Biochem. J. (1980) **187**, 429–436
Printed in Great Britain

Human FAD-Dependent NAD(P)H Diaphorase

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(*Received 21 September 1979*)

A newly discovered human diaphorase, designated diaphorase-4, which accounts for a major part of the diaphorase activity of most tissues but does not occur in erythrocytes, is described. In contrast with other human diaphorases, it is dependent on FAD for activity after electrophoresis, inhibited by low concentrations of dicoumarol and shows a marked affinity for Cibacron Blue. The molecular weight was estimated to be 49000 ± 1800 by gel filtration. Diaphorase-4 appears to show person-to-person quantitative variation, so that about 4% of the population lack appreciable enzyme activity, but it is not yet clear whether this variation is of genetic or non-genetic origin.

A complex series of enzymes catalysing the oxidation of NAD(P)H and using the quinoid redox dye 2,6-dichlorophenol-indophenol as electron acceptor have been described in human tissues (Leroux & Kaplan, 1972; Fisher *et al.*, 1977). Since most tissues contain a heterogeneous collection of such diaphorases, analysis of crude material by spectrophotometric assay can be misleading and relatively uninformative. Electrophoretic analysis, on the other hand, provides excellent resolution of the individual enzymes and allows their characterization without further purification. By the use of electrophoretic procedures three human diaphorases, designated diaphorase-1, diaphorase-2 and diaphorase-3, determined by independent gene loci, have been recognized and characterized by their genetic, biochemical and physical properties. Each of these diaphorases is detected after electrophoresis as a series of active components. Detailed genetic and biochemical studies have shown that in all three cases the component isoenzymes are determined by the same gene and represent the major gene product and a series of chemically modified forms generated post-translation (Fisher *et al.*, 1977; Edwards *et al.*, 1979.) The physiological substrates of the various diaphorases have not yet been identified with certainty, but in erythrocytes diaphorase-1 is the major cytochrome b_5 reductase, with an important role in methaemoglobin reduction (Hultquist & Passon, 1971; Leroux *et al.*, 1975). Diaphorase-2 is the major erythrocyte isoenzyme catalysing the oxidation of NAD(P)H, and diaphorase-3 appears to be the principal source of oxidized coenzyme in sperm (Caldwell *et al.*, 1976).

Although the diaphorases are generally regarded as flavin-containing proteins (Martius, 1963; Williams, 1976), the addition of exogenous flavin is not necessary for the detection of diaphorase-1, diaphorase-2 and diaphorase-3 after electrophoresis. The present paper describes a newly discovered set of human diaphorase isoenzymes that, in contrast with the others, is dependent on the presence of flavin for activity. This group of isoenzymes, designated diaphorase-4, is determined by a separate autosomal gene locus (Povey *et al.*, 1980), and is further distinguished from other diaphorases by molecular size, subunit structure, inhibition by the vitamin K antimetabolite dicoumarol and unusually high affinity for the adsorption reagent Cibacron Blue. Diaphorase-4 appears to show common person-to-person differences in activity, but the cause and significance of this variation are not yet fully understood.

Materials and Methods

Samples

Adult tissues obtained at autopsy and foetal material from abortions (10–24 weeks' gestation) were stored at −20°C until required. Extracts were prepared by homogenizing 1 vol. of tissue in an equal volume of cold distilled water. The cell debris was removed by centrifugation at 10000 ***g*** for 30 min at 4°C.

Electrophoresis

Electrophoretic analysis was carried out with starch gels (11%) and two types of buffer system. For Tris/citrate buffer, pH 7.4, a stock solution containing 0.1 M-Tris and 24 mM-citric acid was used

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undiluted in the electrode compartments and diluted 1:5 for preparation of the gel. For Tris/borate buffer, pH 8.6, a solution containing 0.1 M-Tris and 50 mM-boric acid was used as electrode buffer and diluted 1:5 for the gel. In some experiments both the cathodal electrode buffer and the gel buffer were made 70 μM with respect to NADH. Electrophoresis was performed at 4–5 V/cm for 17 h at 4°C. Diaphorase isoenzymes were detected by immersing the cut gel into a staining solution containing 100 ml of 25 mM-Tris/HCl buffer, pH 8.5, 20 mg of NADH, 2 mg of FAD, 3.0 ml of Methyl Thiazolyl Blue (5 mg/ml stock solution) and 2.0 ml of 2,6-dichlorophenol-indophenol (1 mg/ml stock solution in 25 mM-Tris/HCl buffer, pH 8.5, filtered immediately before use). Samples (40–50 μl) were applied to gels on filter-paper inserts (Whatman no. 17).

Menadione, cytochrome b_5, folic acid, dihydrofolic acid or tetrahydrofolic acid at a concentration of 0.5 mM were sometimes substituted for 2,6-dichlorophenol-indophenol as electron acceptor in the stain. Cytochrome b_5 was prepared from human liver by using the procedure described by Omura & Takesue (1970), and the final concentration was estimated by measuring the absorption at 556 nm and 423 nm and by using molar absorption coefficients ε_{556} 25 600 and ε_{423} 171 000 $M^{-1} \cdot cm^{-1}$.

Chromatography

The molecular size of diaphorase-4 was estimated by gel-filtration chromatography on Sephadex G-150 or G-200 in a 2.5 cm × 90 cm column. The gel was equilibrated and eluted with 50 mM-Tris/HCl buffer, pH 7.5, containing 0.1 M-NaCl and 2 μM-FAD. The column was calibrated with a series of proteins of known molecular size (lactate dehydrogenase, mol.wt. 135 000; nucleoside phosphorylase, mol.wt. 84 000; malate dehydrogenase, mol.wt. 70 000; phosphoglycerate mutase, mol.wt. 60 000; cytochrome *c*, mol.wt. 12 000).

The affinity characteristics for Cibacron Blue were assessed by column chromatography. Blue Sepharose CL 6B (Pharmacia) was equilibrated with 10 mM-Tris/HCl buffer, pH 7.5, and packed into a small column (1 cm × 5 cm). A 0.5–0.7 ml portion of sample was applied to the column, which was then washed with equilibration buffer and eluted with 10 mM-Tris/HCl buffer, pH 7.5, containing 1 mM-FAD, 1 mM-NADH and increasing concentrations of NaCl (0–2.0 M). The NaCl in the eluate was removed by dialysis against 10 mM-Tris/HCl buffer, pH 7.5, containing 0.5 mM-NADH and 0.5 mM-FAD. Each fraction was concentrated by ultrafiltration on a UM 10 membrane in an Amicon pressure filtration apparatus.

Thermostability studies

The thermostability of diaphorase-4 was compared with that of the other diaphorases by heating strips of starch gel on hot-plates after electrophoresis, but before staining for diaphorase activity (McAlpine *et al.*, 1970).

Results

Electrophoretic analysis

A number of different electrophoretic buffer systems successfully resolve the multiple forms of human diaphorase. For example, by using Tris/citrate buffer, pH 7.4, with 0.3 mM-NADH as substrate in the stain, two sets of diaphorase isoenzymes present in aqueous extracts of testis, those of diaphorase-1 and diaphorase-3, are clearly separated (Fig. 1*a*). In these circumstances there are no isoenzymes with cathodal mobility, but if 25 μM-FAD is included in the stain a series of intensely stained cathodal components, labelled diaphorase-4 in Fig. 1(*b*), become visible. When 70 μM-NADH is included in the electrophoretic media the same set of isoenzymes can be recognized, but with slightly increased cathodal mobility (Fig. 1*c*), in contrast with the isoenzymes of diaphorase-1 and diaphorase-3, which show greater anodal mobility in these conditions. The dependence of diaphorase-4 on FAD appears to be decreased when electrophoresis is performed in the presence of NADH, since weak diaphorase-4 activity is distinguishable even if FAD is not included in the stain (Fig. 1*d*). Similar results have been obtained with other electrophoretic buffer systems, and of these a Tris/borate buffer, pH 8.6, with 70 μM-NADH included in both the gel and the cathodal electrode buffers has proved particularly useful since it gives good resolution of most human diaphorases (as shown in Fig. 2).

Tissue distribution

Diaphorase-4 occurs in a wide range of human tissues (Fig. 2), including cultured fibroblasts and lymphoblastoid cells, and accounts for a large proportion of diaphorase activity in most tissues. The diaphorase-4 isoenzymes are particularly active in adult kidney, brain and testis but are not demonstrable in erythrocytes or sperm.

A similar pattern of distribution was observed in material from foetuses between the 16th and 24th weeks of gestation, and there is no evidence for foetal-specific forms of diaphorase-4.

Substrate specificity

Diaphorase-4 catalyses the oxidation of both NADH and NADPH at pH 8.5 with equal facility,

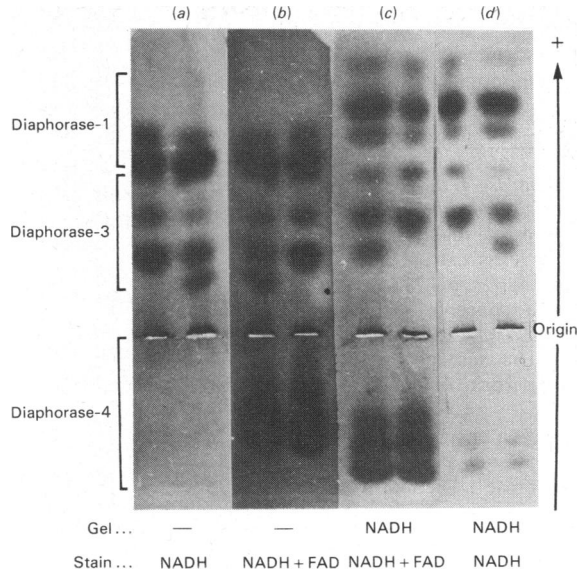

Fig. 1. *Human diaphorase isoenzymes seen in the presence and absence of FAD*
Starch-gel electrophoresis of testis extracts was performed in Tris/citrate buffer, pH 7.4. NADH (70 μM) was added to the gel and cathodal electrode buffers of gels shown in (*c*) and (*d*), but not in (*a*) and (*b*). FAD (25 μM) was included in the stain for gels (*b*) and (*c*), but not (*a*) and (*d*). Full experimental details are given in the text. The testes samples are from two individuals of different diaphorase-3 phenotype. The sample to the left in (*a*) and (*d*) and to the right in (*b*) and (*c*) is diaphorase-3 phenotype 1. The other sample in each case is diaphorase-3 phenotype 2–1.

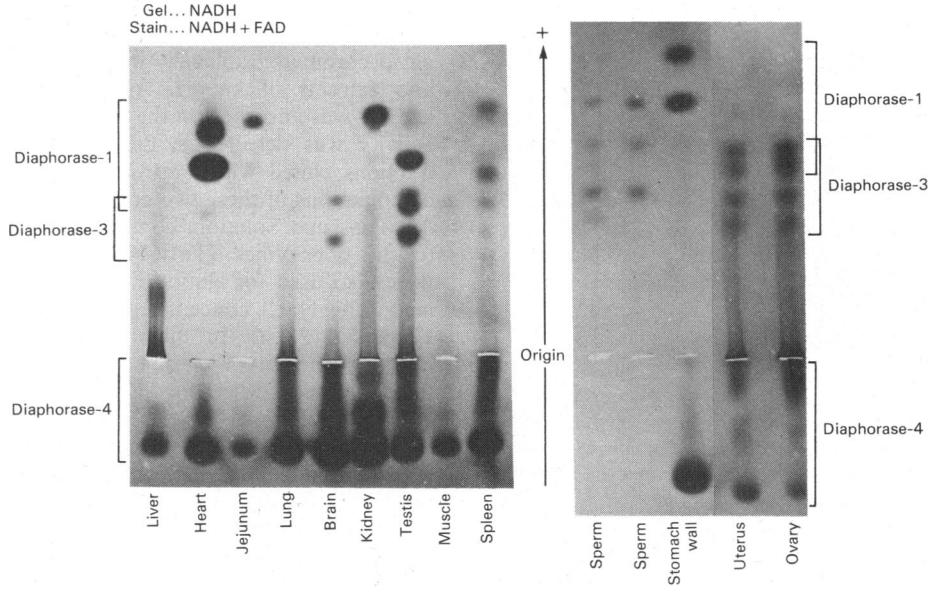

Fig. 2. *Diaphorase isoenzyme patterns in a variety of human tissues*
Starch-gel electrophoresis of human tissue extracts was performed in Tris/borate buffer, pH 8.6, with NADH (70 μM) added to the gel and cathodal electrode buffers. The stain includes NADH (300 μM) and FAD (25 μM). Full experimental details are given in the text. Diaphorase-4 is the major diaphorase of lung, brain, kidney and muscle, but is not detected in sperm.

as judged by staining intensity after electrophoresis. Both reactions are dependent on the presence of FAD, and the mononucleotide FMN cannot substitute.

Table 1. *Relative activities of diaphorase-4, diaphorase-1 and diaphorase-3 with various electron acceptors*

The relative activities of diaphorase-4, diaphorase-1 and diaphorase-3 detected in a testis extract after electrophoresis with Tris/citrate buffer, pH 7.4, and various electron acceptors at a concentration of 0.5 mM in the stain are shown. Full experimental details are given in the text. The activities were judged visually simply by comparison of staining intensity.

Electron acceptor	Relative activity		
	Diaphorase-4	Diaphorase-1	Diaphorase-3
2,6-Dichlorophenol-indophenol	++++	+++	++
Menadione	+++	+++	+++
Cytochrome b_5	++	++	–
Folic acid	++	+	+
Dihydrofolic acid	++	+	+
Tetrahydrofolic acid	++	+	+

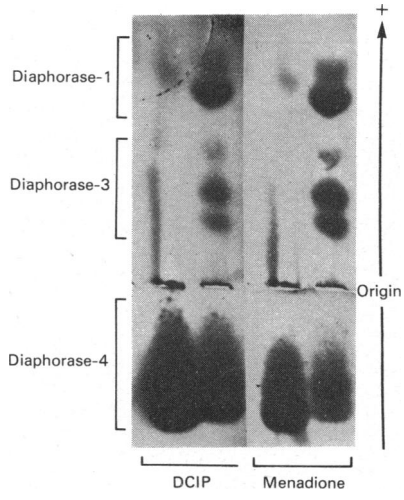

Fig. 3. *Comparison of the relative activities of diaphorase-4, diaphorase-1 and diaphorase-3 with 2,6-dichlorophenol-indophenol or menadione as electron acceptor*

Starch-gel electrophoresis of kidney and testis extracts was performed in Tris/citrate buffer, pH 7.4. The kidney sample is to the left of the testis sample. The gel stain included NADH (300 μM) and FAD (25 μM). One half of a gel was stained with 2,6-dichlorophenol-indophenol (DCIP) (0.5 mM) as electron acceptor and the other half with menadione (0.5 mM). full experimental details are given in the text.

A wide variety of different electron acceptors were utilized by diaphorase-4, including menadione (vitamin K_3), cytochrome b_5, folic acid, dihydrofolic acid and tetrahydrofolic acid, and the results are summarized in Table 1. Menadione is apparently a relatively good substrate for diaphorase-4, although the staining intensity is less than with 2,6-dichlorophenol-indophenol (Fig. 3). Diaphorase-1 and diaphorase-3 also apparently exhibit strong menadione reductase activity. The four other electron acceptors were relatively poor substrates for diaphorase-4 and for diaphorase-1 and diaphorase-3, with the exception of cytochrome b_5, which was appreciably reduced by diaphorase-1.

Inhibition studies

The observation that diaphorase-4, diaphorase-1 and diaphorase-3 all exhibit menadione reductase activity prompted an investigation of the inhibitory effect of the vitamin K antimetabolite dicoumarol. Dicoumarol at concentrations in the range 0.2–2.0 mM was applied in 10 ml of 25 mM-Tris/HCl buffer, pH 8.5, to the cut surface of gel for 10 min before the application of the usual stain. This led to complete inhibition of diaphorase-4 at all the concentrations of dicoumarol tested, whereas the activities of diaphorase-1 and diaphorase-3 were only slightly affected (Fig. 4).

Blue Sepharose chromatography

The affinity of the diaphorase-4 isoenzymes for the adsorption reagent Cibacron Blue was investigated chromatographically by using Blue Sepharose and extracts of kidney. All of the diaphorase-4 activity was adsorbed on the Blue Sepharose, and no activity was detected in the starting buffer or in fractions eluted with 1 mM-NADH, 1 mM-FAD or combinations of these two coenzymes each at 1 mM, 2 mM or 5 mM. Solutions containing NaCl in addition to the coenzymes (1 mM-NADH or 1 mM-FAD) were also used for elution in a stepped gradient of increasing NaCl concentration (0.1 M, 0.2 M, 0.5 M, 1.5 M and 2.0 M). In these conditions some diaphorase-4 activity was eluted with 0.5 M-NaCl, but the major part was eluted with 1.5 M-NaCl. However, the presence of the coenzymes FAD and NADH, even at the low concentration of 1 mM, appeared to be necessary to stabilize diaphorase-4, since no activity was recovered if elution was attempted with NaCl alone.

Diaphorase-4 appears to show an unusually high affinity for Blue Sepharose, and contrasts with diaphorase-1 and diaphorase-3, which are completely eluted with 0.2 mM-NADH in the absence of NaCl (Edwards *et al.*, 1979). The interaction between diaphorase-4 and the other diaphorases and Blue Sepharose appears to be relatively specific, since

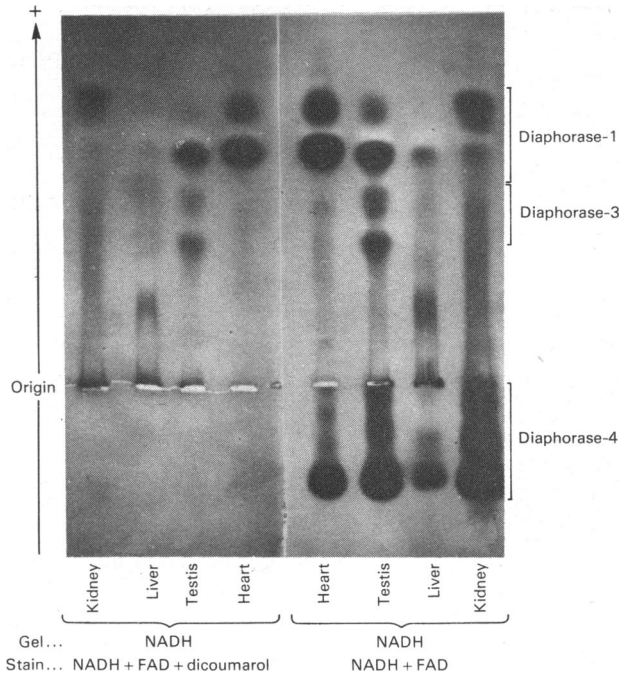

Fig. 4. *Inhibition of diaphorase-4 by dicoumarol*

Starch-gel electrophoresis of human tissue extracts was performed in Tris/borate buffer, pH 8.6, containing NADH (70 μM). One half of the gel was stained in the usual way (see the Materials and Methods section), and the other half was preincubated with 0.2 mM-dicoumarol in 25 mM-Tris/HCl buffer, pH 8.5, for 10 min at 37°C before being stained. Full experimental details are given in the text.

none of these enzymes is adsorbed by unmodified Sepharose.

Molecular size

The molecular size of diaphorase-4 was estimated by gel-filtration chromatography on Sephadex G-150 and G-200. The elution position of the diaphorase-4 isoenzymes was determined by starch-gel electrophoresis of the column fractions, and a typical elution profile is shown in Fig. 5. Eight separate determinations gave an estimate of 49000 ± 1800 for the molecular weight.

In addition to diaphorase-4, a number of other components with diaphorase activity were fractionated from kidney extracts by gel filtration. Diaphorase-1 was eluted, as expected, in a volume corresponding to a molecular weight of 30000 (Fisher *et al.*, 1977), and two other diaphorases, designated diaphorase-5 and diaphorase-8, were eluted at positions corresponding to molecular weights close to 135000 and 115000 respectively. These two diaphorases have so far only been detected in kidney, and diaphorase-5 is exceptional since it apparently does not utilize 2,6-dichlorophenol-indophenol and is only detected with menadione as electron acceptor.

Thermostability studies

Diaphorase-4 appears to be considerably more thermolabile than either diaphorase-1 or diaphorase-3, since all its activity was lost after heating the gel strips for 30 min at 47°C, whereas these conditions did not affect the activity of other diaphorases (Fig. 6). Although the presence of 0.1 mM-NADH in the electrophoresis media protected diaphorase-4 from heat denaturation, so that no activity was lost after 70 min at 47°C, the presence of 0.1 mM-FAD was without effect.

Quantitative variation

The diaphorase-4 isoenzyme patterns in tissue extracts from a population of 628 unrelated British (516 testes samples; 112 kidneys from females) were analysed and no electrophoretic variant was identified. However, there was striking evidence of quantitative variation that appeared to be characteristic of the individual. About 4% of the population sampled exhibited no diaphorase-4 isoenzymes, but normal activities of the other diaphorases. Examination of material from the individuals found to exhibit no diaphorase-4 in homogenates of testis or kidney showed that the diaphorase-4 activity was similarly absent from a wide range of tissues.

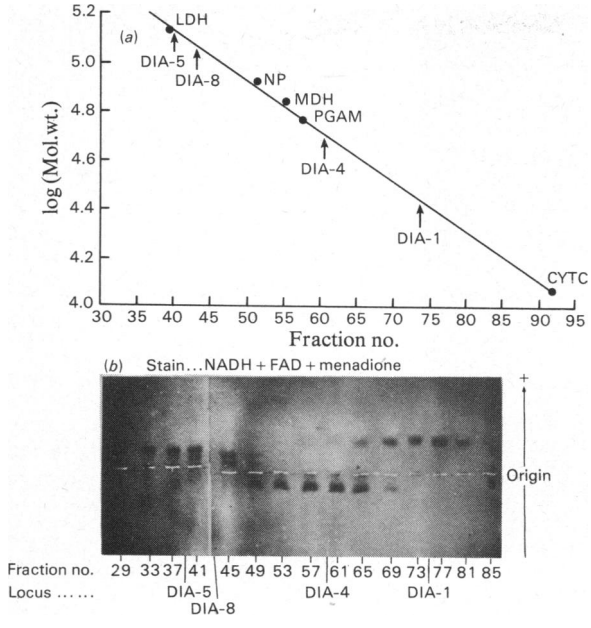

Fig. 5. *Resolution of human diaphorase isoenzymes by gel filtration on Sephadex G-150*

(*a*) shows a plot of log (mol.wt.) versus fraction no. (fraction size approx. 2.5 ml) showing the elution position of the marker proteins [lactate dehydrogenase (LDH), mol.wt. 135000; nucleoside phosphorylase (NP), mol.wt. 84000; malate dehydrogenase (MDH), mol.wt. 70000; phosphoglycerate mutase (PGAM), mol.wt. 60000; cytochrome *c* (CYTC), mol.wt. 12000] and of diaphorase-4 (DIA-4), estimated mol.wt. 50000, diaphorase-1 (DIA-1), mol.wt. 30000, diaphorase-5 (DIA-5), estimated mol.wt. 135000, and diaphorase-8 (DIA-8), estimated mol.wt. 115000. (*b*) shows the starch-gel electrophoresis of fractions eluted after gel filtration on Sephadex G-150. Full experimental details are given in the text. The estimated positions of peak activity for diaphorase-4, diaphorase-1, diaphorase-5 and diaphorase-8 are indicated. Menadione was used as electron acceptor in the stain so that diaphorase-5 could be detected.

A careful analysis of specimens that did show diaphorase-4 activity provided some evidence of two categories, those with relatively high activity being classified as normal and those with relatively low activity classified as intermediate. This latter group accounted for at least 11% of the population sampled. The level of diaphorase-4 activity observed appears to be a constant feature of tissue homogenates prepared from a single individual, as shown in Fig. 7, whether in the normal, intermediate or absent category, and bore no relationship to the activities of the other diaphorases. In Fig. 7 the diaphorase-1 and diaphorase-3 activities of the testis sample from the individual with no diaphorase-4 activity are rather low. However, this was not always the case, and, if, for example, the diaphorase-1 activities of the spleen samples are compared, the individual with no active diaphorase-4 shows the highest activity in this particular instance. It was difficult to distinguish with certainty the normal from the intermediate group simply on the basis of staining intensity, so that estimates of the numbers of individuals in these two classes are probably imprecise.

Discussion

The present paper describes a human diaphorase designated diaphorase-4 that occurs in significant amounts in most tissues, with the exception of erythrocytes. These isoenzymes may be homologous to an enzyme, also fortuitously termed diaphorase-4, whose structural gene has recently been assigned to chromosome 16 by the analysis of somatic cell hybrids (Grzeschik, 1979). The enzyme described by Grzeschik (1979) shows cathodal electrophoretic mobility at pH 8.6 and catalyses the oxidation of both NADH and NADPH. In support of the view that the two enzymes are the same is the recent assignment of the diaphorase-4 described in the present paper to chromosome 16 (Povey *et al.*, 1980).

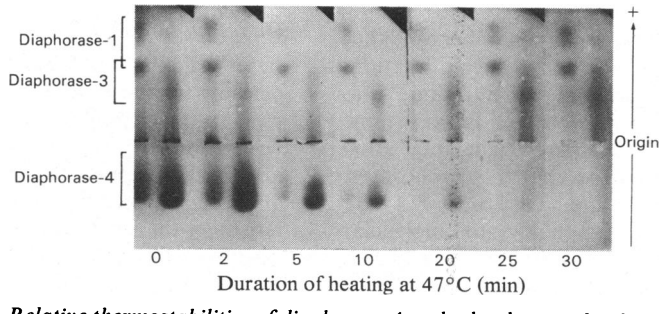

Fig. 6. *Relative thermostabilities of diaphorase-4 and other human diaphorases*

Electrophoresis of human kidney and testis extracts was performed in Tris/citrate buffer, pH 7.4. Strips of gel were treated on hot plates at 47°C for various times (0 to 70 min) before the staining. Full experimental details are given in the text. All diaphorase-4 activity is lost after 30 min at 47°C.

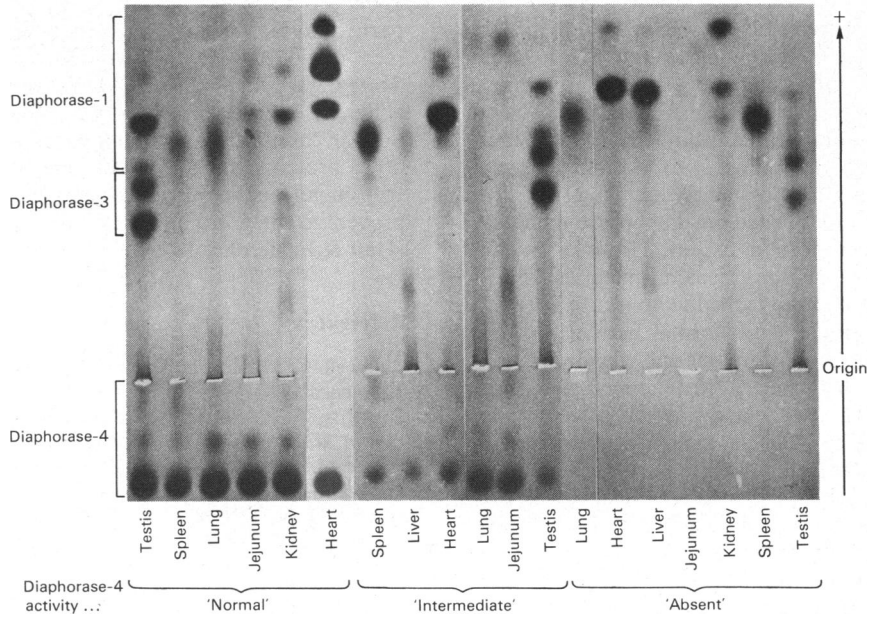

Fig. 7. *Person-to-person quantitative variation of diaphorase-4*
Starch-gel electrophoresis of a variety of human tissue extracts was performed in Tris/borate buffer, pH 8.5, containing NADH (70 μM). Full experimental details are given in the text. The tissues were from three different individuals, who were classified as diaphorase-4 'absent', 'intermediate' and 'normal' in a preliminary survey of testis and kidney extracts. The activities of diaphorase-1 and diaphorase-3 are normal in all three sets of tissues.

Diaphorase-4 is clearly distinguished from other human diaphorases by its dependence on FAD for activity after electrophoresis, which presumably reflects a rather weak association with the co-enzyme, by its inhibition with dicoumarol, by its high affinity for Cibacron Blue and by its molecular size. The molecular weight of diaphorase-4 is estimated to be close to 50000, whereas diaphorase-1, diaphorase-2 and diaphorase-3 have molecular weights of 30000, 18000 and 30000 respectively (Fisher *et al.*, 1977). Furthermore, somatic cell hybrid studies (Grzeschik, 1979; Povey *et al.*, 1980) suggest a dimeric structure for diaphorase-4 that is in contrast with the other diaphorases, which are monomeric. These differences in the catalytic and physical properties indicate that diaphorase-4 is not closely related in its evolutionary origin to the other forms of human diaphorase.

Diaphorase-1 and diaphorase-3 are also distinguished from diaphorase-4 by genetic properties. These enzymes each show common genetic variation that is independent of other diaphorases, including diaphorase-4, and the structural gene locus for diaphorase-1 has been assigned to chromosome 22 (Fisher *et al.*, 1977).

Diaphorase-4 appears to exhibit person-to-person quantitative variation such that about 4% of the population lack this enzyme activity. This variation has been studied only in post-mortem material, and it is uncertain whether it has a genetic origin or is a secondary consequence of some non-genetic event.

If the variation in diaphorase-4 activity is genetic then one simple explanation might be that individuals lacking diaphorase-4 are homozygous for a variant allele determining an inactive or unstable enzyme molecule and that those with intermediate activity are heterozygous carriers of the variant allele. However, since 4% of the population are homozygous for the variant allele, one might expect about 30% to be heterozygotes on the basis of a Hardy–Weinberg distribution, and it is clear that the observed number of individuals in the intermediate category does not fulfil this expectation. However, the method used for categorizing samples into the intermediate or normal groups is crude and based simply on a comparison of staining intensities, and is clearly rather unreliable. A more accurate method of assessing diaphorase-4 activity in tissue extracts is required. Preliminary experiments suggest that an assessment of the amount of NAD(P)H diaphorase activity sensitive to dicoumarol may form the basis of a suitable assay procedure.

The possibility that the quantitative variation shown by diaphorase-4 involves some non-genetic phenomenon has also been considered. There is

apparently no relationship between the activity and the age and sex of the individual or the reported cause of death, nor is activity affected by differences in time elapsing between death and post-mortem examination (usually 1–3 days). The marked sensitivity of diaphorase-4 to the anticoagulant dicoumarol raises the possibility that drug treatment before death may influence the diaphorase-4 activity observed in post-mortem tissues. However, a preliminary survey of the medical histories of 18 patients whose tissues lacked diaphorase-4 and a control group of 21 with normal activities of the enzyme did not provide any clear evidence for such a relationship. However, it was noticed that three individuals with no diaphorase-4 activity had positive histories of anticoagulant treatment, whereas none was found in the control group. In this context it is noteworthy that previous reports (Motulsky, 1964; Vesell & Page, 1968) have demonstrated a marked variation in the rate of dicoumarol metabolism in man that is predominantly under genetic control. The biochemical site of action of the genes involved is unknown, and the possiblity that diaphorase-4 may be implicated indirectly or directly remains to be investigated.

Flavoproteins that are extremely sensitive to dicoumarol and catalyse the oxidation of NAD(P)H and reduction of menadione have been detected in a wide variety of mammals, including ox, pig, rabbit (Martius, 1963) and rat (Ernster *et al.*, 1972). The degree of homology between these enzymes and the human diaphorase-4 isoenzymes is uncertain, but the flavoprotein isolated from bovine liver has a similar molecular weight, namely 52000, and is also a basic protein (Märki & Martius, 1960). The basic nature of diaphorase-4 and its absence from erythrocytes is strongly suggestive of a mitochondrial location, but diaphorase-4 is very readily solubilized in aqueous media, and preliminary subcellular fractionations by differential centrifugation indicate that diaphorase-4 is cytoplasmic in origin.

The very strong affinity exhibited for Cibacron Blue is an unusual characteristic of diaphorase-4 that we may be able to exploit in its purification. Our experiments suggest that the binding is, at least in part, 'non-biospecific', since the dye–enzyme complex could not be broken by a combination of 5mM-FAD and 5mM-NADH and elution was achieved only at NaCl concentrations above 0.5M. Similar 'non-specific' binding of Cibacron Blue to other proteins, such as serum albumin, transferrin, trypsin and chymotrypsin, has been described and proved to be a valuable tool in protein isolation (Haff & Easterday, 1978).

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