldehyde in a place well removed from the laboratory where determinations were carried out. All dilutions from pure acetaldehyde were carried out in the alternative storage place and only very dilute acetaldehyde solutions were kept in the laboratory. If these precautions were not taken, interference with both gas chromatographic and enzymic assays of acetaldehyde occurred by the presence of acetaldehyde in the air, distilled water, on glassware and in laboratory reagents.

#### 6.3.1.8. Standardisation of the fluorometer.

Neither NADH, because of its instability, nor acetaldehyde, because of its volatility, were good standards for setting the fluorometer. A quinine sulphate solution,  $0.025 \mu\text{g}/\text{cm}^3$ , made up in sulphuric acid was therefore used to calibrate the instrument. A blank of sulphuric acid and a quinine sulphate solution kept in sealed fluorometer tubes could be used for up to six weeks without any variation in fluorescence.

#### 6.3.2. Final method for assay of acetaldehyde.

##### 6.3.2.1. Assay procedure.

The fluorometer was used at the following settings:

sensitivity ..... high  
 range ..... x 100, x 300.  
 quinine sulphate ..... set at 50 on x 100 range.

The enzyme solution used was cytoplasmic sheep liver aldehyde dehydrogenase in 0.05 M phosphate buffer, pH 7.3, containing 0.1% mercaptoethanol, with activity between 500 and 2000 absorbance units/ $\text{cm}^3$  and a protein concentration of 2 - 5  $\text{mg}/\text{cm}^3$ .

The reagents used per tube were as follows:

deionised water .....  $1.2 \text{ cm}^3$   
 pyrophosphate buffer,  
 pH 9.3, 33 mM .....  $1.0 \text{ cm}^3$

NAD<sup>+</sup>, 1.67 mM ..... 0.05 cm<sup>3</sup>  
 enzyme ..... 0.05 - 0.1 cm<sup>3</sup>

The required volumes of pyrophosphate buffer, NAD<sup>+</sup>, water and enzyme solution were thoroughly mixed, and 2.7 cm<sup>3</sup> was pipetted into the required number of fluorometer tubes. The pipette was rinsed several times in the assay mixture before transferring the aliquot, with care being taken to avoid frothing.

After addition of the assay mixture to all tubes, they were read in the fluorometer. Any giving more than 3 - 4 fluorescence units variation from the average when read at a sensitivity of  $\times 300$  were discarded. The remainder were then used for the assay, which could be carried out by diffusion, or by direct addition of solutions to the assay mixture.

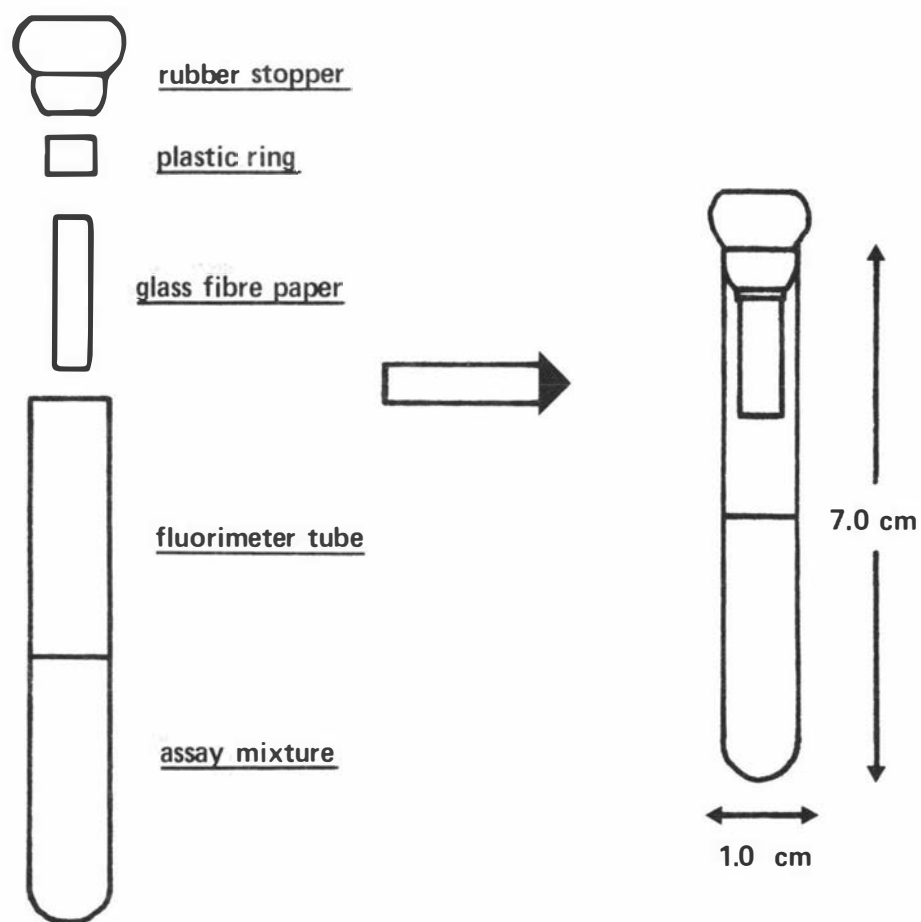
(a) Direct addition assays.

In the case of clean, aqueous solutions of acetaldehyde, estimations could be carried out by direct addition of the solution to the assay system. Sample sufficient to give a total amount of acetaldehyde between 0.25 and 2.5 nmoles (100 - 200  $\mu$ l) was added to the assay system, and mixed by tapping the tube. Samples, together with suitable blank tubes to which no acetaldehyde had been added, were left 5 - 10 minutes to react and the fluorescence was read against the blank tubes. Readings were continued until a constant value, within 3 - 4 fluorescence units on range  $\times 300$ , was obtained for each sample after which tubes were stored for a further period, preferably in the dark, when they were re-checked. Providing that the readings were constant, the assay was considered to be complete.

(b) Diffusion assays

The modified diffusion apparatus is shown in Figure 6.6. Acetaldehyde solutions were pipetted onto the glass fibre paper in a cold room, with 100  $\mu$ l being the maximum volume used. The glass fibre paper wicks were then inserted into fluorometer tubes containing assay mixture, and these, with suitable blanks, were placed in an incubator for 30 - 40 minutes at 32°C. Following incubation, the contents of the tubes with the glass fibre paper wicks still in place were mixed by tapping, and the fluorescence of samples was read against the blanks. Two blanks were usually used in diffusion assays; a tube with no paper wick, and a second with a paper wick on which had been placed 50 - 100  $\mu$ l of

FIGURE 6.6 DIAGRAM OF THE APPARATUS USED FOR THE ASSAY OF ACETALDEHYDE BY DIFFUSION



Glass fibre paper, about 5mm x 20mm was inserted into the plastic ring, which in turn was fitted into the base of the rubber stopper. After pipetting of a sample onto the paper, the rubber stopper was inserted into the top of the fluorimeter tube.

deionised water. Readings of each sample were continued until several values within 3 - 4 fluorescence units of each other were obtained. The tubes were replaced in the incubator and re-read when convenient within the next 2 hours. If readings agreed with the previous set, then the assay was considered to be complete.

#### 6.3.2.2. Calculations.

To confirm linearity of the assays, graphs of known amounts of acetaldehyde against fluorescence were constructed (see Section 6.3.3.1, following). Determination of the acetaldehyde concentration of unknown samples, however, was carried out by comparison of the sample fluorescence with the fluorescence of a standard obtained from the same assay run. The inclusion of standards with each run, rather than the use of a pre-determined standard curve, allowed for both variations in fluorescence arising from different enzyme preparations, or slight variations in temperature, which could affect fluorescence levels. If the standard was widely different from the expected value, then the run was repeated. Calculation of the acetaldehyde level in an unknown sample was carried out as follows:

(a) Direct addition assay.

$$\text{AcoH}_t = \text{AcoH}_s \times \frac{F_t \times \frac{V_t \text{ assay}}{V_b \text{ assay}}}{F_s \times \frac{V_s \text{ assay}}{V_b \text{ assay}}} \times \frac{V_s}{V_t}$$

or:

$$\text{nmoles AcoH}_t = \text{nmoles AcoH}_s \times \frac{F_t \times \frac{V_t \text{ assay}}{V_b \text{ assay}}}{F_s \times \frac{V_s \text{ assay}}{V_b \text{ assay}}}$$

(b) Diffusion assay

$$\text{AcoH}_t = \text{AcoH}_s \times \frac{F_t}{F_s} \times \frac{V_s}{V_t}$$

or:

$$\text{nmoles AcoH}_t = \text{nmoles AcoH}_s \times \frac{F_t}{F_s}$$

The symbols used above are as follows:

$\text{AcH}_t$	.....	acetaldehyde in test sample
$\text{AcH}_s$	.....	acetaldehyde in standard
$F_t$	.....	fluorescence of test assay
$F_s$	.....	fluorescence of standard assay
$V_t$ assay	.....	total volume of test assay
$V_s$ assay	.....	total volume of standard assay
$V_b$ assay	.....	total volume of blank assay
$V_t$	.....	volume of sample added to assay
$V_s$	.....	volume of standard solution added to assay

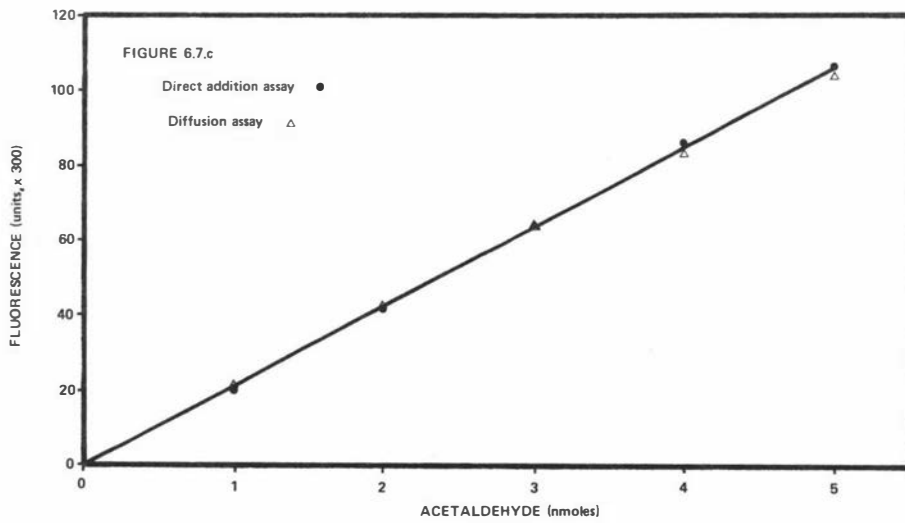
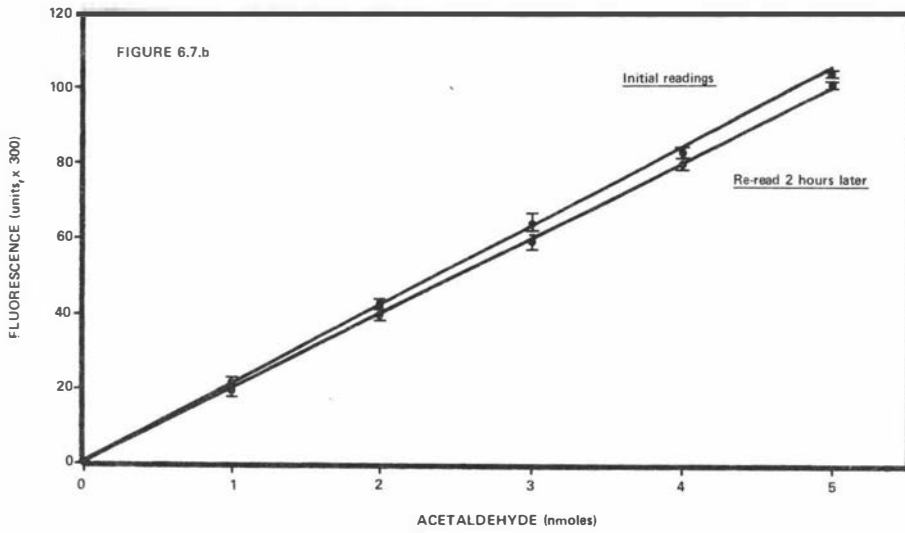
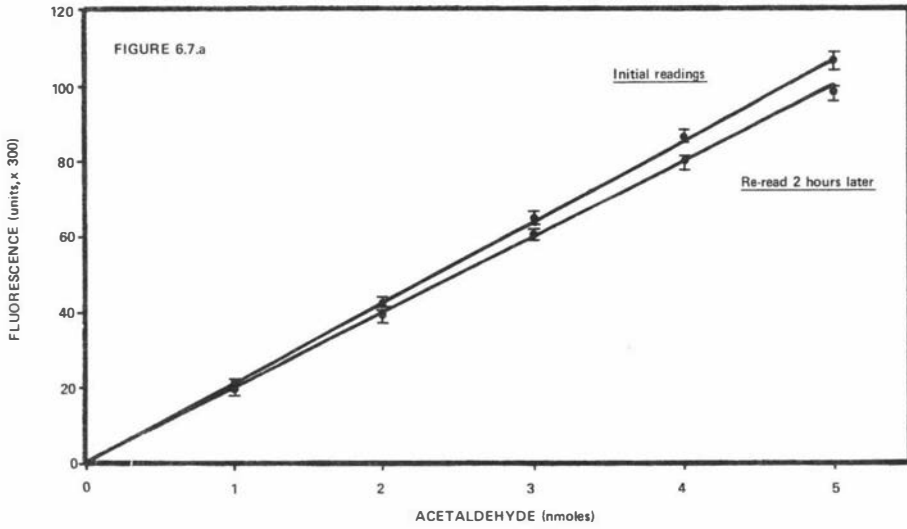
The more complicated calculations of acetaldehyde levels in the direct addition assays arise from the slight dilution of each assay by sample addition. With diffusion assays, where the sample was not added to the assay mix there was no significant dilution. The readings finally used in calculations were always an average of several successive readings of the fluorescence level in the sample. This was considered necessary to compensate for minor instrument fluctuations.

### 6.3.3. Standardisation of the assay

#### 6.3.3.1. Standard curves obtained using diffusion and direct addition assays.

Standard curves were obtained using both direct addition and diffusion assays (Figures 6.7.a and b). Both curves were constructed using 20 - 100  $\mu\text{l}$  of 0.05  $\mu\text{M}$  acetaldehyde solution, which represented from 1 - 5 nmoles of total acetaldehyde. Since values as low as 5 fluorescence units could be read, it was possible to estimate the acetaldehyde content of samples containing as little as 0.25 nmoles of acetaldehyde (which is equivalent to the acetaldehyde contained in 0.1  $\text{cm}^3$  of a 0.01 mg% solution).

The graphs obtained for diffusion and direct addition assays were almost identical (Figure 6.7.o), indicating that acetaldehyde had completely reacted in the diffusion assay. Re-reading of both standard curves after 2 hours showed a slight drop in fluorescence (Figures 6.7 a and b), but no further reaction had occurred in either case except for a slight relative increase in the two highest values for the



Figures 6.7 a and b show standard curves for 1-5nmoles of acetaldehyde, constructed by (a) direct addition of acetaldehyde to the assay, and (b) diffusion of acetaldehyde into the assay using the apparatus shown in Figure 6.6. Bars shown indicate the variation obtained on re-reading each assay several times, and the final points are averages of all the values obtained on re-reading. The lower curve in each case resulted from re-reading the same sets of tubes 2 hours after the initial readings of the assays. Figure 6.7.c shows the two curves from the initial readings shown in Figures 6.7.a and b superimposed, to give a direct comparison between the diffusion and direct addition results.

diffusion curve. The use of volumes larger than 100  $\mu$ l in the diffusion assays caused a delay in reaction with samples giving noticeably higher fluorescence values when read for a second time.

To test the validity of the assay over a wider range of concentrations of acetaldehyde, a standard curve was constructed using the direct addition method for up to 60 nmoles of acetaldehyde (Figure 6.8.a). Non-linearity occurred above 20 nmoles, or an assay concentration of about 9  $\mu$ molar. This was comparable to the NADH concentration at which non-linearity of fluorescence yield occurred (Figure 6.8.b) and it seemed that the assay method was valid for all acetaldehyde concentrations in the range where fluorescence was proportional to NADH concentration.

#### 6.3.3.2. Measurement of NADH produced during acetaldehyde assays.

Since the reaction of acetaldehyde to acetate is irreversible, the assay should go to completion, and the amount of NADH produced would be equal to the amount of acetaldehyde initially present. Standard curves for NADH were constructed by adding a known amount of NADH to 2.7  $\text{cm}^3$  of pyrophosphate buffer, and were prepared over a wide concentration range to determine the point at which fluorescence ceased to be linear in relation to NADH concentration. Figures 6.8.b and 6.9 show these curves, with amounts of NADH expressed as nmoles/assay, covering the range of 1 - 60 nmoles.

The addition of  $\text{NAD}^+$  to the NADH samples did not alter the fluorescence, but the addition of enzyme noticeably reduced NADH fluorescence. This was due possibly either to the presence of finely suspended particulate matter in the enzyme sample, or to the presence of catalase, which absorbs at 400 nm and could absorb some of the light emitted at 468 nm. Because of the reduced fluorescence yield that could occur in the presence of enzyme, to calculate the amount of NADH formed from acetaldehyde during the assay it was necessary to construct a standard curve for NADH in the presence of exactly the same amount of enzyme as in a corresponding acetaldehyde standard curve. This was done for the range of 0 - 3 nmoles of NADH, as illustrated in Figure 6.9. A comparison of fluorescence yields from the quenched standard curve with those from an acetaldehyde standard curve covering a similar range and using the same enzyme sample (Figure 6.7.c) indicated that there was close to a 100% correlation in the conversion of  $\text{NAD}^+$  to NADH, with the NADH recovered totalling 96% of the acetaldehyde added.

FIGURE 6.8.a

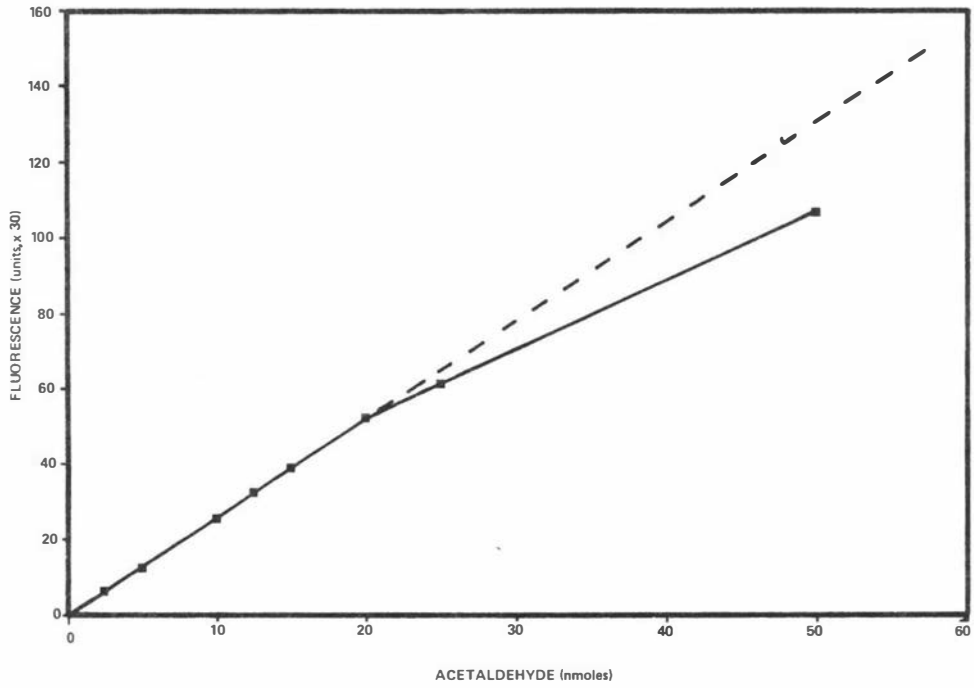
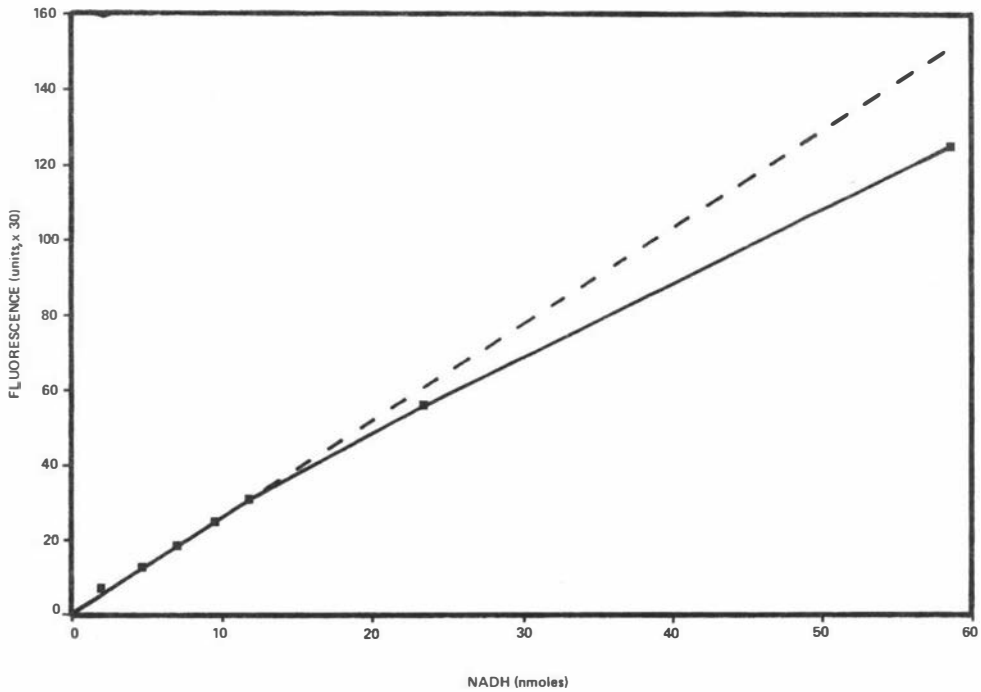


FIGURE 6.8.b



Figures 6.8 a and b show standard curves for (a) nmol of acetaldehyde and (b) nmol of NADH plotted against fluorescence, in the range of 2.5 – 60 nmol. The acetaldehyde standard curve was constructed by direct addition of known amounts of acetaldehyde to the usual assay mixture. The NADH curve was constructed by addition of known amounts of NADH (determined by reading the absorbance of the NADH solution on a Hitachi spectrophotometer at 340nm and using the extinction coefficient of NADH to calculate its concentration) to 2.7cm<sup>3</sup> of pyrophosphate buffer, pH 9.3.

To construct the standard curve for higher concentrations of acetaldehyde (Figure 6.8.a), enzyme sample which had a very low level of catalase contamination and had been centrifuged for 30 minutes at 15,000 g prior to the assay was used. In this case 100% agreement was obtained between fluorescence produced by the reaction of acetaldehyde and that of the same amount of standard NADH, even without enzyme added to the NADH.

No further checks on the relationship of the amount and quality of the enzyme used to the degree of quenching were carried out, but there was sufficient evidence to show that quenching did occur, and that its extent varied with the purity of the enzyme preparation. This had to be allowed for by inclusion of a standard with each set of unknowns, as described earlier.(Section 6.3.2.2.)

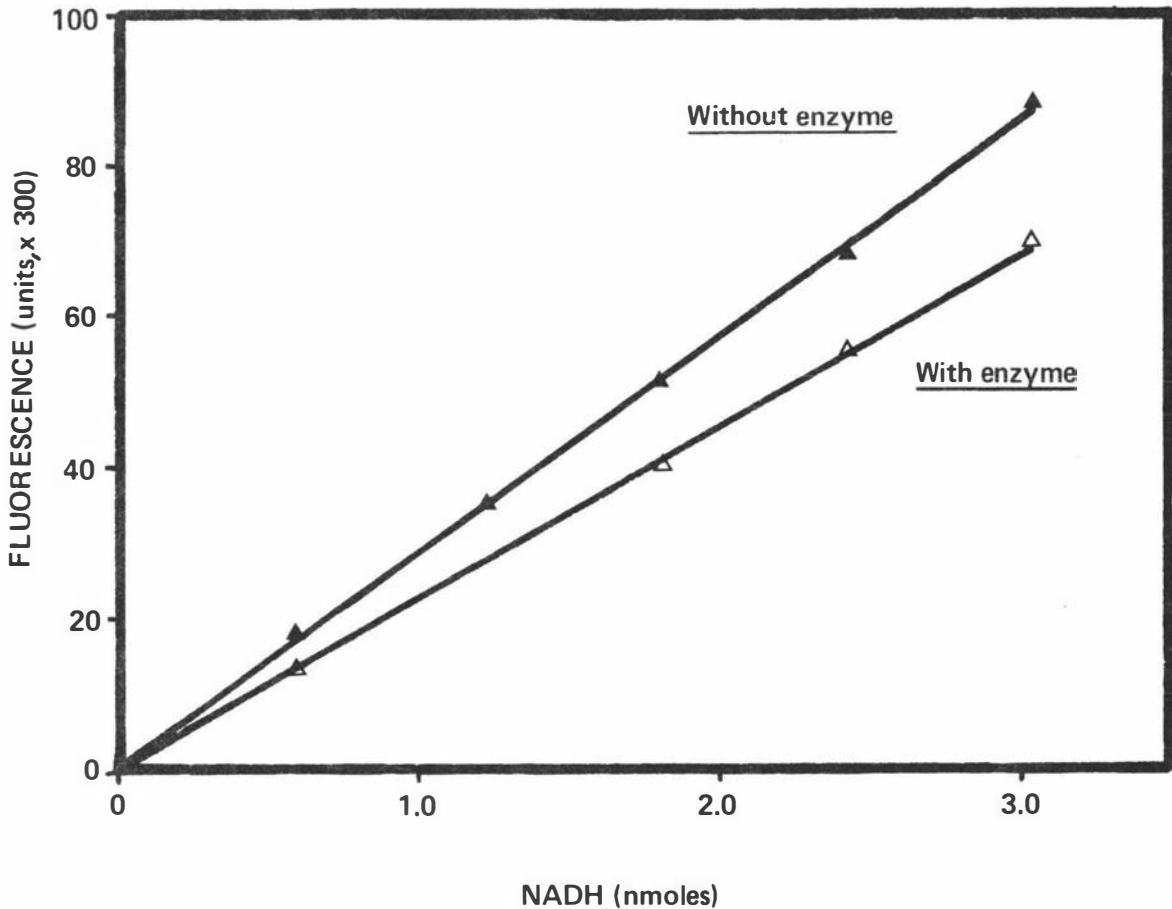
#### 6.4. DISCUSSION

The results obtained showed that acetaldehyde could be satisfactorily assayed in aqueous solution using sheep liver aldehyde dehydrogenase. Diffusion of acetaldehyde into the assay mixture was found to give as complete a reaction as obtained by direct additions. Nearly all the acetaldehyde added to an assay could be accounted for by NADH formed, which confirmed that the reaction of the enzyme with acetaldehyde was complete.

Table 6.1 gives a summary of some of the methods reported in the literature for determination of acetaldehyde in blood samples, and provides a comparison of these with the enzymic method developed in the present study and the gas chromatographic method developed by K.Couchman (1974).

The sensitivity of the enzymic assay compared favourably with that of the gas chromatographic method finally developed in this laboratory (Couchman, 1974). The latter method detected as little as 0.01 mg% acetaldehyde and required a 500  $\mu$ l sample. The enzymic assay could detect the same level of acetaldehyde in only 100  $\mu$ l of sample. The enzymic method of Lundquist (1958) could also detect as little as 0.01 mg% acetaldehyde, but required 2 cm<sup>3</sup> of sample or a minimum of 5 nmoles of acetaldehyde. Bergmeyer (1965) quoted levels of about 0.02 mg% as detectable by an assay using yeast alcohol dehydrogenase, which required 2 cm<sup>3</sup> of sample. Two other enzymic assays, using

FIGURE 6.9 STANDARD CURVES FOR NADH: QUENCHED AND UNQUENCHED



Standard curves are shown for 1 - 3 nmoles of NADH in the presence and absence of aldehyde dehydrogenase. Known amounts of NADH were added to  $2.7\text{cm}^3$  of pyrophosphate buffer, pH 9.3, to construct the upper curve, and to  $2.7\text{cm}^3$  of buffer/enzyme mix (containing approximately  $0.1\text{cm}^3$  enzyme/ $2.7\text{cm}^3$ ), for the lower curve. The number of moles of NADH present was calculated by reading the absorbance of the NADH solution on a Hitachi spectrophotometer at 340nm, and using the extinction coefficient to calculate the concentration.

TABLE 6.1

## SUMMARY OF METHODS FOR ACETALDEHYDE DETERMINATION

Method	Sample Volume	Minimum Level of Acetaldehyde Detectable	Reference
Chemical - p-hydroxybiphenyl	12 cm <sup>3</sup>	0.02 mg%	Stotz, 1943
Chemical - semicarbazide	2 cm <sup>3</sup>	0.04 mg%	Burbridge <u>et al.</u> , 1950
Enzymic - yeast alcohol dehydrogenase	-	20 nmoles	Racker, 1957
Enzymic - ox liver aldehyde dehydrogenase	-	20 nmoles	Racker, 1957
Enzymic - yeast alcohol dehydrogenase	2 cm <sup>3</sup>	0.018 mg% (8 nmoles)	Bergmeyer, 1965
Enzymic - yeast aldehyde dehydrogenase	2 cm <sup>3</sup>	0.01 mg% (5 nmoles)	Lundquist, 1958
Enzymic - sheep liver aldehyde dehydrogenase	0.1 cm <sup>3</sup>	0.01 mg% (0.25 nmoles)	This study
Gas chromatographic - direct injection of whole blood	2 - 7 $\mu$ l	0.04 mg%	Baker <u>et al.</u> , 1969
Gas chromatographic - direct injection of deproteinised supernatants	0.05 cm <sup>3</sup>	0.4 mg%	Roach and Creaven, 1968
Gas chromatographic - direct injection of deproteinised supernatants	-	0.01 mg%	Majchrowicz and Mendelson, 1970
Gas chromatographic - head space gas above a deproteinised blood sample	0.5 cm <sup>3</sup>	0.01 mg%	Couchman, 1974

yeast alcohol dehydrogenase (Racker, 1957) and liver aldehyde dehydrogenase (Racker, 1957) were less sensitive, both requiring a lower limit of 0.02  $\mu$ moles of acetaldehyde per assay.

Other reported methods of acetaldehyde determination using gas chromatography have varied in their sensitivities. Methods described by Duritz and Truitt (1964) and Coldwell et al (1971), who compared the method of Duritz and Truitt with that of Boiteau and Moussion (1968) gave no detailed information on lower limits of sensitivity. Roach and Creaven (1968) stated that their method could detect down to 4  $\mu$ g/cm<sup>3</sup>, or 0.4 mg%, using only 50  $\mu$ l of blood. This method was subsequently used, with some modification, for measurement of levels as low as 0.01 mg% acetaldehyde in blood (Majchrowicz and Mendelson, 1970). That of Baker et al. (1969) could detect less than 0.04 mg% in whole blood using 2 - 7  $\mu$ l of sample.

The enzymic method developed in the present work compared favourably for sensitivity with all the above methods. The chemical methods of Stotz (1943) and Burbridge et al., (1950) were less sensitive. The Stotz method could detect down to 0.02 mg% acetaldehyde, but required 12 cm<sup>3</sup> of blood to do so. The Burbridge method was more sensitive, being able to detect down to about 0.04 mg% acetaldehyde using only a 2 cm<sup>3</sup> blood sample, but the size of the blood sample could not be increased any further to allow for estimation of lower levels.

Gas chromatographic methods of acetaldehyde determination have a major advantage over enzymic and chemical methods in specificity. Both chemical methods mentioned are subject to possible interference by compounds other than acetaldehyde, and all three enzymes used in enzymic assays will react with a wide range of aldehydes. The enzymic method developed in this work is an improvement on the others described, in that its range is limited to volatile aldehydes, of which acetaldehyde is the only one normally present in blood samples. On occasions, formaldehyde may be present and when its presence is suspected results for samples assayed for acetaldehyde by the enzymic method could be erroneous.

## SECTION 7

ESTIMATION OF ACETALDEHYDE LEVELS IN BLOOD SAMPLES OF  
SUBJECTS FOLLOWING ETHANOL INTAKE

7.1 INTRODUCTION

The enzymic assay for acetaldehyde developed as described in Section 6 showed suitable sensitivity for use in the detection of acetaldehyde in blood samples following ethanol administration to human subjects. While the enzymic assay was being developed, a satisfactory procedure for gas chromatographic determination of acetaldehyde had been established, and this was being used routinely to estimate acetaldehyde levels in blood samples taken from volunteers given a dose of ethanol. The gas chromatographic method (Couchman, 1974) used head-space gas over a deproteinised blood sample to which sodium sulphate was added to displace the volatile constituents from the aqueous phase. There was some doubt (a) whether such treatment of blood samples left the original acetaldehyde level unchanged, and (b) if the gas chromatograph was accurately estimating the final level of acetaldehyde in the perchlorate supernatants. The enzymic assay was used in an attempt to resolve these problems and provide results which could be quoted as the blood acetaldehyde level of a sample, by two independent methods.

Some comparisons of two different methods for measurement of acetaldehyde concentrations had previously been reported by Lundquist (1958) who compared plasma levels of acetaldehyde from his enzymic method with those obtained from the chemical method of Burbridge *et al.* (1950). Truitt (1970) also compared acetaldehyde release from blood using a gas chromatographic method and the chemical methods of Burbridge (1950) and Stotz (1943). In each case, these comparisons involved only a few samples, and were not used to establish levels of acetaldehyde in subjects following ethanol intake.

The wide variations in blood acetaldehyde in human subjects reported in the literature indicated that a study which confirmed such levels using two independent methods would make a worthwhile contribution to research on the metabolism of ethanol and acetaldehyde.

## 7.2 METHODS

### 7.2.1. Administration of ethanol to subjects.

Ethanol, in the form of Vodka, was given to volunteers at a dose rate of 0.5 g ethanol/kg body weight, and the full amount was consumed in 10 minutes or less.

### 7.2.2. Blood samples.

Blood samples were taken under medical supervision by means of an indwelling cannula inserted in an arm vein. This was kept clear between samples by the use of heparinised saline. The first few  $\text{cm}^3$  of blood, containing heparin, were discarded, and a fresh syringe used to take 8  $\text{cm}^3$  of blood. Three  $\text{cm}^3$  of blood were immediately blown through the needle into 3  $\text{cm}^3$  of ice-cold 1 M perchlorate and well mixed. The remainder was placed in a bottle containing heparin, and both specimens were stored on ice until required.

### 7.2.3. Correction for blood volumes and dilution factors.

Inaccuracies in the amount of blood taken into perchlorate were compensated for by weighing the sample bottle before and after the blood sample was added. If too little blood was taken, a correction factor was used in subsequent calculations, and if too much blood was taken, more perchlorate was added to give the correct dilution.

When whole blood, plasma, or red blood cells were subjected to perchloric acid treatment, equal volumes of 1.0 M perchloric acid and sample were mixed, but the volume of solid removed during centrifugation of the treated samples left final dilution factors of 1:1.85, 1:1.94 and 1:1.71 respectively. These figures were obtained from average values for the percentage of water in blood fractions (Geigy, 1970).

### 7.2.4. Acetaldehyde assays.

The estimation of acetaldehyde in samples was carried out by the diffusion assay described in Section 6. Some readings were taken at shorter incubation times than described in the method to enable changes in fluorescence to be followed more closely. Incubation times and volumes of samples used are given in the appropriate results sections. All gas chromatography estimations were carried out by K.Couchman.

### 7.3. RESULTS

#### 7.3.1. Estimation of acetaldehyde in whole blood.

Samples of whole blood taken after ethanol administration and assayed for acetaldehyde using the diffusion assay showed a continuous increase in fluorescence, indicating an apparent production of acetaldehyde.

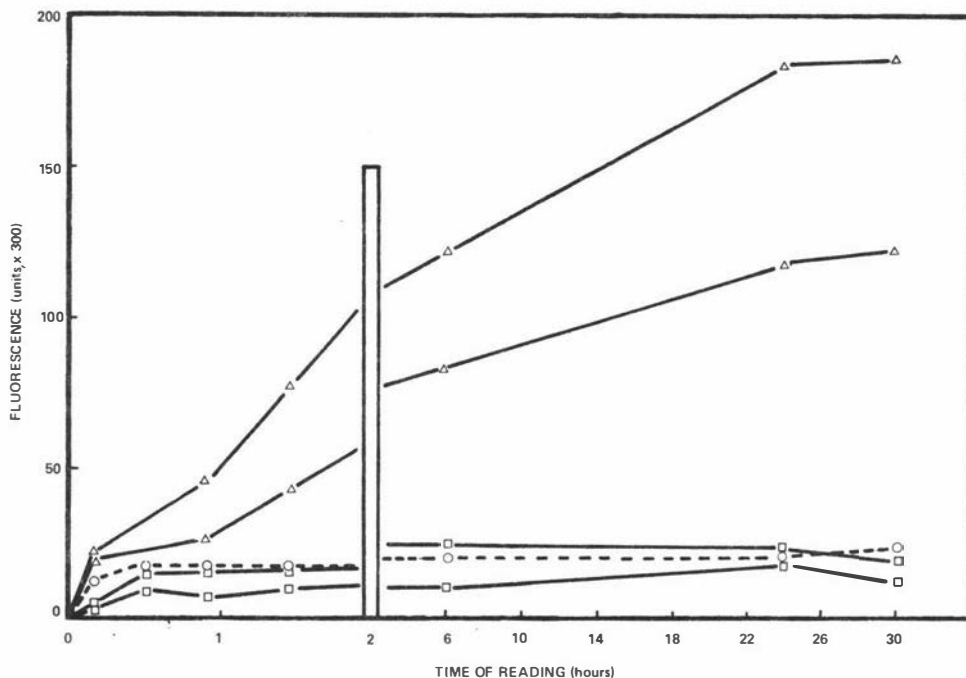
Duplicate samples (50  $\mu$ l) of whole blood taken prior to and 30 minutes after the administration of ethanol to subject 11 were assayed and the fluorescence produced was followed for 24 hours (Figure 7.1.a). Although there was considerable variation between the duplicates, the post-ethanol samples showed a continuous increase in fluorescence whilst the blood samples taken before ethanol administration were relatively stable.

Figure 7.1.b shows results for the same blood samples assayed after 1 and 3 days storage at 4°C. After storage for 1 day, the increase in fluorescence for the post-ethanol sample was similar to that observed previously and after 3 days storage this sample produced much less acetaldehyde. It was also noticeable in blood samples 1 and 3 days old that there appeared to be no initial acetaldehyde and production commenced after 10 minutes incubation (Figure 7.1.b). With fresh blood, however, there was a significant level of acetaldehyde present after the assay system had been incubated for only 10 minutes (Figure 7.1.a). Extrapolation of the acetaldehyde production curve for fresh blood back to zero time provided an estimate of the initial level of acetaldehyde in the blood sample.

To confirm the findings for subject 11, whole blood from three further subjects was assayed. Figures 7.2.a, b and c show acetaldehyde production from the blood of subjects 10, 16 and 20. In each case the blood samples used were fresh, and were taken before, and at 30 minutes after, ethanol administration.

Results for rates of acetaldehyde production from blood of all four volunteers (10, 11, 16 and 20), with details of acetaldehyde levels for the same samples determined by gas chromatography, are shown in Table 7.1. The rates were calculated over similar time ranges in each case, using the appropriate standard readings to convert fluorescence units to acetaldehyde concentration. Calculation in terms of nmoles of

FIGURE 7.1.a DIFFUSION ASSAY OF WHOLE BLOOD SAMPLES (subject 11)



Whole blood samples (50  $\mu$ l), taken before (  $\square$  ) and after (  $\Delta$  ) a volunteer (subject 11) was given ethanol, were assayed for acetaldehyde by the diffusion method. Each assay, including an accompanying acetaldehyde standard, (50  $\mu$ l, 0.11mg%) (  $\circ$  ), was re-read over a period of several hours. The fluorescence readings obtained for each sample and for the standard are plotted against the time over which the assay was followed.

FIGURE 7.1.b DIFFUSION ASSAY OF STORED WHOLE BLOOD SAMPLES (subject 11).

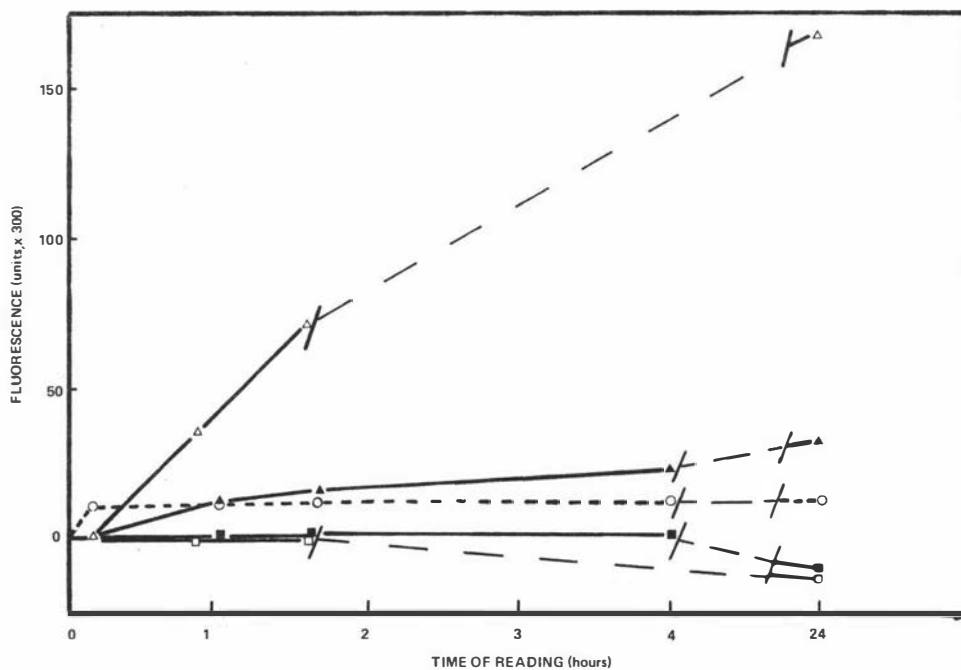
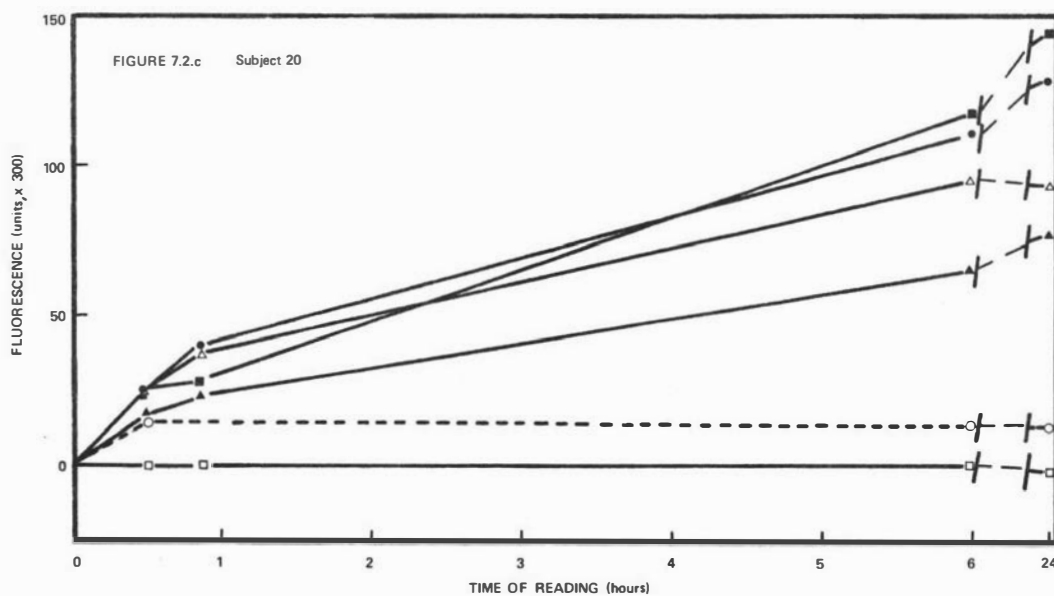
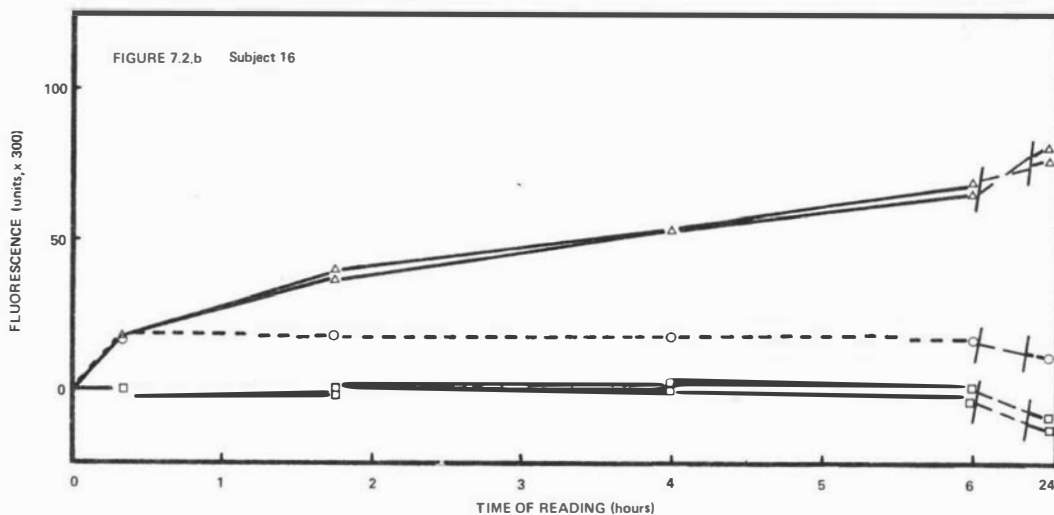
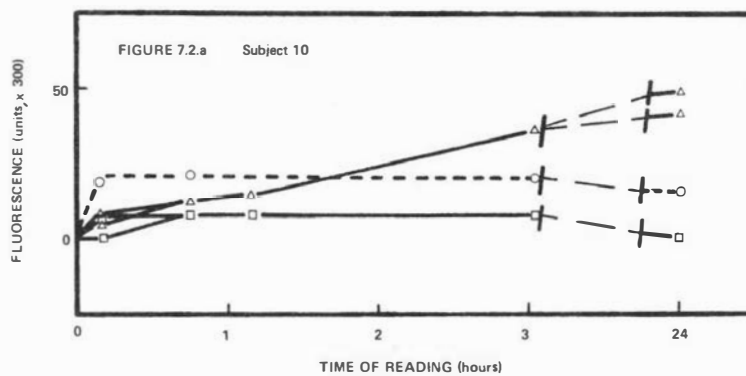


Figure 7.1.b shows the same blood samples as those in Figure 7.1.a, assayed for acetaldehyde by diffusion assay following storage at 4°C for 1 day (pre-ethanol  $\square$  post-ethanol  $\Delta$ ) and three days (pre-ethanol  $\blacksquare$  post-ethanol  $\blacktriangle$ ). The assays and an accompanying standard (  $\circ$  ) were again re-read over several hours, and the readings plotted against time over which the assay was followed.

FIGURES 7.2.a, b and c. DIFFUSION ASSAYS OF WHOLE BLOOD (subjects 10, 16 and 20)



Figures 7.2.a, b and c show whole blood samples (50  $\mu$ l) taken before ( $\square$ ) and after ( $\Delta$ ) ethanol was administered to three volunteers (subjects 10, 16 and 20), assayed for acetaldehyde by diffusion assay. Each assay was followed for several hours, and the fluorescence changes observed are plotted against time. Figure 7.2.c also shows post ethanol blood samples assayed with PCMB ( $\blacktriangle$ ), pyrazole ( $\bullet$ ) and water ( $\circ$ ) added to the paper wick. A standard acetaldehyde sample ( $\circ$ ) was included with each set of assays.

acetaldehyde produced per  $\text{cm}^3$  of blood per minute showed similar rates for all samples. Pre-ethanol blood samples were relatively stable in all cases.

In each of the cases for which fresh blood was used, there appeared to be a small amount of acetaldehyde present in the post-ethanol blood sample initially. Initial acetaldehyde levels shown in Table 7.1 were obtained by extrapolating fluorescence increase curves back to zero time, and calculating the amount present at this point by comparison with the fluorescence level of the standard. Results for initial acetaldehyde levels in the pre- and post-ethanol samples were similar by both gas chromatographic and enzymic assay.

Figure 7.2.c also shows acetaldehyde production curves obtained when whole blood was incubated on the paper wick in the presence of p-chloromercuribenzoate (PCMB), a sulphhydryl reagent, and pyrazole, a specific alcohol dehydrogenase inhibitor. With PCMB, acetaldehyde production was only slightly inhibited, and pyrazole had no effect.

### 7.3.2. Estimation of acetaldehyde in untreated plasma.

Plasma samples (50  $\mu\text{l}$ ) from subjects 10 and 11, taken before and after ethanol administration, were assayed for acetaldehyde. The results are shown in Figures 7.3.a and b. Until 25 minutes, no significant change was apparent in the case of samples from subject 11. Samples from subject 10 showed a slight increase in fluorescence at 10 and 40 minutes, which indicated levels of about 0.01 mg% in the pre-ethanol plasma sample, and 0.02 - 0.03 mg% in the post-ethanol sample. The latter results were 2 - 3 times less than the results shown by gas chromatography for the 30 minute post-ethanol blood sample from subject 10, but were only slightly lower than levels found by extrapolating enzymic assay curves to zero time (Table 7.1). There was apparently no acetaldehyde readily diffusible from either pre- or post-ethanol plasma samples for subject 11, which contrasted with results for both gas chromatographic determinations and extrapolation of the diffusion assay of whole blood (Table 7.1). Continued re-reading showed an increase in fluorescence of the post ethanol sample which began between 1 and 2 hours after starting the incubation, and which continued for over 9 hours (Figure 7.3.b).

TABLE 7.1

LEVELS OF ACETALDEHYDE IN WHOLE BLOOD

Fresh blood was collected before and 30 minutes after ethanol intake. Samples were assayed using enzymic diffusion assay. The rate of acetaldehyde production by the samples was calculated from the rate of increase of fluorescence on re-reading the assays over the time intervals indicated. Values (a) and (b) are duplicates of the same blood sample. The initial acetaldehyde level of the samples was estimated by extrapolating the fluorescence increase curves back to zero time. Levels from gas chromatography were determined using perchlorate supernatants of whole blood.

---

Subject No.	Rate of production of acetaldehyde in assay (nmoles AcH/cm <sup>3</sup> blood/minute 30 min sample)	Time range (hours)	Initial acetaldehyde level (mg %)		Gas chromatography level (mg %)	
			0 time	30 min	0 time	30 min
11	(a) 0.19 (b) 0.25	1-6	.03-.06	.10	.03	.11
10	(a) 0.21 (b) 0.21	0.75-3	0-.03	.03-.04	0	.06
16	(a) 0.25 (b) 0.21	1.75-6	0	.07	.03	.05
20	(a) 0.36	1-6	0	.08-.12	.01	.09

---

The two plasma samples assayed as described were 1 day old, and had been stored at 4°C after being separated from the red blood cells. In case this had influenced the results, plasma samples from the next subject (subject 13) were assayed without delay. Results still showed very low acetaldehyde levels in plasma samples obtained after ethanol had been ingested. Plasma samples taken at several time intervals after ethanol administration were assayed for subject 13, and the acetaldehyde levels found were very much lower than those for corresponding whole blood samples determined by gas chromatography (Table 7.2). Table 7.2 also shows that the plasma samples did not show any significant increase in their fluorescence in the first hour of readings, as the 10 minute results are very similar to the 60 minute results.

### 7.3.3. Estimation of acetaldehyde in perchloric acid supernatants of whole blood.

The addition of 0.5 M perchloric acid to a standard acetaldehyde solution did not affect either the direct addition or the diffusion assay of acetaldehyde.

#### (a) Direct addition assays.

Since the perchloric acid supernatants of whole blood were clear, samples (100 µl) were added directly to assays mixtures, and the resultant fluorescence levels were read within 5 minutes. Readings were continued for up to 24 hours after the start of the assay, and showed large changes in levels of fluorescence. Samples fluoresced more than blanks initially, and after 3 hours gave levels below the blank reading, but after 24 hours were again well above the blank level. Both pre- and post-ethanol samples showed these fluctuations, and, although the post-ethanol samples did give a slightly higher level of fluorescence initially, no satisfactory estimate of acetaldehyde levels could be obtained.

#### (b) Diffusion assays

Samples (100 µl) of perchlorate supernatants of pre- and post-ethanol blood samples from subject 15 were assayed by the normal diffusion assay procedure. An increase in fluorescence occurred between the 15 minute and 1 hour readings of the assay (Figure 7.4) but this appeared to occur in both the samples and standards, indicating that diffusion was not complete at 15 minutes. From 1 hour onwards, however, only minor variations occurred in the fluorescence level of either

TABLE 7.2

ACETALDEHYDE LEVELS IN UNTREATED PLASMA  
- SUBJECT 13

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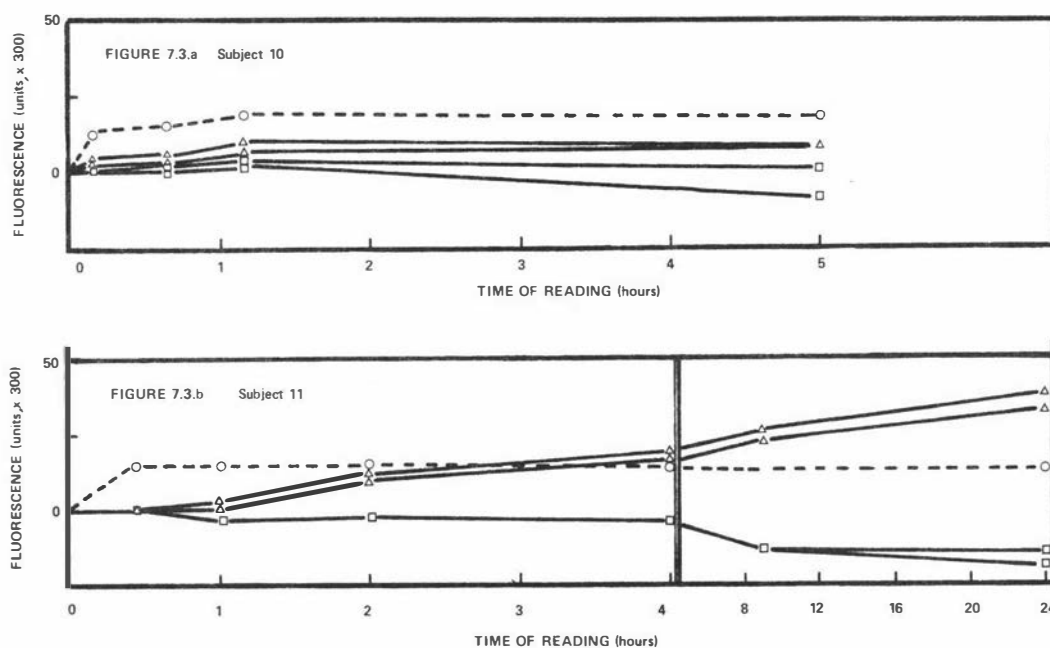
Levels of acetaldehyde in untreated plasma samples obtained at the times specified after ethanol intake were assayed using the enzymic assay. Levels of acetaldehyde in whole blood samples obtained at corresponding times were determined using gas chromatography. The same set of enzymic assays were read several times to check that no increase in fluorescence occurred with time. Values shown below were calculated from the first and second readings, taken at 10 minutes and 60 minutes respectively.

---

time of sampling	acetaldehyde level in untreated plasma enzymic assay (mg %)		acetaldehyde level in whole blood - gas chromatograph assay (mg %)
	Reading 1	Reading 2	
	0	<0.01	
15	0.0	<0.01	0.06
30	0.019	0.026	0.06
60	0.019	0.029	0.10
90	0.020	0.018	0.10
120	0.030	0.032	0.08
150	0.020	0.022	0.07
180	0.015	0.022	0.07

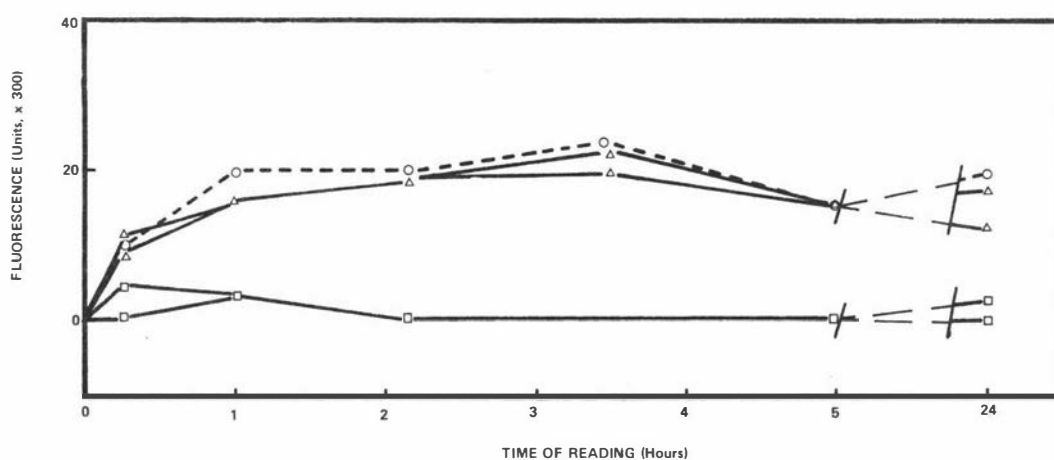
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FIGURES 7.3 a and b DIFFUSION ASSAYS OF PLASMA SAMPLES



Figures 7.3 a and b show plasma samples ( $50 \mu\text{l}$ ) taken from two subjects (10 and 11) before ( $\square$ ) and after ( $\Delta$ ) ethanol was administered. The samples were assayed for acetaldehyde using the diffusion assay, and fluorescence values found on re-reading the assays over several hours are plotted against time, together with readings for an acetaldehyde standard ( $\circ$ ), ( $50 \mu\text{l}$ ,  $0.11\text{mg}\%$ ).

FIGURE 7.4. DIFFUSION ASSAY OF PERCHLORATE SUPERNATANTS OF WHOLE BLOOD



Perchlorate supernatants ( $100 \mu\text{l}$ ) of whole blood taken from a volunteer (subject 15) before ( $\square$ ) and after ( $\Delta$ ) ethanol intake were assayed for acetaldehyde using the diffusion assay. Fluorescence values obtained on re-reading the assay over several hours are plotted against time, together with readings for an acetaldehyde standard ( $\circ$ ), ( $100 \mu\text{l}$ ,  $0.11\text{mg}\%$ ).

standards or samples. The concentration of acetaldehyde was calculated from the 1 hour reading as 0.077 mg%, for duplicate samples of the post-ethanol blood, and as 0.015 or 0.02 mg% for the pre-ethanol blood. There was good agreement with the gas chromatography value of 0.08 mg% for the post-ethanol sample, but no acetaldehyde was detected in the pre-ethanol sample by gas chromatography.

#### 7.3.4. Comparison of results for whole blood, plasma, and perchlorate supernatants.

As a final comparison of the three different sample types that had been used, whole blood, plasma, and perchlorate supernatant from a single blood sample were assayed. Figure 7.5 shows results for subject 16 for duplicate samples of:

- (a) post-ethanol blood sample, whole blood.
- (b) post-ethanol blood sample, untreated plasma.
- (c) post-ethanol blood sample, supernatant from perchlorate-treated whole blood.

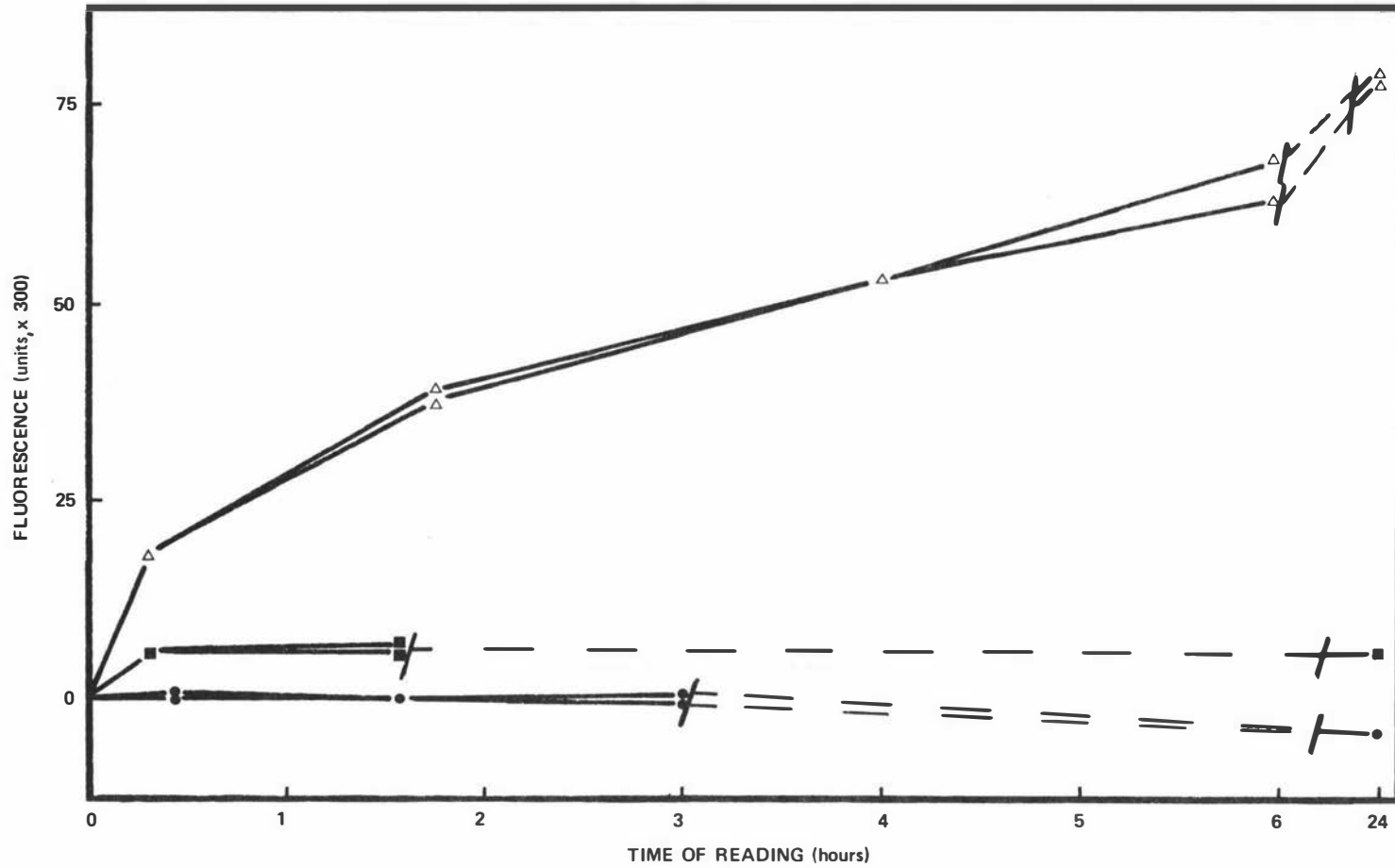
The perchlorate supernatant of the post-ethanol sample in this case showed a fairly low level of acetaldehyde, but the level on re-reading the assay was constant. Calculation from the 20 minute reading gave a level of 0.03 mg%, showing reasonable agreement with the gas chromatography determination, of 0.05 mg%. Untreated plasma showed an insignificant level of acetaldehyde, and whole blood (untreated) showed the usual continuous acetaldehyde production (the whole blood results for subject 16 were also shown in Figure 7.2.b).

Since the use of perchlorate supernatants appeared to be satisfactory in that it gave similar results to those from gas chromatography and that there appeared to be no continuing reaction once the initial acetaldehyde had diffused into the assay system, the method was used in estimating acetaldehyde levels during subsequent stages of the project.

The assay using whole, untreated blood was not continued because, although the extrapolation of the fluorescence increase back to zero time was probably a reasonably valid procedure for estimating initial acetaldehyde levels, it was too time consuming to be used routinely.

The production of acetaldehyde from whole blood containing ethanol has been reported by Truitt (1970) and has also been observed in this laboratory (Couchman, 1974). In both these cases estimations were carried out by gas chromatography, which provided positive identification

FIGURE 7.5 DIFFUSION ASSAY OF WHOLE BLOOD, PLASMA, AND PERCHLORIC ACID SUPERNATANT OF WHOLE BLOOD FROM A SINGLE SUBJECT



Whole blood, (  $\Delta$  ), 50  $\mu$ l, plasma (  $\bullet$  ) 50  $\mu$ l, and perchloric acid supernatant of whole blood (  $\blacksquare$  ), 100  $\mu$ l, were assayed for acetaldehyde. The samples all came from a single blood sample taken from subject 16, 30 minutes after ethanol was administered. Duplicates of each sample were assayed, and the fluorescence changes found after successive readings of each assay are plotted against time.

of the acetaldehyde formed, so, although acetaldehyde was not identified as the cause of the fluorescence increase produced in the assay of whole blood, it appears to be the most probable explanation. Attempts to inhibit this acetaldehyde production were not successful; a sample incubated in the presence of pyrazole still gave a high rate of fluorescence increase (Figure 7.2.c) and although a sample incubated with PCMB gave a lower rate, the difference between this and untreated samples was not significant. The decrease in initial acetaldehyde levels in whole blood after 1 day, and the decrease in rate of its production after 3 days, indicated that studies on acetaldehyde metabolism using whole blood should be carried out solely on fresh samples.

The assay of untreated plasma showed very little free acetaldehyde, and did not give satisfactory agreement with results for whole blood levels determined by gas chromatography. Since at this stage no reason for these very low levels could be found, this assay was not continued, although levels of acetaldehyde in plasma were reinvestigated subsequently (Section 7.3.6.).

#### 7.3.5. Determination of acetaldehyde levels in human subjects following ethanol administration.

Table 7.3 (Figures 7.6 and 7.7) shows results obtained for levels of acetaldehyde in whole blood from 6 subjects following ethanol intake, determined by both enzymic and gas chromatographic assays using perchlorate supernatants of whole blood. Three of the subjects were male (17, 19 and 20) and three were female (21, 22 and 23). Results obtained by the two methods used showed reasonable agreement, with enzymic assays tending in most cases to be 10 to 50% higher than gas chromatographic assays. The exception was the case of subject 17 (Figure 7.6.a), where the enzymic assay results were lower.

Consistent differences between the two methods are probably related to the dependence of the enzymic assay results on the level of fluorescence of an accompanying standard. Variations in fluorescence of the standard would result in a consistently raised or lowered level for all assays in one set, and would explain differences of this type that occurred between the two assays, for example in Figure 7.6.b. Such variation as seen between the 30 minute samples of Figure 7.6.d and the 90 minute samples of Figure 7.7.b could be due to random error in either of the methods. Detection of these variations illustrates the

TABLE 7.3

BLOOD ACETALDEHYDE LEVELS (mg %) IN PERCHLORATE  
SUPERNATANTS OF WHOLE BLOOD, BY ENZYMIC  
AND GAS CHROMATOGRAPHY ASSAYS

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Whole blood was taken directly into perchlorate at zero time (before ethanol intake) and at the specified time intervals after ethanol had been ingested. Perchlorate supernatants were assayed for acetaldehyde by enzymic and gas chromatography methods. Samples from subject 21 were assayed twice by the enzymic method, with results given as 21 a and 21 b below. Acetaldehyde levels are given in mg % in the table below.

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## (a) Enzymic assay

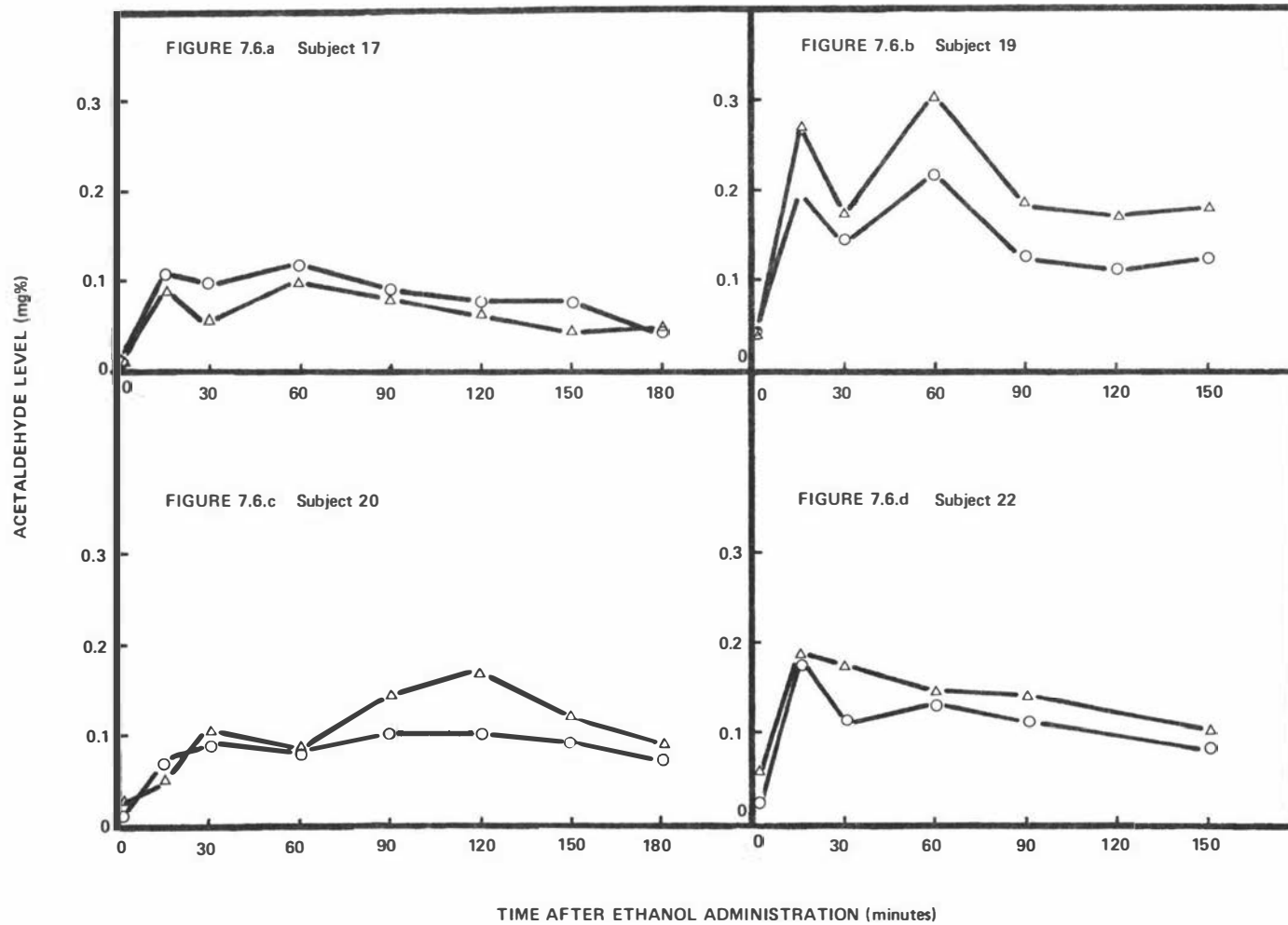
Time (minutes)	0	15	30	60	90	120	150	180
Subject No.								
17	0.0	0.095	0.056	0.098	0.081	0.060	0.042	0.051
19	0.044	0.275	0.179	0.308	0.182	0.170	0.180	-
20	0.027	0.048	0.107	0.086	0.148	0.170	0.120	0.109
21 a	0.0	0.102	0.122	0.245	0.190	0.176	0.105	0.200
b	0.0	0.124	0.131	0.247	0.187	0.174	0.130	0.180
22	0.025	0.188	0.175	0.148	0.143	-	0.106	-
23	0.0	0.085	0.117	0.141	0.181	0.101	0.124	0.082

## (b) Gas chromatographic assay

Time (minutes)	0	15	30	60	90	120	150	180
Subject No.								
17	0.01	0.11	0.10	0.12	0.09	0.08	0.08	0.04
19	0.05	0.20	0.14	0.22	0.12	0.11	0.12	-
20	0.01	0.07	0.09	0.08	0.10	0.10	0.09	0.07
21	0.02	0.08	0.07	0.22	0.13	0.14	0.08	0.15
22	0.00	0.18	0.11	0.13	0.11	-	0.08	-
23	0.01	0.06	0.09	0.13	0.10	0.06	0.09	0.04

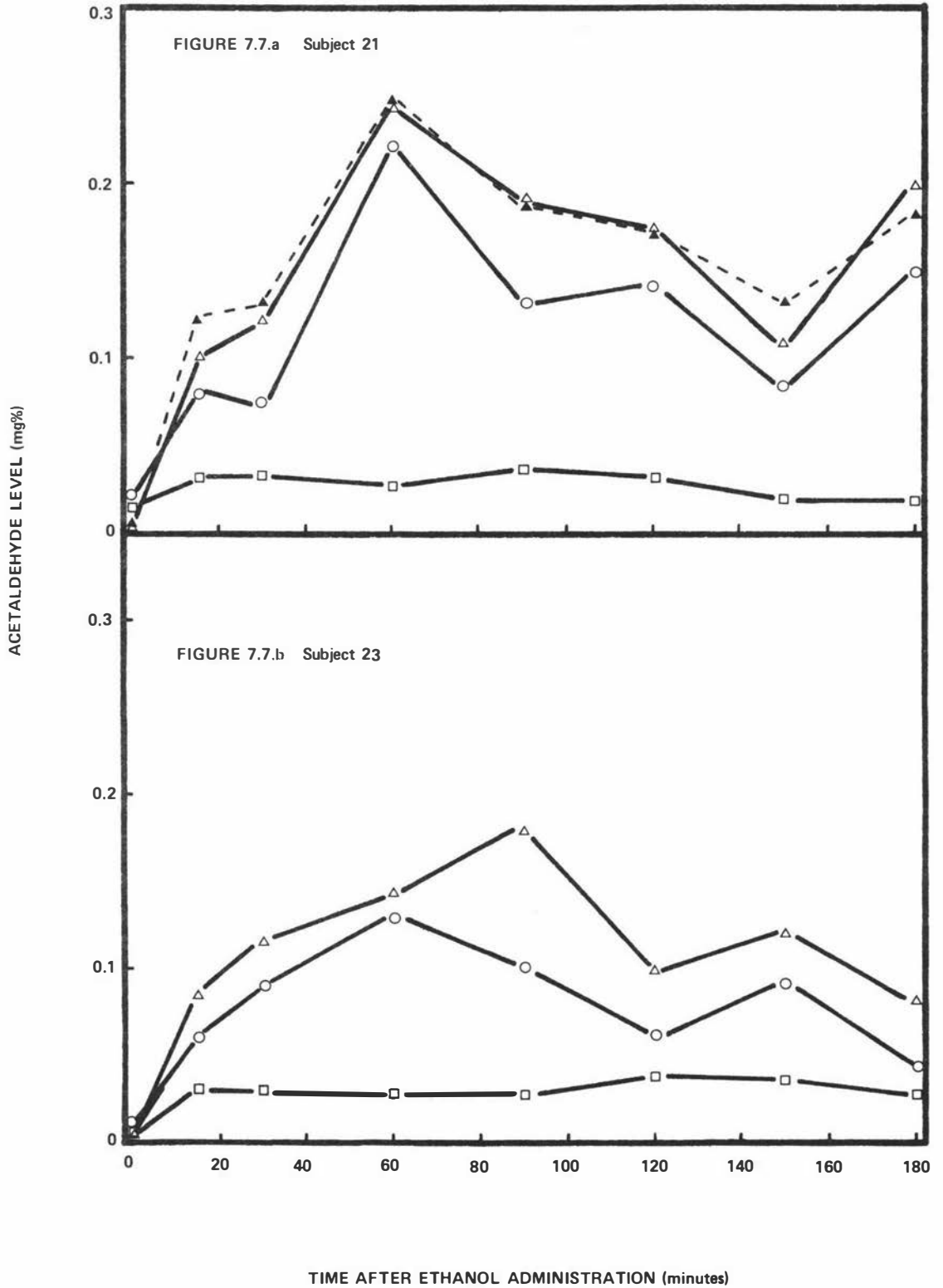
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FIGURE 7.6 a, b, c and d. BLOOD ACETALDEHYDE LEVELS, DETERMINED USING PERCHLORATE SUPERNATANTS OF WHOLE BLOOD, BY ENZYMIC AND GAS CHROMATOGRAPHIC ASSAYS.



Figures 7.6 a to d show acetaldehyde levels (mg%) in perchloric acid supernatants of whole blood determined by both gas chromatographic ( O ) and enzymic ( Δ ) assay. The data used to plot these graphs are also given in Table 7.3. The times at which the blood samples were taken following ethanol administration are shown on the horizontal axis.

FIGURES 7.7 a and b WHOLE BLOOD AND PLASMA ACETALDEHYDE LEVELS



Figures 7.7 a and b show whole blood ( $\Delta$ ) and plasma ( $\square$ ) acetaldehyde levels (mg%) determined by enzymic assay. The whole blood acetaldehyde levels were also determined by gas chromatography ( $\circ$ ). Figure 7.7a also shows a repeat run on whole blood acetaldehyde levels ( $\Delta$ ) determined by enzymic assay, to give an indication of the reproducibility of results. The times at which blood samples were taken following ethanol administration are shown on the horizontal axis.

advantage of having two methods for the determination of acetaldehyde, as clearly, more significance can be attached to fluctuations in acetaldehyde levels if these are confirmed by two different methods.

With the exception of one or two points, the overall shapes of the acetaldehyde curves determined by each assay were similar. There were, however, some variations between subjects - subjects 19 and 21 tended to show fluctuations in levels, with no definite downward trend with time, whereas subjects 17 and 22 showed an initial increase in acetaldehyde, followed by a fairly steady decline, with only minor fluctuations. Subjects 20 and 23 showed moderate fluctuations, with some tendency for levels to decrease in later samples. Subject 19 showed in both assays a high initial level of acetaldehyde, and also the highest levels seen following ethanol intake. This subject had been drinking the night before, and commenced the test with a blood alcohol level of 32 mg% at 9.0 am.

#### 7.3.6. Levels of acetaldehyde in plasma following ethanol intake.

At this stage, although satisfactory agreement between the enzymic and gas chromatography methods had been found for whole blood levels of acetaldehyde, no explanation had been found for the failure of untreated plasma, assayed enzymically, to give results which agreed with those for whole blood levels of acetaldehyde assayed by gas chromatography. Such inconsistent results, illustrated for subject 13 by Table 7.2, were also found for subject 20, where gas chromatography whole blood levels were confirmed by enzymic assay (Table 7.4). To test whether treatment of plasma with perchloric acid would elevate the acetaldehyde levels to give agreement with the whole blood levels, fresh blood samples were taken from subject 21, kept on ice until all samples were taken, then centrifuged to obtain plasma. The plasma was treated with perchloric acid, and supernatants assayed for acetaldehyde.

The results showed very little acetaldehyde in the perchlorate-treated plasma samples, and no increase in fluorescence was noted when the assays were re-read. Table 7.5 and Figure 7.7.a show the differences found between acetaldehyde levels in whole blood and plasma from subject 21. To confirm this finding, plasma was also obtained from blood samples from subject 23. In this case, whole blood was centrifuged immediately after it was taken from the volunteer, and the plasma

TABLE 7.4

ACETALDEHYDE LEVELS IN UNTREATED PLASMA  
- SUBJECT 20

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Blood samples were taken before, (0 minutes), and at the times given below, after ethanol ingestion. Whole blood samples were taken directly into perchlorate, and the supernatants were assayed for acetaldehyde by both the enzymic and gas chromatography methods. Plasma samples, not treated, were assayed by the enzymic assay.

---

Time of sampling (minutes)	Plasma acetaldehyde (mg %)	Whole blood acetaldehyde enzymic assay (mg %)	gas chromato- graphic assay (mg %)
0	0	0.027	0.01
30	0.021	0.107	0.09
60	0.029	0.086	0.08
90	0.029	0.148	0.10

---

TABLE 7.5

WHOLE BLOOD AND PLASMA LEVELS OF ACETALDEHYDE FOLLOWING  
ETHANOL INTAKE, DETERMINED BY ENZYMIC ASSAY

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Blood samples were taken before, (0 minutes) and at the specified times after ethanol was ingested. Whole blood was taken directly into perchlorate, and at the same time, a further whole blood sample was taken, and centrifuged to obtain plasma. Centrifugation was carried out as soon as the samples were taken in the case of subject 23, and at the completion of the entire run in the case of subject 21. Plasma samples were treated with perchloric acid, and supernatants of these and of whole blood samples were assayed for acetaldehyde.

---

Time of sampling (minutes)	Subject 21		Subject 23	
	whole blood (mg %)	plasma (mg %)	whole blood (mg %)	plasma (mg %)
0	0	0.018	0	0
15	0.102	0.032	0.085	0.032
30	0.122	0.032	0.117	0.027
60	0.245	0.025	0.141	0.027
90	0.190	0.035	0.181	0.026
120	0.176	0.030	0.101	0.037
150	0.105	0.018	0.124	0.035
180	0.200	0.018	0.082	0.028

---

separated and stored on ice until required. The results for subject 23 are shown in Table 7.5 and Figure 7.7.b, and again, the levels of acetaldehyde in perchlorate supernatants of plasma were very much lower than in perchlorate supernatants of whole blood. The levels of acetaldehyde in the plasma samples of subject 23 were confirmed by gas chromatography, which also showed that ethanol levels were the same in both whole blood and in plasma.

#### 7.3.7. Distribution of acetaldehyde in blood.

To determine the distribution of acetaldehyde in fractions of blood, 1.0 cm<sup>3</sup> samples of whole blood from subjects 25 and 26 were centrifuged to obtain plasma and red blood cells. Samples of these fractions were then treated with perchloric acid, and the supernatants assayed for acetaldehyde, in parallel with assays of perchlorate supernatants from corresponding whole blood samples. The results are shown in Table 7.6. Plasma samples again showed low levels of acetaldehyde, but red blood cell levels showed an increase when compared with whole blood. In subject 25, red blood cell levels were 1.38 and 1.44 times the acetaldehyde level of whole blood at 60 and 120 minutes after drinking ethanol. In subject 26, red blood cell levels were 1.63 and 1.62 times whole blood levels at 30 and 120 minutes after drinking ethanol.

Recoveries for acetaldehyde, shown in Table 7.7, were calculated assuming red blood cells comprised 42% of the blood volume (Geigy, 1970. The subjects were both female.) and showed that from 64 - 76% of the acetaldehyde found in the whole blood samples was regained by assaying fractionated blood. Reasons for the recovery not being 100% were possibly (a) that blood samples being fractionated were stored on ice for 2 hours, and during this time were centrifuged, whereas the whole blood samples were added to perchlorate immediately, and (b) that there was a small amount of plasma left above the red blood cell pellet. In spite of the low recovery of acetaldehyde, this experiment showed that there was more acetaldehyde in red blood cells than in plasma (Table 7.7).

#### 7.3.8. Distribution and recovery of acetaldehyde added to whole blood.

A fresh blood sample of 7 cm<sup>3</sup> was divided into two 3.5 cm<sup>3</sup> portions, to one of which sufficient acetaldehyde was added to give a final concentration of 0.15 mg% (sample B). This sample was well mixed, and

TABLE 7.6

LEVELS OF ACETALDEHYDE IN WHOLE BLOOD, PLASMA  
AND RED BLOOD CELLS FROM VOLUNTEERS AFTER ETHANOL INTAKE

---

Blood samples were taken before (zero time) and at the time intervals shown, after subjects were given ethanol. One whole blood sample was taken directly into perchlorate, and another which was kept on ice until all samples were collected, was fractionated to give plasma and red blood cells. Plasma and red blood cell samples were treated with perchloric acid, and the supernatants together with whole blood perchlorate supernatants were assayed for acetaldehyde. The levels in whole blood perchlorate supernatants were determined by gas chromatography as well as by the enzymic assay.

---

	Whole blood acetaldehyde enzymic assay (mg %)	gas chromato- graphic assay (mg %)	Plasma acetaldehyde (mg %)	Red blood cell acetaldehyde (mg %)
Subject 25				
zero time	0.011	0.01	<0.010	<0.010
60 minutes	0.142	0.14	0.014	0.195
120 minutes	0.089	0.06	0.023	0.128
Subject 26				
zero time	0.000	0.01	0.000	0.000
30 minutes	0.131	0.10	0.000	0.213
120 minutes	0.136	0.13	0.012	0.220

---

TABLE 7.7

RECOVERY AND PERCENTAGE OF ACETALDEHYDE IN PLASMA  
AND RED BLOOD CELLS

---

Acetaldehyde recovery from whole blood was calculated from the addition of acetaldehyde levels for plasma and red blood cells given in Table 7.6, assuming red blood cells to comprise 42% of the blood volume (Geigy, 1970 page 556. Both subjects were female.). Levels of acetaldehyde in plasma and red blood cells are given both as the percentage of the original whole blood level found in each fraction, and as the percentage of the recovered acetaldehyde level found in each fraction.

---

	% recovery from whole blood	% of initial acetaldehyde in each fraction		% of recovered acetaldehyde in each fraction	
		plasma	red blood cells	plasma	red blood cells
Subject 25					
60 minutes	64	6	58	9	91
120 minutes	76	15	61	19	81
Subject 26					
30 minutes	69	0	69	0	100
120 minutes	73	5	68	7	93

---

kept on ice for 30 minutes to allow the acetaldehyde to distribute throughout the sample. Sample A, containing no added acetaldehyde, was also kept on ice for this time, after which  $0.5 \text{ cm}^3$  of each sample was treated with perchloric acid. A further  $1.0 \text{ cm}^3$  of each blood sample was centrifuged to give plasma and red blood cells, then  $0.3 \text{ cm}^3$  of each of these fractions was treated with perchloric acid. All the perchloric acid treated samples were centrifuged, and the supernatants assayed for acetaldehyde. The remainder of each blood sample was incubated at  $32^\circ\text{C}$  for 1 hour, then the treatments described above were repeated.

The results of this experiment are shown in Table 7.8. There was no acetaldehyde initially present in sample A. The recovery of added acetaldehyde from sample B was 69% prior to the separation of plasma and red blood cells and incubation at  $32^\circ\text{C}$ . The recovery estimated from the addition of plasma and red blood cell levels was lower, the acetaldehyde level being  $0.07 \text{ mg}\%$ , giving 48% recovery of added acetaldehyde or 69% recovery when compared with the assay of unfractionated blood. The value for acetaldehyde concentration obtained from the plasma and red blood cell levels was calculated assuming red blood cells to comprise 47% of the blood volume (Geigy, 1970. The subject in this case was a male.).

The initial loss of acetaldehyde from the level of  $0.15 \text{ mg}\%$  actually added to  $0.103 \text{ mg}\%$  determined by assaying whole blood was not unexpected. The continued loss of acetaldehyde following fractionation of the blood sample was less expected, although it gave a similar recovery from whole blood to that found in earlier experiments. The time between perchlorate treatment of the initial whole blood sample, and perchlorate treatment of the plasma and red blood cells was, in this experiment, only 10 minutes. In this interval, however, the sample was centrifuged in a bench centrifuge, and the plasma removed. During these steps, the temperature of the sample would have increased, and this could possibly have accelerated the removal of acetaldehyde.

The distribution of acetaldehyde added to this blood sample was not the same as that of acetaldehyde produced from drinking ethanol (Section 7.3.7.). Red blood cell acetaldehyde was actually lower than plasma levels in these recovery experiments, instead of higher as found in blood from subjects who had taken ethanol. Similar recoveries

TABLE 7.8

LEVELS OF ACETALDEHYDE FOUND AFTER ADDITION  
TO WHOLE BLOOD

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Sufficient acetaldehyde was added to a fresh whole blood sample to give a level of 0.15mg % acetaldehyde in the sample. This blood (sample B), with an accompanying blank sample (sample A) was left for 30 minutes on ice, then samples of whole blood, plasma, and red blood cells from both the blank blood and the acetaldehyde-containing blood were treated with perchloric acid, and the supernatants were assayed for acetaldehyde. The remainder of each whole blood sample was incubated for 1 hour at 32°C, then was fractionated, and the plasma and red blood cells, as well as a whole blood sample, were treated with perchlorate, and the supernatants assayed for acetaldehyde.

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	Whole Blood (mg %)	Plasma (mg %)	Red Blood Cells (mg %)
Initial level added			
sample A	0	-	-
sample B	0.15	-	-
Levels in unincubated blood			
sample A	0	<0.01	0
sample B	0.103	0.083	0.057
Levels in incubated blood			
sample A	0.045	0.037	0.033
sample B	0.043	0.045	0.034

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of acetaldehyde were noted following fractionation in each case, the 69% observed in this experiment being in the range of 64 - 76% found for the earlier samples.

Following incubation of the whole blood samples at 32°C for 1 hour, the levels of acetaldehyde in sample B had dropped further. Sample A appeared to have produced acetaldehyde, so that both samples exhibited similar levels. The reason for such a production of acetaldehyde is not known, but it could possibly result from the presence of endogenous ethanol. Levels of acetaldehyde did not differ significantly between whole blood, plasma and red blood cells in either of the incubated samples.

#### 7.3.9. Contamination of enzyme sample with trace amounts of alcohol dehydrogenase.

In Section 6.3.1.3., the endogenous activity caused by the presence of alcohol dehydrogenase in aldehyde dehydrogenase preparations was described. During two sets of assays on perchloric acid supernatants of whole blood (subjects 21 and 22) a continuous increase in fluorescence was observed. The enzyme sample used did not show greater than normal endogenous activity, so that initially, the increase was thought to be due to acetaldehyde production by the perchloric acid supernatants, possibly similar to that observed by Sippel (1973). However, a set of standard acetaldehyde solutions, also containing ethanol, were assayed using the same enzyme sample, and a continuous increase in fluorescence was seen, similar to that observed with the perchloric acid supernatants of blood. This indicated that the enzyme mixture and not the samples produced the fluorescence increase, and the successful inhibition of this increase with pyrazole showed that the enzyme sample was probably contaminated with alcohol dehydrogenase. The presence of this alcohol dehydrogenase could not be detected using the spectrophotometric assay for the enzyme, but a check showed that one fraction from a gel filtration column which had contained a small amount of alcohol dehydrogenase had been added to the enzyme sample used for these assays. The level of alcohol dehydrogenase in the sample had been just detectable spectrophotometrically when the column run was completed 2 months earlier. The amount of alcohol dehydrogenase present was not sufficient to show an appreciable endogenous rate with mercaptoethanol, but was enough to give a measurable rate with ethanol, for which the maximum rate was

greater. The rate of increase was only equivalent to about 0.01 - 0.02 mg% of acetaldehyde in 30 minutes, so that for the purpose of calculating acetaldehyde levels from the initial 30 minute readings of samples it could be neglected. From results for standard solutions, it was found that each sample showed a constant rate of increase once the diffusion of existing acetaldehyde was complete. Extrapolation of the straight line obtained by plotting successive readings to zero time gave a reading which permitted calculation of the correct acetaldehyde concentration for the standard. Accordingly, an enzyme sample slightly contaminated with alcohol dehydrogenase could be used for acetaldehyde assays, but both the process of plotting a graph for each assay, and the necessity to read each assay 6 or 8 times to obtain the final rate of increase were time consuming. The failure to detect the contaminating alcohol dehydrogenase in the aldehyde dehydrogenase sample described above by spectrophotometric assay showed the importance of completely removing alcohol dehydrogenase during the initial purification of aldehyde dehydrogenase samples.

#### 7.4 DISCUSSION

##### 7.4.1. Production of acetaldehyde by whole blood.

Whole blood samples which contained ethanol were found to give increasing levels of fluorescence with time of incubation in the enzymic assay, and similar samples were found to produce acetaldehyde when assayed by gas chromatography during incubation at similar temperatures. Acetaldehyde was presumably, therefore, the cause of the fluorescence increase in the enzymic assay.

Such production of acetaldehyde had been noted previously by Truitt (1970) who claimed that there were two types of acetaldehyde production in blood containing ethanol. One type of acetaldehyde production, from untreated whole blood, occurred at a rate parallel to that of hemolysis, and this appeared to agree with results found by K.Couchman (1974) and those from the enzymic assays. The second type of acetaldehyde production, however, occurred after the incubation of blood precipitated with  $ZnSO_4 - Ba(OH)_2$ . This production could not be reproduced using blood precipitated with accepted  $ZnSO_4 - Ba(OH)_2$  concentrations, but appeared to occur in blood precipitated at the concentrations of these reagents used by Truitt, which did not give

complete deproteinisation (Couchman, 1974). From experiments with deuterium labelled ethanol, Truitt concluded that acetaldehyde formed in precipitated blood came from a source other than ethanol, and that ethanol merely catalysed its release. Possible sources of the acetaldehyde were suggested by Truitt as "The split off of an acetaldehyde grouping from some protein or a release of bound acetaldehyde ....." . Release of acetaldehyde from precipitated blood had been noted much earlier by both Barker (1941) and Stotz (1943), the release being catalysed by either ethanol, or copper sulphate-calcium hydroxide reagent.

In 1973, the oxidation of ethanol by human erythrocytes was reported (Smalldon, 1973) and the reaction was stated to be catalysed by oxyhemoglobin. It is not clear at present how such oxidation of ethanol by erythrocytes relates to the release of acetaldehyde from blood reported by Truitt (1970), Barker (1941), Stotz (1943) and in this study. It is probable, however, that there are two distinct mechanisms involved.

The release of acetaldehyde from unprecipitated blood may be explained by the oxidation of ethanol to acetaldehyde by oxyhemoglobin. Alternatively, catalase, which is capable of catalysing ethanol oxidation in liver (Oshino et al., 1973), and which occurs in red blood cells, could explain the production of acetaldehyde from unprecipitated blood containing ethanol. Ethanol release from precipitated blood, however, may be due as suggested by Truitt (1970) to protein breakdown, or release of bound acetaldehyde. If there are, in fact, two types of acetaldehyde production, this would make the results of Truitt (1970) very difficult to interpret, as the method used for blood treatment did not give complete precipitation, so that both types of production could have been observed simultaneously.

The formation of acetaldehyde, independent of ethanol, by erythrocyte ghosts has been reported (Lionetti et al., 1964) but the reaction could not explain acetaldehyde production from whole blood observed in this study as this did not occur in the absence of ethanol, to any significant extent.

#### 7.4.2. Relative levels of acetaldehyde found in plasma and red blood cells.

The low levels of acetaldehyde found in plasma not treated with perchloric acid were initially considered due to some discrepancy in results between gas chromatography and enzymic assays, or to a difference between perchlorate treated and untreated samples. The two assays were found to agree on whole blood samples, however, and assay of supernatants from perchlorate treated plasma showed that low levels still occurred. It appeared, therefore, that plasma exhibited a significantly lower level of acetaldehyde than did whole blood. The finding of elevated levels of acetaldehyde in red blood cells compared with whole blood confirmed that loss of acetaldehyde from plasma was unlikely to explain the low levels.

Variations in acetaldehyde levels in plasma and red blood cells might explain some of the variation in acetaldehyde levels in the literature. Lundquist and Wolthers (1958) who have quoted some of the lowest levels for blood acetaldehyde following ethanol intake, used plasma rather than whole blood to obtain these values. The levels reported by these workers corresponded fairly closely to the levels of acetaldehyde found in plasma in this study. For a blood alcohol level of 30 - 50 mg%, Lundquist and Wolthers gave plasma acetaldehyde levels of 0.0 - 0.03 mg%. In the present work, for similar blood alcohol levels, plasma acetaldehyde levels of 0.0 - 0.035 mg% were observed, with whole blood levels being considerably higher (0.05 - 0.25 mg%). Lundquist and Wolthers (1958) calculated theoretical levels of acetaldehyde that should appear in the blood following ethanol intake, and found values very close to their experimental plasma levels. For a blood ethanol concentration of 46 mg%, and assuming the velocity of ethanol removal to be equal to the velocity of acetaldehyde removal, a theoretical blood acetaldehyde concentration of 0.029 mg% was estimated. If the velocity of acetaldehyde removal was assumed to be faster, the theoretical acetaldehyde level would be lower still. The agreement between Lundquist and Wolthers calculated blood acetaldehyde concentrations and the experimental plasma levels could indicate that the elevated acetaldehyde levels found in red blood cells were due to an aspect of metabolism or an artefact of sample treatment not as yet accounted for.

There are several possibilities to explain the means by which red blood cells could obtain an elevated acetaldehyde level, for example:

(a) The increased level of acetaldehyde in red blood cells may be related to the oxidation of ethanol observed in erythrocytes (Smalldon, 1973) and/or to the ethanol-induced production of acetaldehyde seen in whole blood in this study and by Truitt (1970). The situation of such acetaldehyde producing mechanisms within the red blood cell would mean that acetaldehyde in blood, rather than being a product of ethanol metabolism in the liver, would be a product of ethanol metabolism or action within the red blood cell. For this explanation to apply, however, it would be necessary for a barrier to exist against acetaldehyde diffusion from the red blood cell to the plasma.

(b) Red blood cells might be capable of selective uptake of acetaldehyde, although results on the addition of acetaldehyde to whole blood made this appear unlikely. Formaldehyde uptake by red blood cells has been reported for dogs blood (Malorny et al, 1965) and human blood (Jung and Onnen, 1955), but Freundt (1970) found that red blood cells did not take up acetaldehyde.

(c) If plasma proteins bound acetaldehyde and proteins in the red blood cell did not, a difference in free acetaldehyde levels in the two places could possibly result.

(d) Perchloric acid could possibly interact with something present in red blood cells, but not in plasma, to produce acetaldehyde in the presence of ethanol.

With present evidence, the first explanation would appear to be most likely, although no evidence has been found for a barrier to diffusion of acetaldehyde from the red blood cell. An alternative possibility to a diffusion barrier would be the existence of an acetaldehyde breakdown mechanism operative in the red blood cell membrane. The existence of such a mechanism was suggested by Lionetti et al. (1964). These workers found turnover of acetaldehyde formed from deoxynucleoside metabolism in erythrocyte ghosts, indicating that as well as a production mechanism there existed an effective removal system for acetaldehyde in the red blood cell membrane. This could mean that acetaldehyde produced in the red blood cell matrix would be metabolised as it diffused out through the red blood cell membrane, thus never reaching the plasma in concentrations similar to those in the red blood cell.

The final explanation for elevated acetaldehyde levels in red blood cells may be any one, or a combination of several of the above possibilities, but obviously at this stage there is a clear requirement for further investigation into this aspect of acetaldehyde metabolism.

#### 7.4.3. Loss of acetaldehyde from whole blood.

The experiment attempting to recover acetaldehyde added to whole blood illustrated that loss of acetaldehyde occurred even when blood was kept on ice. No evidence of selective uptake of acetaldehyde by red blood cells was seen, the concentration being less in this fraction than in plasma from blood separated after 30 minutes on ice. Complete removal of acetaldehyde did not occur following incubation at 32°C for 1 hour, but a sample of blood to which no acetaldehyde had been added showed the same level as the blood to which acetaldehyde had been added. This appeared to indicate that a small amount of acetaldehyde production could occur in blood without added ethanol present, but that all the exogenous acetaldehyde had been removed.

Acetaldehyde loss from whole blood has been reported previously (Stotz, 1943; Duritz and Truitt, 1964). Stotz found that loss was much more rapid from whole blood than from plasma, although he found that there was no significant loss from whole blood stored on ice for 20 minutes, a 40 - 50% decrease in levels taking as much as 14 hours. Separated plasma only lost 6 - 10% of its acetaldehyde in 14 hours. Acetaldehyde added to plasma was quantitatively recovered by Lundquist (1958) who did not, however, show recoveries for acetaldehyde added to whole blood.

Evidence for an aldehyde dehydrogenase in non-nucleated erythrocytes from various species, including humans, was presented by Matthies (1957; 1958). The enzyme was mainly assayed using formaldehyde rather than acetaldehyde, and appeared to be in the matrix rather than in the red blood cell membrane. The relationship of this apparent aldehyde dehydrogenase activity to the acetaldehyde loss from whole, human blood samples still remains to be elucidated. Catalase has also been suggested as responsible for the removal of formaldehyde from blood (Malorny *et al.*, 1965), but Freundt (1970) stated that the catalase inhibitor 3-amino 1,2,4 triazole did not influence acetaldehyde turnover in blood in vivo or in vitro.

#### 7.4.4. Levels of acetaldehyde in perchlorate supernatants of whole blood following ethanol intake.

Following the discovery that levels of acetaldehyde in red blood cells and plasma differ, the significance of whole blood levels of acetaldehyde is somewhat doubtful. Such levels could differ depending on the degree of extraction of acetaldehyde from red blood cells during perchlorate precipitation. Also, since the source of acetaldehyde in whole blood is in doubt, levels cannot be related to any other metabolic parameters determined during the testing programme with any real meaning. For example, it would not be relevant to compare individual rates of ethanol breakdown with blood levels of acetaldehyde until there is some evidence that acetaldehyde in blood results from the same reaction pathway as does the majority of ethanol removal from the body. It is interesting, however, to compare the levels of acetaldehyde found in whole blood samples in this study with others for similar studies reported in the literature.

In the present study, for blood alcohol levels of between 20 and 80 mg% (Couchman, 1974), blood acetaldehyde levels were between 0.05 and 0.25 mg%. This places the results among the lowest in the literature, with the exception of Lundquist and Wolthers results (1958) where "blood" levels of acetaldehyde are actually plasma levels, and a study by Truitt (1971), where whole blood levels of 0.005 - 0.01 mg% were reported. The latter result is difficult to reconcile with another report by the same author (Truitt and Walsh, 1971) where a range of 0.01 - 0.056 mg% was given, and levels in the earlier report were referred to as less than 0.1 mg%.

In a recent study on blood levels of acetaldehyde in alcoholics (Majchrowicz and Mendelson, 1970) levels of 0.04 - 0.20 mg% were found by gas chromatographic assay of zinc sulphate-barium hydroxide precipitated blood, for ethanol levels of 100 - 400 mg%. For subjects drinking grain ethanol, at 1 - 100 mg% ethanol, acetaldehyde levels were about 0.03 - 0.05 mg% - lower than the levels found for the normal subjects used in this work. In an even more recent study comparing alcoholics and non-alcoholics, Korsten *et al.* (1975) found levels of 0.1 - 0.16 mg% acetaldehyde in normal subjects, and 0.16 - 0.22 mg% in alcoholics, at 100 - 200 mg% ethanol. Perchlorate supernatants of whole blood were used in this study, and acetaldehyde levels for alcoholics were higher than those of Majchrowicz and Mendelson (1970).

Korsten et al (1975) found a steady decrease in levels of acetaldehyde with decreasing ethanol, below 100 mg%. Between 20 and 100 mg% ethanol, levels of acetaldehyde found for normal subjects were 0.01 - 0.13 mg%, and for alcoholics, 0.02 - 0.21 mg%, the range of values for normal subjects being again slightly lower than that found in the present study. In the present study, however, no definite trend for the acetaldehyde level to decrease with decreasing ethanol level was seen. Acetaldehyde levels tended to remain constant over the time range studied, while blood ethanol levels fell, on an average, from 50 - 15 mg% (Couchman, 1974). Any drop in acetaldehyde level that was seen in the present study was not nearly as marked as that seen by Korsten et al. (1975). This could be due to the fact that ethanol was infused for several hours in the latter study, prior to beginning measurement of ethanol and acetaldehyde levels in blood. The maximum ethanol level used by Korsten et al. was also higher, being raised to a constant level of about 200 mg% before the experiment began.

Acetaldehyde production from ethanol, apparently catalysed by ascorbic acid, has been found in perchloric acid extracts of rat blood, and rat liver homogenates (Sippel, 1973). There was no evidence of acetaldehyde production by perchloric acid supernatants of whole blood in either the enzymic or the gas chromatographic assays in the present study, but Korsten et al. (1975) found acetaldehyde production when ethanol was added to perchloric acid extracts of whole blood from human subjects. Both Sippel and Korsten et al. used incubation temperatures of 60°C or more in their assays, whereas temperatures used in this work were much lower. This may explain the absence of acetaldehyde production during assays in the present study, but there is still the possibility that some non-enzymatic ethanol oxidation occurred in the perchlorate extracts prior to their incubation. Korsten et al. added thiourea to their samples, as this was reported by Sippel (1972) to prevent acetaldehyde production, and this may explain the lower range of blood acetaldehyde levels found by these workers. Majchrowicz and Mendelson (1970) used a neutral precipitant, which may explain the very low range of their values for alcoholics.

Considering the results of this study, and the other two recent studies discussed above, it would appear that whole blood levels of acetaldehyde following ethanol intake range between 0.01 and 0.25 mg% and that alcoholics do not consistently show higher levels than normal

subjects when all three sets of results are compared. The range of variation has been reduced compared with earlier studies, but there are still inconsistencies. Such variation might be eliminated if plasma levels of acetaldehyde were studied as well as whole blood levels, and so provide a means of distinguishing between acetaldehyde arising from metabolism in the liver and other tissues, and acetaldehyde arising from the enzymic or other reactions of ethanol in red blood cells.

## SECTION 8

CONTINUING STUDIES8.1 ENZYMOLOGY OF ACETALDEHYDE METABOLISM

## 8.1.1. Significance of microsomal aldehyde dehydrogenase.

The existence of a microsomal aldehyde dehydrogenase could be significant in view of the proliferation of smooth endoplasmic reticulum and the increased activity of microsomal enzymes observed following prolonged ethanol intake (Rubin and Hutterer, 1968; Lieber and DeCarli, 1970 a). The increase in microsomal material has been attributed to the occurrence of a microsomal ethanol oxidising system (Lieber and DeCarli, 1970 b), but the existence of this enzyme as a distinct entity has been questioned (Oshino et al., 1973; Carter and Isselbacher, 1971). Although an apparent separation of the microsomal ethanol oxidising system from catalase and alcohol dehydrogenase has now been achieved (Teschke et al., 1972), the possible importance of the increase in microsomal material in relation to accelerated rates of ethanol metabolism (Lieber and DeCarli, 1972) could justify further investigations of both (a) the microsomal aldehyde dehydrogenase itself, and (b) the effects of acetaldehyde administration on microsomal enzymes.

## 8.1.2. Differences between cytoplasmic and mitochondrial enzymes.

The results of the present investigation and associated studies in the department (T.M.Kitson, A.MacGibbon) on the isolation and characterisation of mitochondrial and cytoplasmic aldehyde dehydrogenases showed that both enzymes were of similar overall charge, molecular weight, and subunit molecular weight, but exhibited some differences in specificity, inhibitor response, and kinetic properties. The two enzymes also exhibited different abilities to give a positive reaction with the PMS-NBT activity stain. Since a proportion of the colour developed with this stain by aldehyde dehydrogenase was possibly due to a sulphhydryl-PMS complex, it may be suggested that there could be a difference in the number and/or location of free sulphhydryl groups in the two enzymes. The mitochondrial and cytoplasmic enzymes have different sensitivities

to disulfiram (T.M.Kitson, personal communication). Since disulfiram reacts with sulphhydryl groups (Niems et al, 1966), this also suggests that the two enzymes may differ in their sulphhydryl group content. Further studies have now been planned to include comparisons of the amino acid composition and sulphhydryl group content of cytoplasmic and mitochondrial sheep liver aldehyde dehydrogenases.

### 8.1.3. Relative significance of cytoplasmic and mitochondrial enzymes.

Eckfeldt et al. (1975) suggest that the cytosolic aldehyde dehydrogenase from horse liver may be of greater importance in the oxidation of acetaldehyde than the mitochondrial enzyme, but this is at variance with the reports by Hassinen et al. (1970), Lindros et al. (1972) and Parrilla et al. (1974) on the relative significance of mitochondrial and cytoplasmic acetaldehyde oxidation in rat liver.

The study of acetaldehyde metabolism in perfused livers or liver homogenates from sheep, from which two aldehyde dehydrogenases (this study) and aldehyde oxidase (Hendtlass, 1973) have now been characterised in detail, could provide more information on the relative contribution of mitochondrial and cytoplasmic aldehyde dehydrogenases to the breakdown of acetaldehyde.

## 8.2 IN VIVO AND IN VITRO STUDIES ON ACETALDEHYDE METABOLISM IN HUMAN SUBJECTS

To obtain more information on possible causes of alcohol addiction, the study of acetaldehyde metabolism in human subjects would seem to be of prime importance. The study of liver aldehyde dehydrogenase from humans would be complicated by the problem of obtaining liver samples fresh enough to enable separation of mitochondria and cytoplasm. Such a separation of intracellular fractions would be essential if a clear picture of acetaldehyde metabolism in human livers was to be obtained. The results for the assay of blood acetaldehyde reported in this study have indicated that the use of whole blood or plasma levels of acetaldehyde in attempts to determine a possible correlation between acetaldehyde and alcohol addiction may not be valid.

For further work in the department, stable isotopes are to be used to study both the turnover rates of acetaldehyde, and the distribution of acetaldehyde in blood samples, following labelled ethanol

administration to human volunteers. Before these studies can commence, however, more information is needed on the mechanisms involved in the production and metabolism of acetaldehyde in human blood, and work is already continuing on this problem area.

The estimation of acetaldehyde levels in the blood of volunteers following ethanol ingestion is to be continued. Plasma levels will also be determined to provide a consistent picture of differences between these and whole blood levels.

## SECTION 9

APPENDIX 1 - THE USE OF DEAE PROTION IN SEPARATION OF ALDEHYDE  
DEHYDROGENASE9.1 INTRODUCTION

Protion is a viscose (regenerated cellulose) ion exchange resin, developed by Grant (1971). It can be manufactured in both carboxymethyl and diethylaminoethyl forms, and because of a rigid granular structure has flow characteristics superior to fibrous cellulose ion exchange resins. The improved flow characteristics provide better packing qualities, and less fines form than with fibrous resins. For these reasons, Protion resins are ideal for large scale protein separations. Since large quantities of aldehyde dehydrogenase were required for both aldehyde assays, and kinetic study by other research workers in the department, Protion was used in initial attempts to purify aldehyde dehydrogenase.

9.2 METHODS9.2.1. Resin preparation - conditioning and regeneration of DEAE Protion and DEAE cellulose.

Initial conditioning of DEAE Protion was carried out by washing the resin successively in 0.5 M HCl, water to a neutral pH, 0.5 M NaOH, and again water to a neutral pH. Regeneration of resin between each run involved washing with 1 M NaCl, water, 0.5 M NaOH, and water to a neutral pH. Equilibration with the desired buffer was carried out by adding the acid component of the buffer, at 0.2 - 0.5 M, lowering the pH to less than 4.5 with HCl, and degassing the resin by evacuation in a Buchner flask. Sufficient of the base component of the buffer was added to bring the resin to the desired pH, and the ionic strength was adjusted in small steps to the level required. The resin was usually fully equilibrated before packing and was always removed from the column before regeneration with NaOH. Washing with 1 M NaCl was usually carried out in the column. DEAE cellulose (Whatman DE 32) was treated in the same way as DEAE Protion, unless otherwise stated.

### 9.2.2. Alkali treatment of Protion.

Protion was treated with NaOH under varying conditions to improve capacity. Exact details of such treatment will be given in the relevant results section.

### 9.2.3. Enzyme preparation.

The enzyme sample used in comparative column work was a 45 - 70% ammonium sulphate precipitate of sheep liver cytosol which was redissolved and extensively dialysed against the initial elution buffer. The sample was always checked for correct ionic strength and pH prior to column runs. Protein was determined by the method of Lowry *et al.* (1951). Aldehyde dehydrogenase activity was estimated as described in Section 2.2.3.(a).

### 9.2.4. Column runs.

Both gradient and stepwise systems were used in comparing DEAE Protion, DEAE cellulose, and NaOH-treated DEAE Protion. All relevant details for each run are stated with tables and figures in the results section.

Protein levels in column effluents were usually determined by absorbance at 280 nm, and aldehyde dehydrogenase activities in fractions by the normal procedure described in Section 2.2.3.(a).

In some cases, absorbance at 400 nm, due largely to catalase, was recorded as an extra guide to column capacity and function. Catalase was eluted just after aldehyde dehydrogenase in gradient runs. If column overloading occurred, catalase would usually be eluted early as well as aldehyde dehydrogenase. Accordingly, monitoring 400 nm absorbance gave an additional guide to capacity of the column. In stepwise runs, catalase remained bound to the column during the initial elution, and measurement of the resultant green band at the top of the column was a further guide to column function.

The phosphate buffers used for column runs were prepared according to Dawson *et al.* (1969, p 490). The concentrations given refer to the initial potassium dihydrogen phosphate and sodium hydroxide solutions, and the actual concentration of phosphate ions in the final buffer is half this value.

### 9.3 RESULTS

While using the initial DEAE Protion product for enzyme purification it became clear that the resin did not have as great a capacity for binding aldehyde dehydrogenase as was observed with DEAE cellulose, although the bovine serum albumin capacities for the two resins were reported to be similar (J.S.Ayers, personal communication). As shown in Figure 9.1 and Table 9.1, DEAE Protion was overloaded by aldehyde dehydrogenase activity at very low protein - resin ratios, an unbound fraction eluting before the main band of activity. Identity of the unbound enzyme was compared with that of the bound enzyme by examining specificities, and the use of gel electrophoresis, and no significant differences were found. Re-running of unbound enzyme after ammonium sulphate precipitation, in an identical column run, showed that the enzyme was bound in the second run. This distinguished it from an unretarded activity found in whole liver homogenates which, when re-run following ammonium sulphate treatment, was still unretarded. The significance of this true unretarded activity was discussed in Section 4.

The capacity of DEAE Protion also seemed to decrease with repeated use of the resin, the deterioration occurring at a much greater rate than any decrease in capacity of cellulose resins. One reason for this may have been fouling of the resin by nucleic acids, since the capacity could be improved to some extent by heating in 1.25 M NaOH for 4 hours and the substances removed appeared to be nucleic acid in nature (Morrison, 1973).

The two defects, namely low capacity and rapid fouling of the resin made the initial Protion product unusable in the present purpose. It was difficult to ascertain readily whether results being obtained were due to resin failure or to genuine properties of the enzyme being purified. On the basis of these results it was decided to stop using the original product until such time as it could be improved, and until it was clear whether the enzyme from pure cytosolic extracts was fully retarded on conventional resins under the same conditions.

Meanwhile, work continuing in the department showed that the capacity of DEAE Protion could be increased by treating it with NaOH, without destroying the excellent flow properties of the resin. This new treated resin was subsequently tested for use in the purification of aldehyde dehydrogenase.

TABLE 9.1

COMPARISON OF GRADIENT RUNS ON ORIGINAL DEAE PROTION, DEAE  
CELLULOSE, AND NaOH TREATED DEAE PROTION

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The column runs shown in this table are illustrated in Figures 9.1 (original DEAE Protion), 9.2 (NaOH treated DEAE Protion) and 9.3 (DEAE cellulose). Resin loading is expressed in terms of g protein/cm<sup>3</sup> resin.

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	Original DEAE Protion	DEAE Protion NaOH treated	DEAE cellulose
Column volume (cm <sup>3</sup> )	480	100	170
Sample volume (cm <sup>3</sup> )	30	70	60
Sample protein (mg/cm <sup>3</sup> )	30	20.5	48.5
Total protein (g)	0.90	1.44	2.91
Resin loading (g/cm <sup>3</sup> )	0.0019	0.014	0.017
Activity overload (%)	10	none	none

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Initially, gradient runs were tried on the new resin to check for improvements in elution patterns, and in binding of aldehyde dehydrogenase. A marked improvement in elution pattern was observed with the new resin, (Figure 9.2) and columns could be loaded to a much higher level of protein before any overload occurred (Table 9.1).

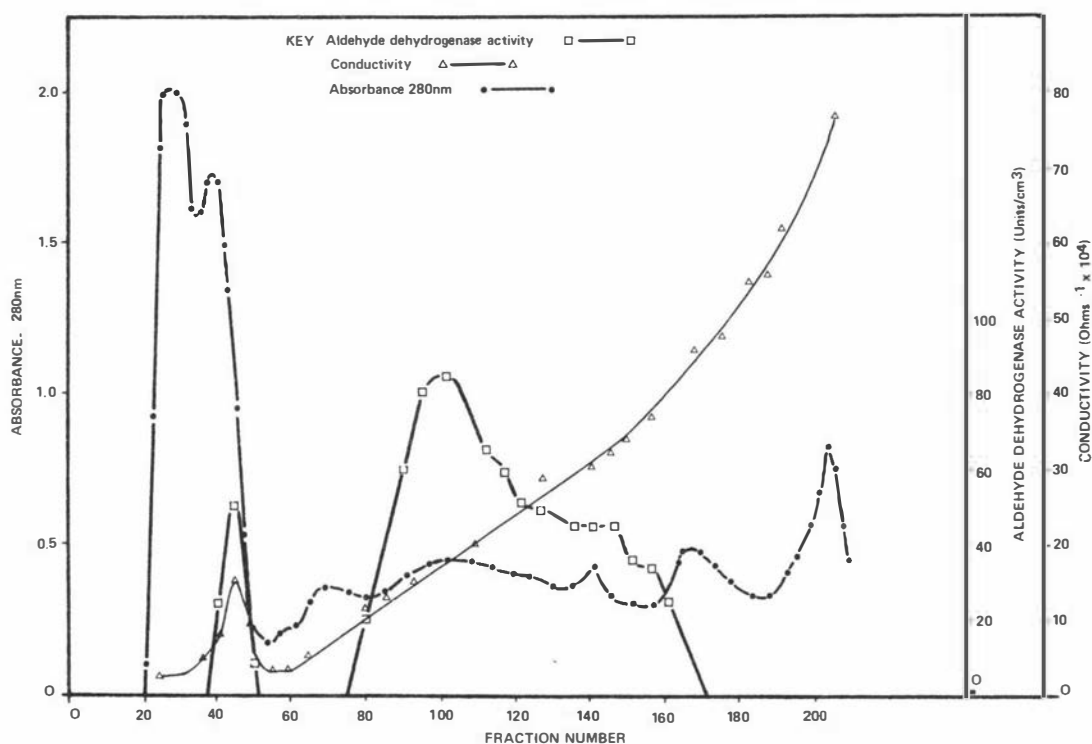
Peak levels of 280 nm absorbance observed during elution of later peaks from the gradient on original DEAE Protion were nearly all less than 25% of the 280 nm absorbance observed in the initial peak (Figure 9.1). However, later peaks eluted during the gradient on DEAE cellulose (Figure 9.3) or from NaOH-treated Protion (Figure 9.2) were above 70% of the levels initially eluted from these resins. The protein profile on the new resin, shown in Figure 9.2, was very similar to that observed with DEAE cellulose (Figure 9.3).

Comparison of two resin treatments, 8% and 10% NaOH, showed little difference between the two. This is illustrated by Figures 9.4 and 9.5. Elution profiles were almost identical in the two gradient runs, and neither column showed any signs of overload. Aldehyde dehydrogenase activity appeared at an eluate conductivity of  $25 \times 10^4 \text{ ohms}^{-1}$  in each case, and peaked at  $41.5 \times 10^4 \text{ ohms}^{-1}$  on the 8% resin, and at  $42.5 \times 10^4 \text{ ohms}^{-1}$  on the 10% resin. Absorption peaks (400 nm) occurred at 30 and  $73.5 \times 10^4 \text{ ohms}^{-1}$  on 8% resin, and at 27.5 and  $72.5 \times 10^4 \text{ ohms}^{-1}$  on the 10% resin. Such close agreement indicated that the improvement from NaOH treatment was reproducible using 8 and 10% NaOH.

The treated resin, when used in stepwise runs for separation of aldehyde dehydrogenase showed similarly improved properties. Overloading occurred at much higher protein levels than in similar runs on the original resin. Figure 9.6 gives an example of stepwise elution patterns which once again compared favourably with a similar DEAE cellulose run (Figure 9.8). The DEAE cellulose shows sharper peaking of 400 nm absorbance at the third buffer change, and a little less 400 nm absorbance with the second buffer, but otherwise the two column runs are very similar. The length of green band remaining bound on the Protion column was 12 cm on a 15.2 cm column, and with the cellulose resin, 6.7 cm in a 15.2 cm column.

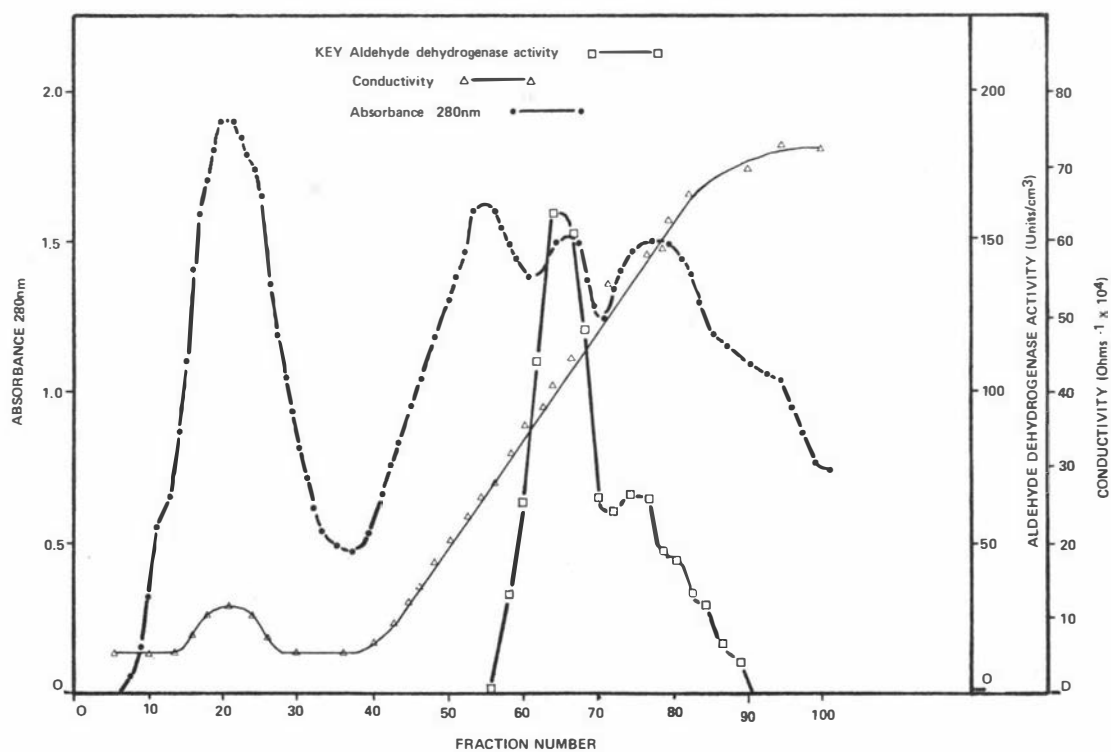
The length of NaOH treatment and the volume of NaOH used influenced the capacity obtained. Treatment with 8% NaOH for 20 minutes, at a ratio of  $5 \text{ cm}^3/\text{g}$  resin, gave a product which had less capacity than one treated at a ratio of  $10 \text{ cm}^3/\text{g}$  resin for 1 hour. This is illustrated

FIGURE 9.1 ELUTION OF ALDEHYDE DEHYDROGENASE ON ORIGINAL DEAE PROTION



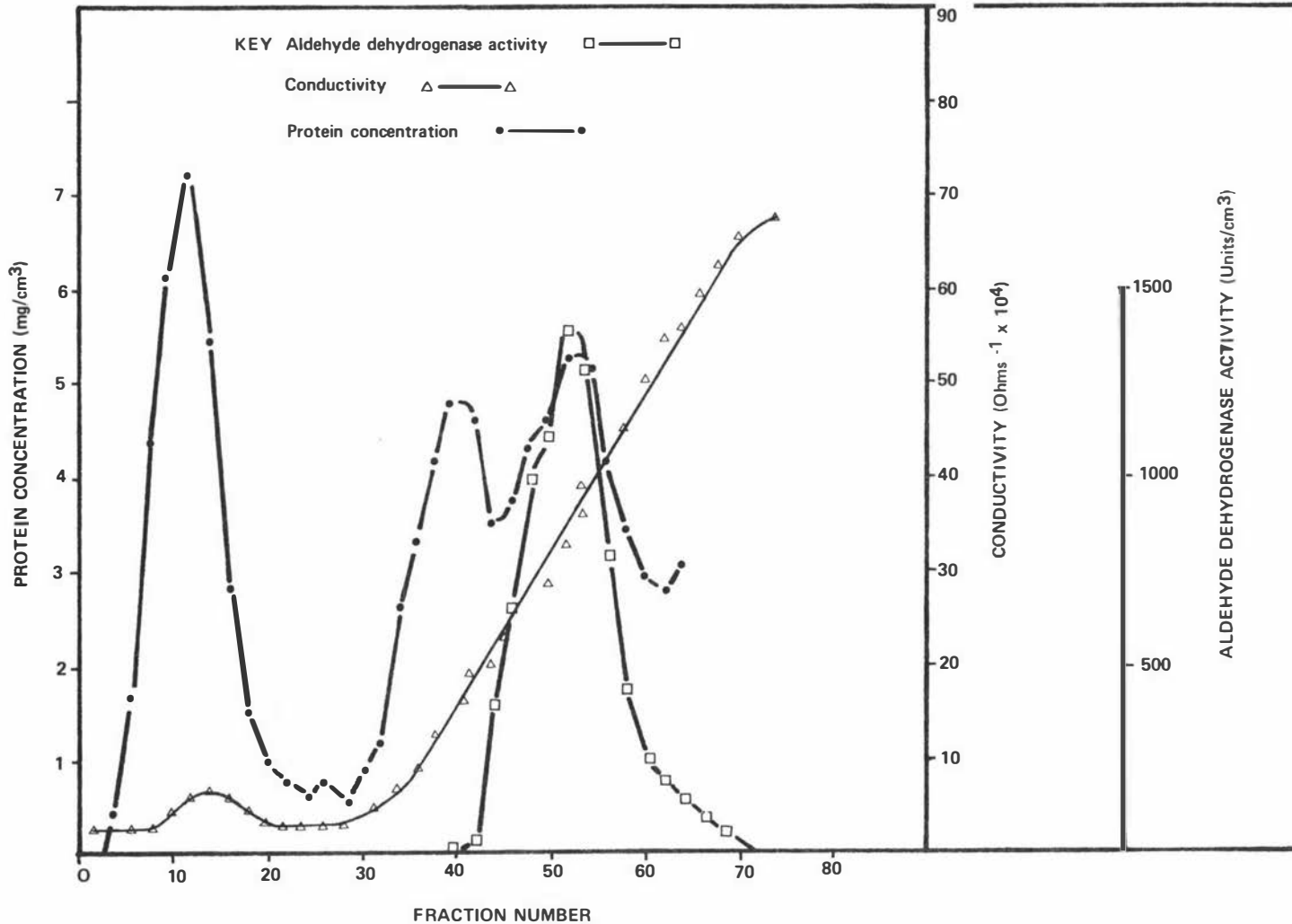
Volume of column, 480 cm<sup>3</sup>; protein load, 0.0019 g protein /cm<sup>3</sup> resin; gradient generated using 500 cm<sup>3</sup> of 0.0025M and 500 cm of 0.1M phosphate buffer, pH 7.3; flow rate, 1.8cm<sup>3</sup>/min. Aldehyde dehydrogenase activity is expressed in absorbance units (1 unit of enzyme activity = 0.001 absorbance units/min.).

FIGURE 9.2 ELUTION OF ALDEHYDE DEHYDROGENASE ON NaOH TREATED PROTION



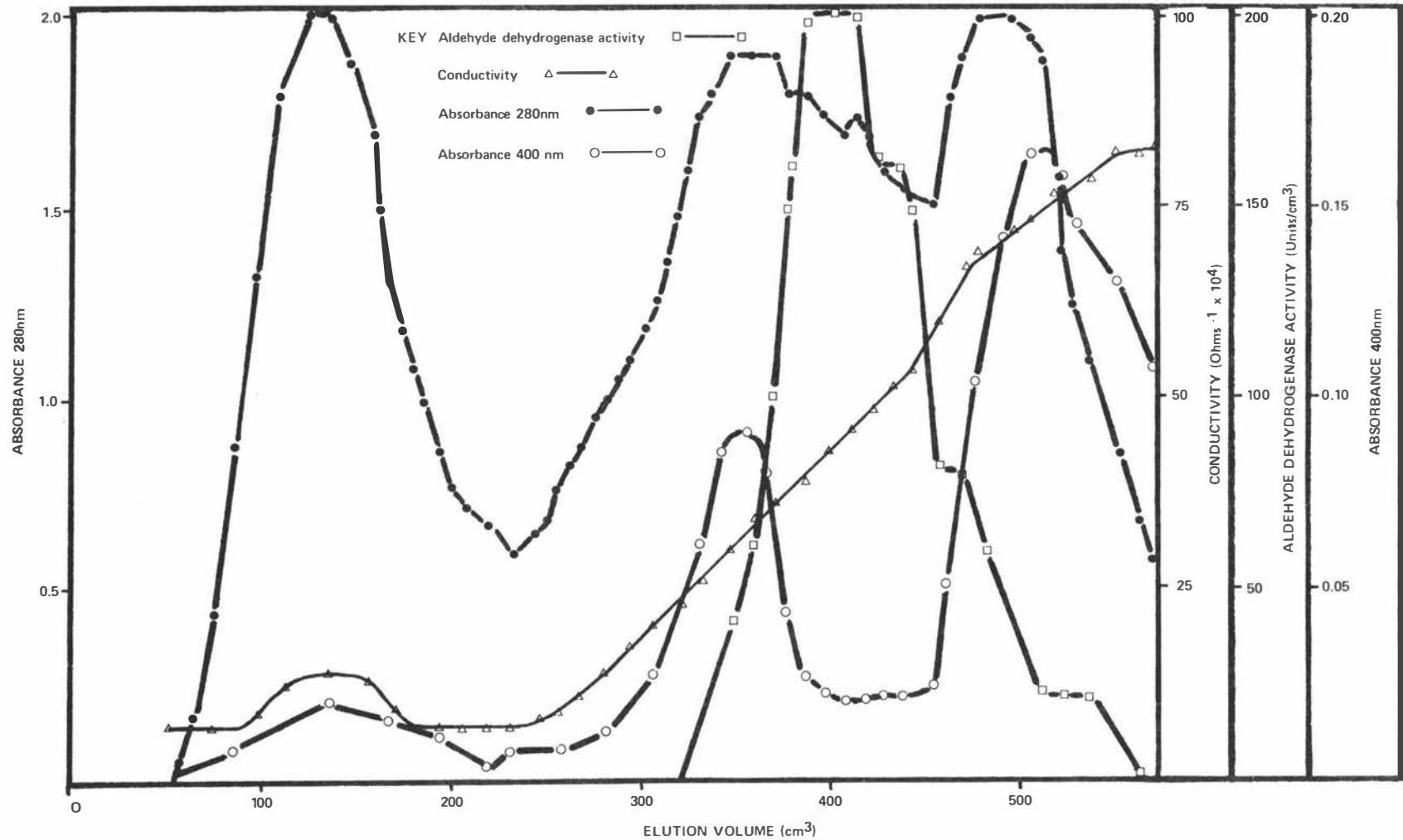
Volume of column, 100cm<sup>3</sup>; protein load, 0.014 g protein /cm<sup>3</sup> resin; gradient generated using 150cm<sup>3</sup> 0.005M and 150 cm<sup>3</sup> 0.1M phosphate buffer, pH 7.3. An initial wash of 80cm<sup>3</sup> of 0.005M buffer was used before starting the gradient. The flow rate was 1.25 cm<sup>3</sup>/min. The protion used was treated with 10% NaOH. Aldehyde dehydrogenase activity is expressed in absorbance units (1 unit of enzyme activity = 0.001 absorbance units/min.).

FIGURE 9.3 ELUTION OF ALDEHYDE DEHYDROGENASE ON DEAE CELLULOSE



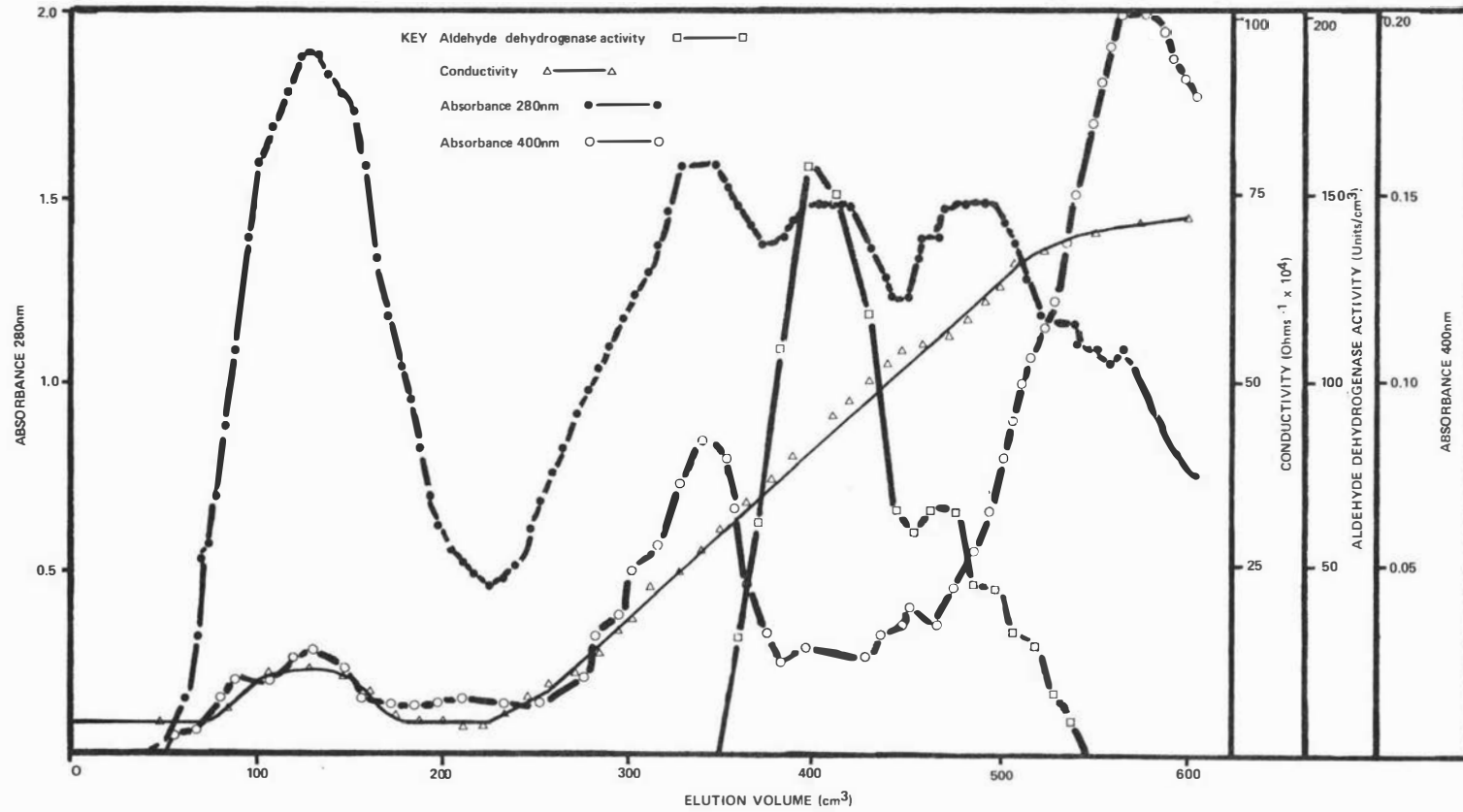
Volume of column, 170cm<sup>3</sup>; protein load, 0.017 g protein /cm<sup>3</sup> resin; gradient generated using 200cm<sup>3</sup> of 0.0025M and 200cm<sup>3</sup> of 0.1M phosphate buffer, pH 7.3. An initial wash of 60cm<sup>3</sup> of 0.0025M buffer was used before starting the gradient. The flow rate was 1.5cm<sup>3</sup>/min. Protein levels were estimated by Folins protein determination, rather than by absorbance at 280nm. This was necessary because of high protein levels eluted, due to a high loading concentration. Aldehyde dehydrogenase activity is expressed in absorbance units (1 unit of enzyme activity = 0.001 absorbance units/min.).

FIGURE 9.4 ELUTION OF ALDEHYDE DEHYDROGENASE ON NaOH TREATED PROTION (8%)



Volume of column, 100cm<sup>3</sup>; protein load, 0.014 g protein /cm<sup>3</sup> resin; gradient generated using 150 cm<sup>3</sup> 0.005M phosphate buffer, pH 7.3, and 150 cm<sup>3</sup> of 0.1M phosphate buffer, pH 7.3. An initial wash of 80cm<sup>3</sup> of 0.005M buffer was used before starting the gradient. The flow rate was 1.25cm<sup>3</sup>/min. The protion used was treated with 8% NaOH. Aldehyde dehydrogenase activity is expressed in absorbance units (1 unit of enzyme activity = 0.001 absorbance units/min).

FIGURE 9.5 ELUTION OF ALDEHYDE DEHYDROGENASE ON NaOH TREATED PROTION (10%)



Volume of column, 100cm<sup>3</sup>; protein load, 0.014 g protein /cm<sup>3</sup> resin; gradient generated using 150cm<sup>3</sup> of 0.005M and 150cm<sup>3</sup> of 0.1M phosphate buffer, pH 7.3. An initial wash of 80cm<sup>3</sup> of 0.005M buffer was used before starting the gradient. The flow rate was 1.25cm<sup>3</sup>/min. The protion used was treated with 10% NaOH. Aldehyde dehydrogenase activity is expressed in absorbance units (1 unit of enzyme activity = 0.001 absorbance units/min). Some of the same data has been used to plot the above graph as shown in Figure 9.2, but it is reproduced to give simpler comparison with Figure 9.4. The column runs represented in Figures 9.4 and 9.5 were carried out under identical conditions. Results are plotted against volume eluted rather than fraction number, to avoid differences due to different fraction volumes. Absorbance at 400nm is included as an extra indication of column function.

by Table 9.2 and by Figures 9.6 and 9.7.

The two 8% NaOH treated resins in these experiments were loaded with a sample at a relatively high protein concentration. To assess the effect of this protein concentration, a similar loading in terms of total protein was applied in a more dilute form, namely  $7.0 \text{ mg/cm}^3$  instead of  $26.5 \text{ mg/cm}^3$ . Figure 9.9 shows DEAE Protion treated with 8% NaOH for 1 hour, and Figure 9.10, DEAE Protion treated with 8% NaOH for 20 minutes. Apart from the broader initial protein peak in Figure 9.9, the elution patterns of Figures 9.6 and 9.9 are very similar. The length of green band bound to the column with dilute loading was 10 cm as compared with 12 cm in the case with the more concentrated protein load. Figure 9.10 compared with Figure 9.7 shows less overload, but the 20 minute treated resin still exhibited a less satisfactory elution pattern than the resin treated for a longer time, even with the dilute protein load.

As the treatment with 8% NaOH for 1 hour gave a resin comparable to DEAE cellulose in elution characteristics, a similar method was used to prepare resin for a large-scale preparative run. This run gave good results (Figure 9.11), similar to earlier small-scale stepwise runs, and indicated that the resin could be successfully treated in larger batches, and that large-scale columns still provided good resolution with good capacity. During subsequent recycling, however, the resin capacity appeared to deteriorate, and by the third run (Figure 9.12) the resin's properties resembled those of a much less treated product, as for example that shown in Figure 9.7. The resin overloaded with aldehyde dehydrogenase activity, and gave high absorbance at 400 nm in both the first and second elution buffers. Since the same resin sample was used in each of these runs, and no other factors were significantly different (Table 9.3), it appeared that the resin had lost much of its capacity after being recycled only twice. The resin showed no change in total volume during the two recycling procedures.

Similar large-scale columns were tried using DEAE cellulose. Runs were longer, as washing rates were limited by the flow rate of the column. During successive runs there was a deterioration observed in the properties of DEAE cellulose also. Overloading did not occur, but 400 nm absorbance in the second buffer wash increased greatly. It appeared that the column was losing capacity for binding catalase during the second buffer wash. The cellulose was being recycled by washing in 1 M NaCl,

TABLE 9.2

NaOH TREATED PROTION - COMPARISON OF TWO  
DIFFERENT TREATMENT PROCEDURES

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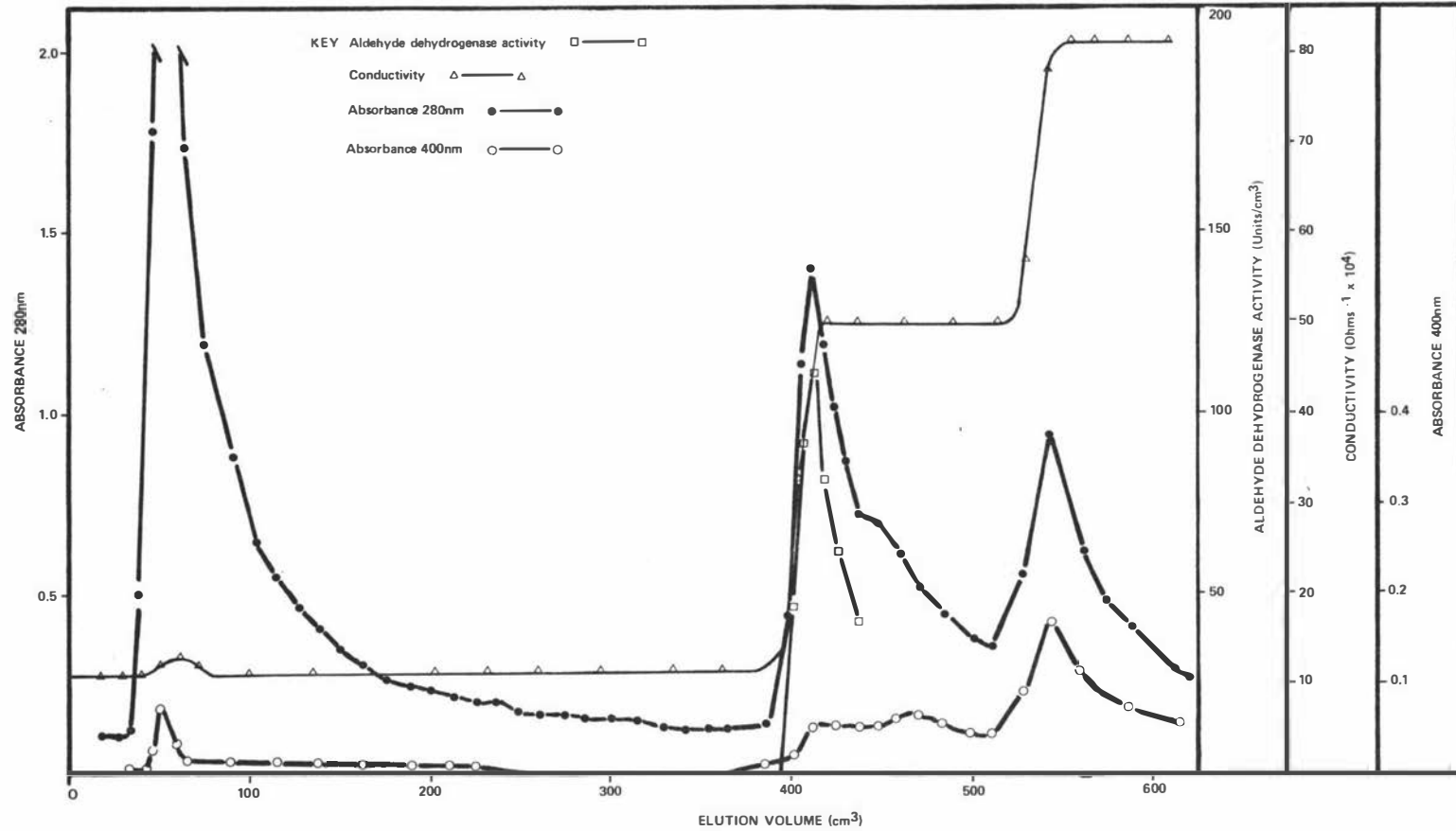
R 1 represents resin swollen in 200cm<sup>3</sup> of 8% NaOH for 20 minutes, and R 11, resin swollen in 400cm<sup>3</sup> of 8% NaOH for 1 hour. In each case, 40g of resin were used. Typical elution patterns are shown in Figures 9.6 and 9.7.

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	R 1	R 11
Column length (cm)	16.5	16.5
Column volume (cm <sup>3</sup> )	38.0	40.0
Sample volume (cm <sup>3</sup> )	14.0	14.0
Protein (mg/cm <sup>3</sup> )	26.5	26.5
Total protein (g)	0.37	0.37
Protein load (g/cm <sup>3</sup> )	0.0098	0.0093
Activity overload (%)	12.0	none
Length of green band (cm)	full length of column	12.0

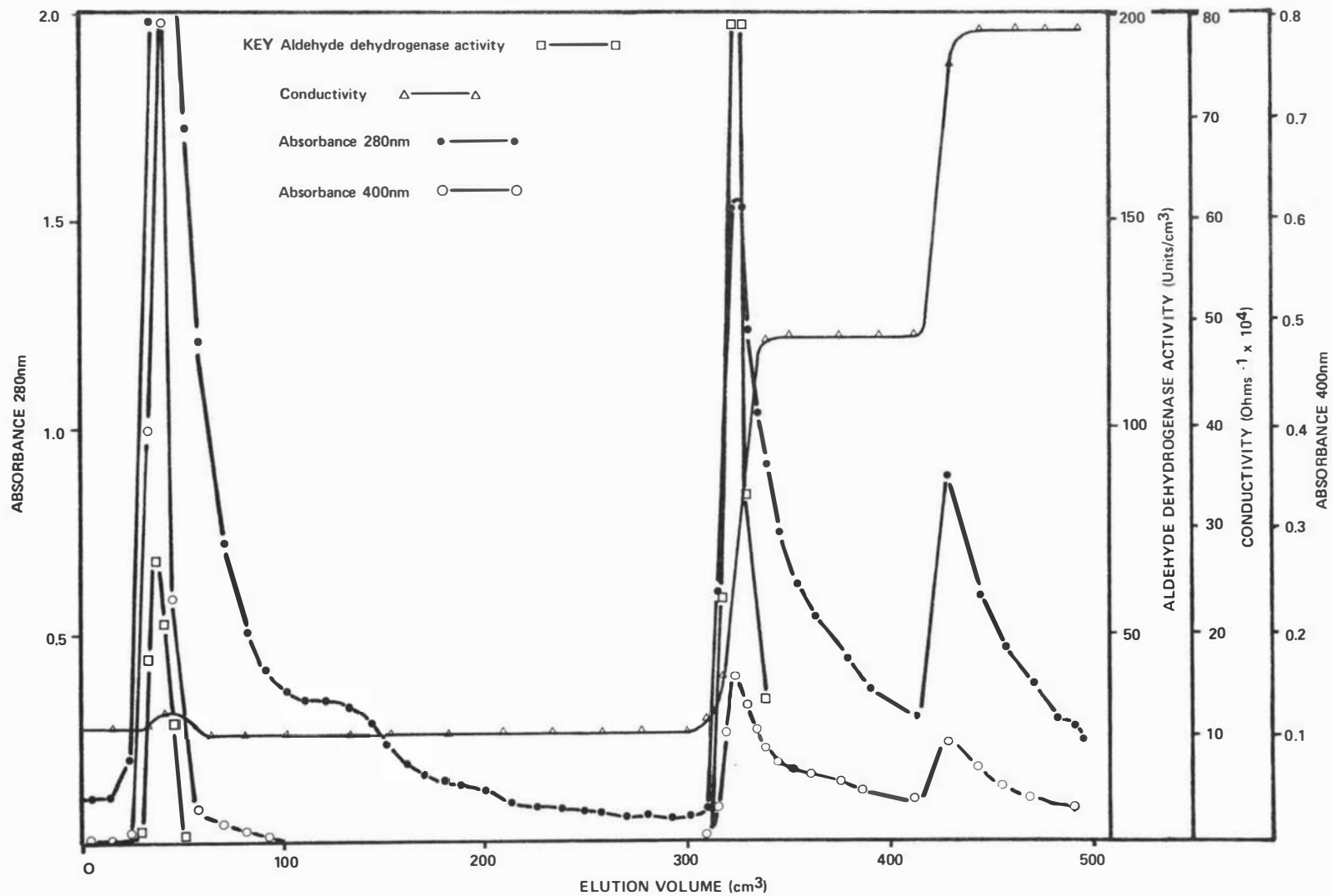
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FIGURE 9.6 STEPWISE ELUTION OF ALDEHYDE DEHYDROGENASE ON NaOH TREATED PROTION (8%, 1 HOUR)



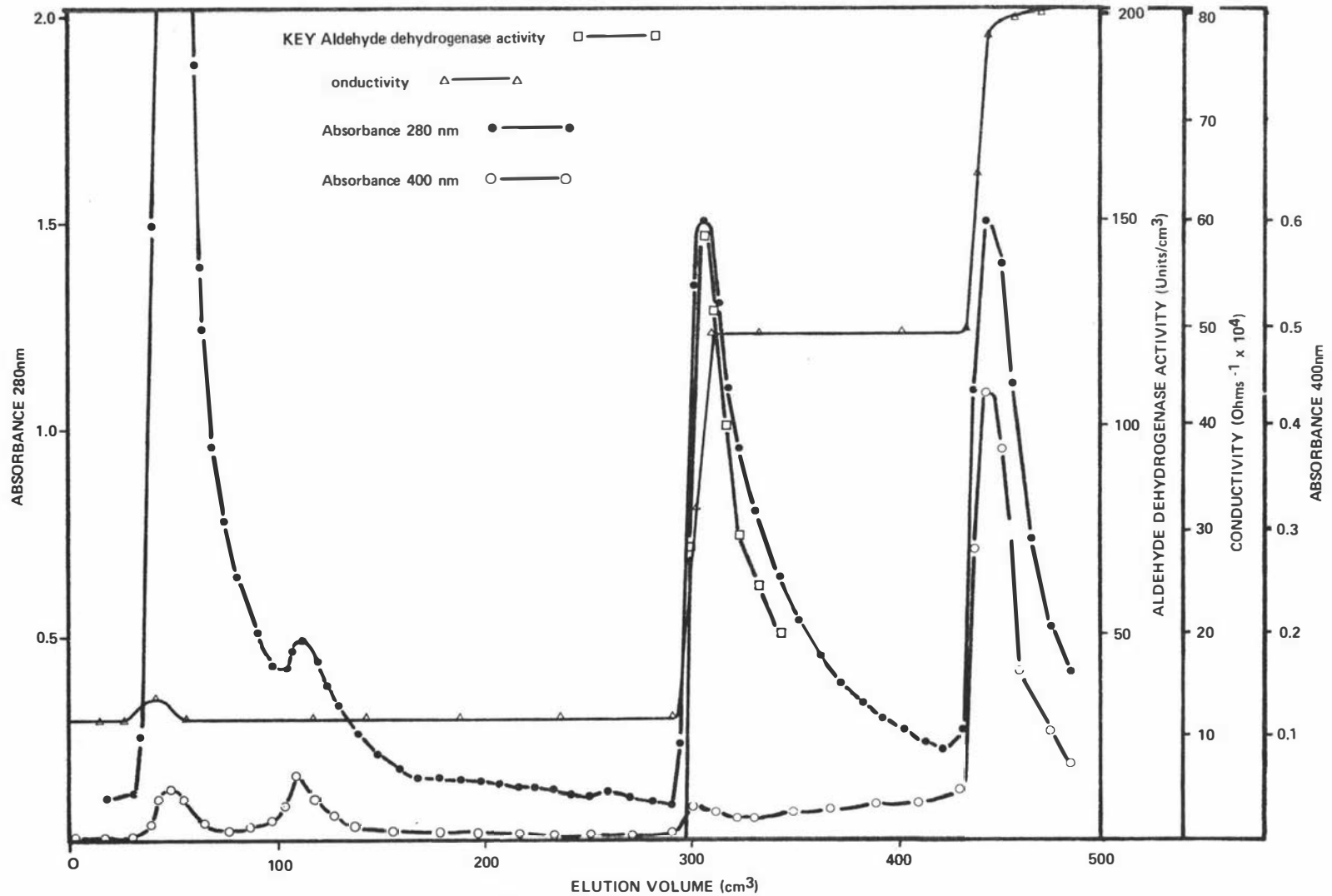
Column volume, 40cm<sup>3</sup>; protein load, 0.0093 g protein/cm<sup>3</sup> resin; initial elution, 0.01M, second elution, 0.05M, and third elution, 0.1M phosphate buffers, pH 7.3; flow rate, 1.0cm<sup>3</sup>/min; protion treatment, 8% NaOH, 10cm<sup>3</sup>/g resin, 1 hour. Aldehyde dehydrogenase activity is expressed in absorbance units (1 unit of enzyme activity = 0.001 absorbance units/min.).

FIGURE 9.7 STEPWISE ELUTION OF ALDEHYDE DEHYDROGENASE ON NaOH TREATED PROTION (8%, 20 MINUTES)



Column volume, 38cm<sup>3</sup>; protein load, 0.0098 g protein/cm<sup>3</sup> resin; initial elution, 0.01M, second elution, 0.05M, third elution 0.1M phosphate buffer, pH 7.3; flow rate, 1cm<sup>3</sup>/min; protion treatment 8% NaOH, 5cm<sup>3</sup>/g resin, 20 minutes. Aldehyde dehydrogenase activity is expressed in absorbance units (1 unit of enzyme activity = 0.001 absorbance units/min).

FIGURE 9.8 STEPWISE ELUTION OF ALDEHYDE DEHYDROGENASE ON DEAE CELLULOSE



column volume, 37cm<sup>3</sup>; protein load, 0.01 g protein/cm<sup>3</sup> resin; first elution, 0.01M, second elution 0.05M, third elution, 0.1M phosphate buffer, pH 7.3; flow rate, 1cm<sup>3</sup>/min. Aldehyde dehydrogenase activity is expressed in absorbance units (1 unit of enzyme activity = 0.001 absorbance units/min).

0.5 M NaOH, and water, as described for DEAE Protion. This regeneration procedure had worked satisfactorily for many cycles with DEAE cellulose on a small scale. It did not, however, appear to provide sufficient regeneration of cellulose on a large scale. The inclusion of an acid wash, followed by a second NaOH wash appeared to give full regeneration of the DEAE cellulose. It is possible that similar treatment might improve the recycling of DEAE Protion, but this has yet to be tested.

#### 9.4 DISCUSSION

The original DEAE Protion appeared to have low capacity for binding aldehyde dehydrogenase from the 45 - 70% ammonium sulphate fraction of sheep liver cytosol. Results also suggested that the resin was only retarding a small percentage of the total protein loaded. Levels of protein at which the original resin overloaded were about 10% of the levels to which DEAE cellulose and NaOH-treated Protion could be loaded, without showing overloading effects.

A possible explanation for the action of NaOH is that the resin particles of DEAE Protion contain pores of a size small enough to limit the access of some larger protein molecules. The action of NaOH may be expanding the particles, to expose previously inaccessible charged groups, so increasing the capacity of the resin for molecules of higher molecular weight. DEAE cellulose, being fibrous, rather than granular, does not have internal structure limiting the access of larger proteins to charged groups. The existence of a limiting pore size could also explain why the two resins appeared to have similar capacities when bovine serum albumin, with a molecular weight of only 68,000 was used to determine the capacity. The two main proteins observed in the experiments on DEAE Protion, aldehyde dehydrogenase and catalase, both have molecular weights of over 200,000.

Although the capacity of the original DEAE Protion has been improved considerably, the NaOH treated sample used in these experiments did not stand up to the recycling procedure. The reasons for this are not clear, but could relate to either nucleic acid fouling, or to a collapse of the resin particles. The former explanation is unlikely to be correct since the capacity loss due to nucleic acid fouling is generally less rapid (Morrison, 1973). The liver extract used to investigate the rate of fouling was not ammonium sulphate treated, but this would

TABLE 9.3

COLUMN RUNS USING A SINGLE BATCH OF NaOH  
TREATED PROTION

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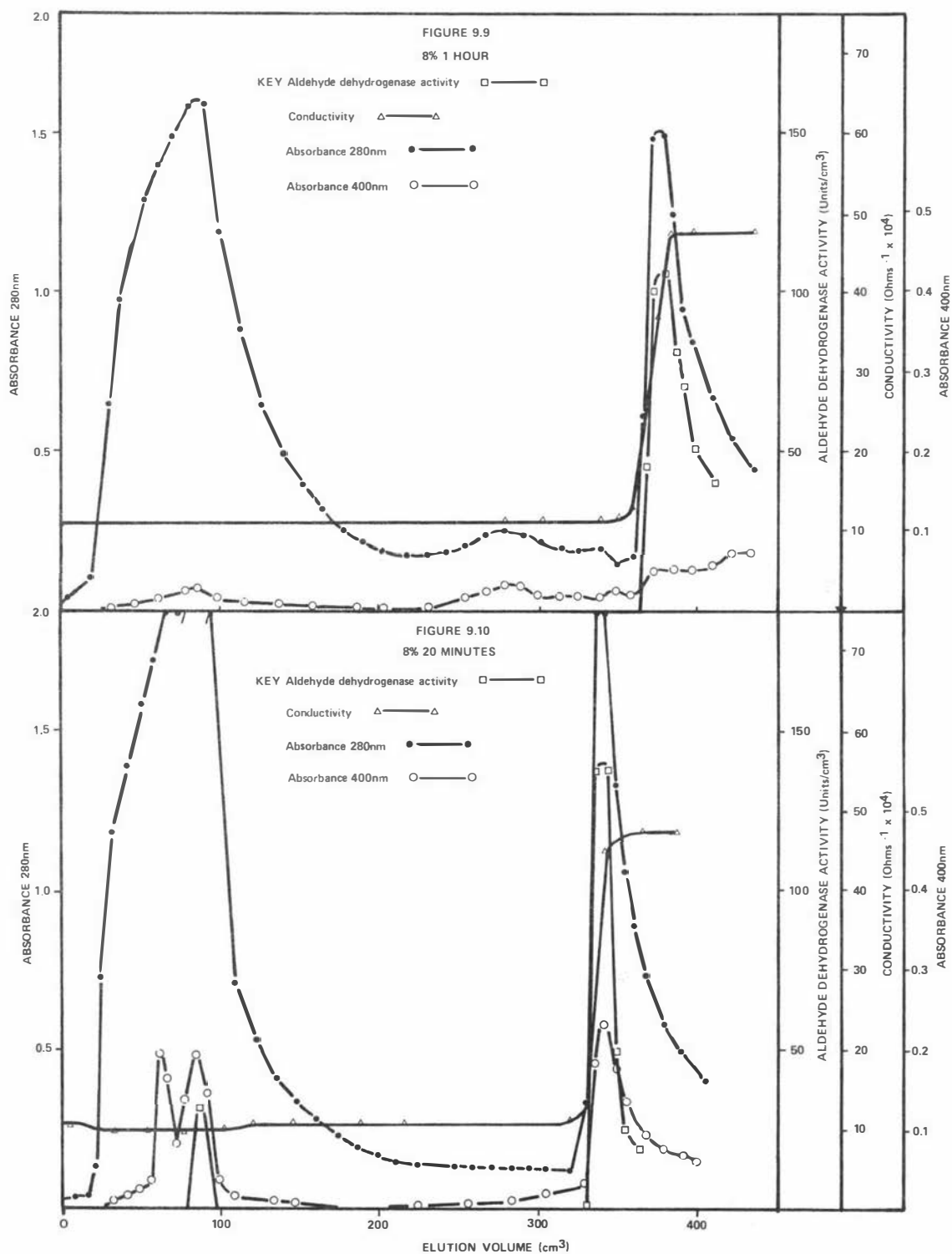
The Protion sample used for these column runs was treated with 8% NaOH for 1 hour, at  $9.0\text{cm}^3/\text{g}$ . The elution patterns of runs 1 and 3 are shown in Figures 9.11 and 9.12.

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	Run 1	Run 2	Run 3
Column length (cm)	40	41	40
Column volume ( $\text{cm}^3$ )	960	970	960
Sample volume ( $\text{cm}^3$ )	300	500	250
Protein concentration ( $\text{mg}/\text{cm}^3$ )	50	16.5	44
Flow rate (loading) ( $\text{cm}^3/\text{minute}$ )	1.75	2.3	2.1
Protein load ( $\text{g}/\text{cm}^3$ )	0.015	0.008	0.011
Length of green band (cm)	19	21	full length of column
Overload (%)	none	none	10

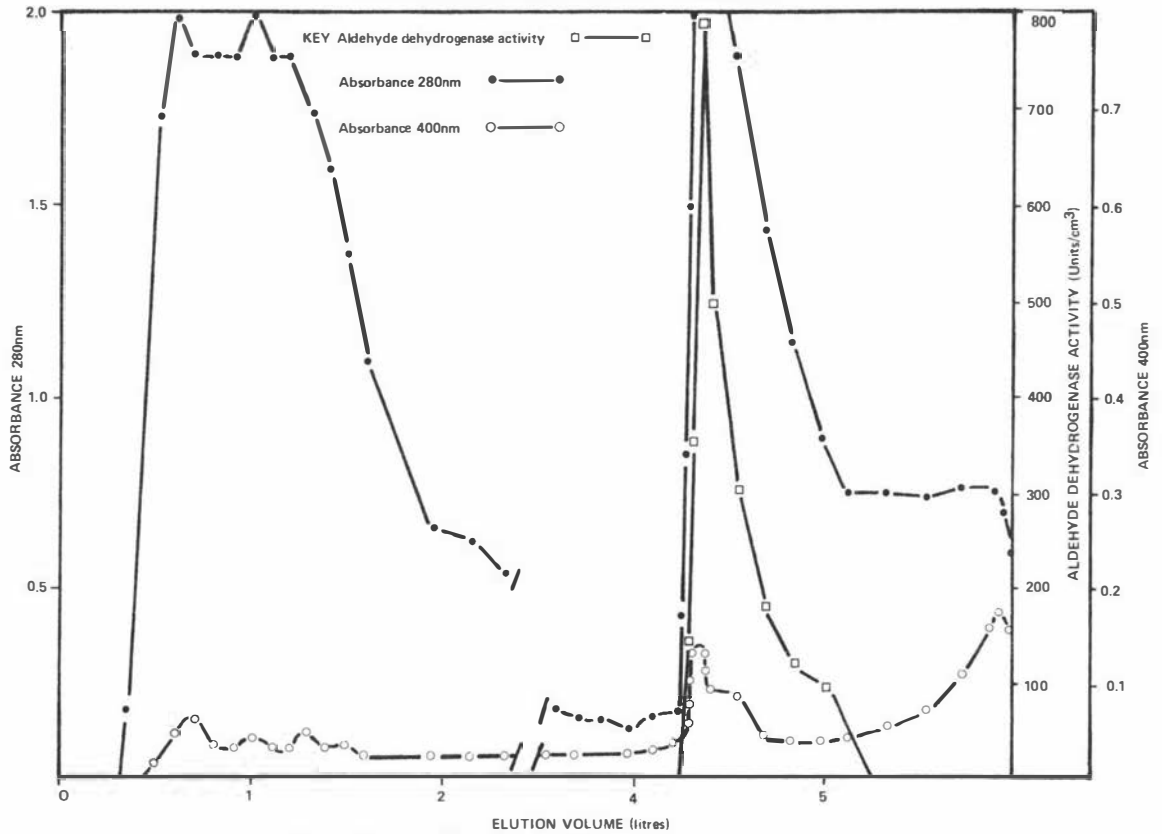
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FIGURES 9.9 and 9.10 STEPWISE ELUTION OF ALDEHYDE DEHYDROGENASE ON NaOH TREATED DEAE PROTION, DILUTE PROTEIN LOADING



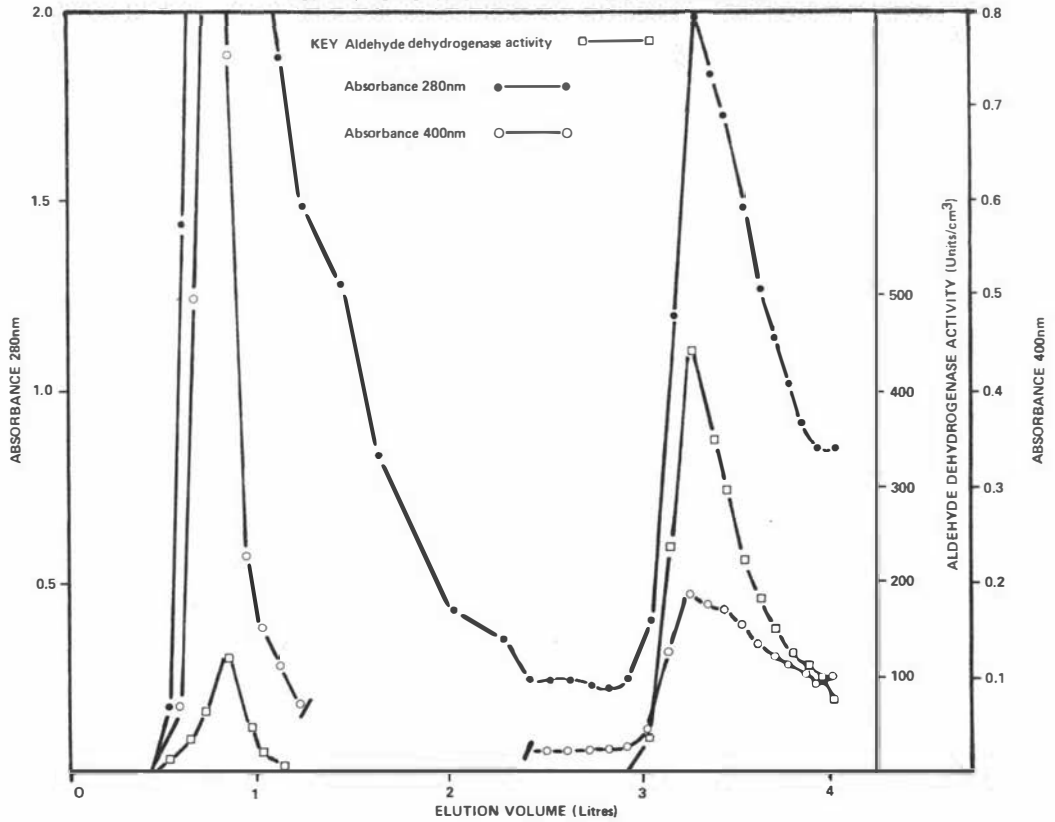
Column volumes, 40cm<sup>3</sup>; protein load, 0.012 g protein/cm<sup>3</sup> resin; elution buffers, 0.01M and 0.05M phosphate, pH 7.3; flow rates, 1cm<sup>3</sup>/min. Aldehyde dehydrogenase activity is expressed in absorbance units (1 unit of enzyme activity = 0.001 absorbance units/min).

FIGURE 9.11 LARGE SCALE OEAE PROTION COLUMN RUN



Column volume, 960cm<sup>3</sup>; protein load, 0.015 g protein/cm<sup>3</sup> resin; loading rate, 1.75 cm<sup>3</sup>/min; running rate, 5cm<sup>3</sup>/min; elution buffers, 0.01M and 0.05M phosphate, pH 7.3; protion treatment, 8% NaOH, 9cm<sup>3</sup>/g resin, 1 hour. Aldehyde dehydrogenase activity is expressed in absorbance units (1 unit of enzyme activity = 0.001 absorbance units/min).

FIGURE 9.12 LARGE SCALE OEAE PROTION COLUMN - THIRD RUN



Column volume, 960cm<sup>3</sup>; protein load, 0.011g protein/cm<sup>3</sup> resin; loading rate, 2.1cm<sup>3</sup>/min; running rate, 5cm<sup>3</sup>/min; elution buffers, 0.01M and 0.05M phosphate, pH 7.3; Protion treatment, 8% NaOH, 9cm<sup>3</sup>/g resin, 1 hour. The resin had been used twice prior to this column run. Aldehyde dehydrogenase activity is expressed in absorbance units (1 unit of enzyme activity = 0.001 absorbance units/min).

only serve to increase the likelihood of fouling due to nucleic acids. Collapse of the resin structure also seems an unlikely explanation for the capacity loss , as the resin had neither lost nor gained volume noticeably.

If the capacity loss observed with DEAE Protion occurs for a similar reason to the loss of resolution seen on DEAE cellulose given similar treatment, then the inclusion of an acid wash in the recycling procedure may be sufficient to allow continuing re-use of the resin. Further work is required to finally solve the problems of recycling DEAE Protion, but if a product that stands recycling can be produced, or a satisfactory method of recycling the existing resin is found, then the properties of this resin will make it ideal for large scale protein separation, and superior for this purpose to any other resins available commercially at the present time.

APPENDIX II.1. Abbreviations.

CM	carboxymethyl
DEAE	diethylaminoethyl
$\epsilon$	extinction coefficient
$E_{1\%}^{1\text{cm}}$	absorbance for a 1% solution through a path length of 1 cm.
$K_M$	Michaelis constant
mg%	mg/100 cm <sup>3</sup>
min.	minute
mercaptoethanol	2 - mercaptoethanol
NAD <sup>+</sup>	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
NBT	nitroblue tetrazolium
nm	nanometers
nmoles	nanomoles
PCMB	p-chloromercuribenzoic acid
PMS	phenazine methosulphate
P <sub>i</sub>	inorganic phosphate
QH	quinoyl hydrazone
R <sub>f</sub>	distance moved relative to dye front
sat <sup>d</sup> .	saturated
SDS	sodium dodecyl sulphate
V	volts
v/v	by volume
w/v	weight/volume

2. Chemicals

Acetaldehyde	BDH Laboratory Chemicals
Acrylamide	Merck
Amido Black	Hopkin and Williams
Ammonium persulphate	BDH Laboratory Chemicals
Ampholytes (Ampholine carrier ampholytes)	LKB-Produkter, Sweden
Bisacrylamide (N,N'-methylene- bisacrylamide)	Eastman Organic Chemicals
Coomassie Blue	Colab laboratories inc., Chicago
2,6-dichlorophenol indophenol	BDH Laboratory Chemicals
D-glucose-6-phosphate	Sigma Chemical Company
L-glutamic acid hydrochloride	BDH Laboratory Chemicals
$\alpha$ -Keto glutaric acid	Sigma Chemical Company
2-mercaptoethanol	Fluka A.G. Buchs S.G., Switzerland
Nicotinamide adenine dinucleotide, Grade AA or Grade III*	Sigma Chemical Company
Nicotinamide adenine dinucleotide reduced form, Grade III	Sigma Chemical Company
Nitroblue tetrazolium, Grade III	Sigma Chemical Company
Phenazine methosulphate	Sigma Chemical Company
Pyrazole	Donated by Dr M.R.Grimmet, University of Otago.
Sodium dodecyl sulphate	BDH Laboratory Chemicals
Sodium pyruvate	Fluka A.G. Buchs S.G., Switzerland
N,N,N',N'- tetramethylethylene- diamine	Koch-Light Laboratories
Quinoyl hydrazine (3-hydrazino- quinoline-di-HCl	Chemicals Procurement laborator- ies, Inc.

\* Grade AA - routine assays.

Grade III -  $K_M$  determinations, development of fluorometric assay.

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