STARCH-GEL INVESTIGATIONS OF THE RELATIONSHIPS BETWEEN DEHYDROGENASE PROTEINS

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SUMMARY

A band which migrates, during starch-gel electrophoresis, to a far cathodal position, and catalyzes the reduction of nitro blue tetrazolium without the addition of substrate, has previously been found to also be associated with alcohol dehydrogenase (alcohol:NAD oxidoreductase, EC 1.1.1.1) and steroid activity. It is shown in the present report to be converted into an anodal glutamate dehydrogenase band (L-glutamate:NAD (P) oxidoreductase (deaminating), EC 1.4.1.3) during storage of liver tissue. The intensity of the cathodal band increases when the concentration of the starch gel is increased, and is not seen after electrophoresis in agarose. Chemical examinations indicate that glutamate is present in the cathodal band. The liberation of glutamate dehydrogenase from the cathodal band is influenced by allosteric effectors. Conformational changes in the glutamate dehydrogenase, caused by these effectors, appear to correlate with the electrophoretic patterns obtained. Evidence is presented which suggests that the cathodal band can also liberate mitochondrial malate dehydrogenase bands (L-malate:NAD oxidoreductase, EC 1.1.1.37). The possible significance of these results is discussed.

INTRODUCTION

During a starch-gel electrophoretic investigation by KOEN AND SHAW[1] of the comparative mobilities of different dehydrogenase isoenzymes, a band was observed which migrated towards the far cathodal end of the gels. It catalyzed the reduction of nitro blue tetrazolium (in the presence of NAD and phenazine methosulphate (PMS)) with any substrate, but the activity was greatest with malate or glutamate. In a subsequent study, SHAW AND KOEN[2] found that this band exhibited activity in the absence of any known substrate. They used the term 'nothing dehydrogenase' in reference to the activity of this band in the apparent absence of substrate. The abbreviation NDH is used in the present communication for reference to this band,

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Abbreviation: PMS, phenazine methosulphate.

but its use is not intended to imply any assumptions about the interpretation, or that known enzymes *plus* bound substrate can be ruled out. SHAW AND KOEN concluded that this NDH consisted primarily of alcohol dehydrogenase (alcohol:NAD oxidoreductase, EC 1.1.1.1), on the basis of the following observations: (1) It corresponded in mobility to the liver alcohol dehydrogenase band of deer mice, even when the mobility was altered by genetic variations, and (2) bands of commercially purified horse liver alcohol dehydrogenase, some of which migrate to the same cathodal position as that of the NDH band, also showed activity without added substrate.

More recently it has been observed by PEITRUSZKO et al.[3] that the most cathodal band of commercial horse liver alcohol dehydrogenase catalyzes the interconversion of certain 3 β-hydroxy and 3-keto steroids. This band constitutes a small percentage of alcohol dehydrogenase activity, but the greater proportion of the steroid activity.

These results suggested that the NDH band may be more complex than had been originally believed. During electrophoretic studies of various dehydrogenases in human tissue homogenates, we found that the NDH can be converted to an anodal glutamate dehydrogenase band (L-glutamate:NAD (P) oxidoreductase (deaminating), EC 1.4.1.3), which does not exhibit activity without the substrate. Allosteric effectors, which influence the state of aggregation and substrate specificity of glutamate dehydrogenase[4,5] were also found to influence the NDH–glutamate dehydrogenase conversion, and the glutamate dehydrogenase electrophoretic patterns. Electrophoretic evidence was found that the NDH can also produce mitochondrial malate dehydrogenase (L-malate:NAD oxidoreductase, EC 1.1.1.37) bands. These results, and a consideration of possible explanations are presented below.

EXPERIMENTAL

The present investigations were conducted with human tissues, obtained at autopsy. The tissues were homogenized in distilled water (1 ml per g of tissue). Starch-gel electrophoresis, and detection of lactate dehydrogenase (L-lactate:NAD oxidoreductase, EC 1.1.1.27) activity were performed as previously described[6]. A potassium phosphate–citrate buffer (pH 7.0) was used for the electrophoresis[7]. Glutamate dehydrogenase or malate dehydrogenase activities were detected by substituting for the lactate, 10 mg per ml of L-glutamate or 2 mg per ml of L-malate, respectively. Alcohol dehydrogenase was detected by the use of 1.5 ml of ethanol per 100 ml of solution.

L-Leucine, when incorporated in the starch gel, was used at a concn. of 0.02 M per l. In cases in which GTP and NADH were incorporated into the gel, the concns. were 0.1 and 0.2 mM per l, respectively. The latter additions were made to the starch solution after the starch had been dissolved by heating, and cooled to about 65°.

L-Glutamate determinations were made on sections cut out of starch gels, by the method described by BERNT AND BERGMEYER[8]. Bands whose positions in the gel had been determined by the staining of adjacent sections, were cut out. The remaining portion of the gel, from which the bands had been removed, was stained for enzyme activity, in order to verify that the correct bands had been obtained. The solution in a starch section was obtained by freezing, thawing and squeezing

out from the starch. Proteins in the solution were removed by precipitation with HClO$_4$ and the procedure continued as described.

When purified bands were obtained for subsequent electrophoresis, a portion of the starch section was inserted directly into a mold in the second gel, without freezing. Agarose electrophoresis was also conducted as previously described[9]. Commercial preparations of purified horse liver alcohol dehydrogenase and glutamate dehydrogenase were obtained from the Sigma Chemical Co.

RESULTS

Conversion of NDH to glutamate dehydrogenase

Fig. 1 presents results obtained with 2 liver homogenates. One sample had been obtained the same day, and the other was 18 days old (stored at 4°). No substrate was used in Fig. 1A, glutamate was used in 1B, and a mixture of glutamate and
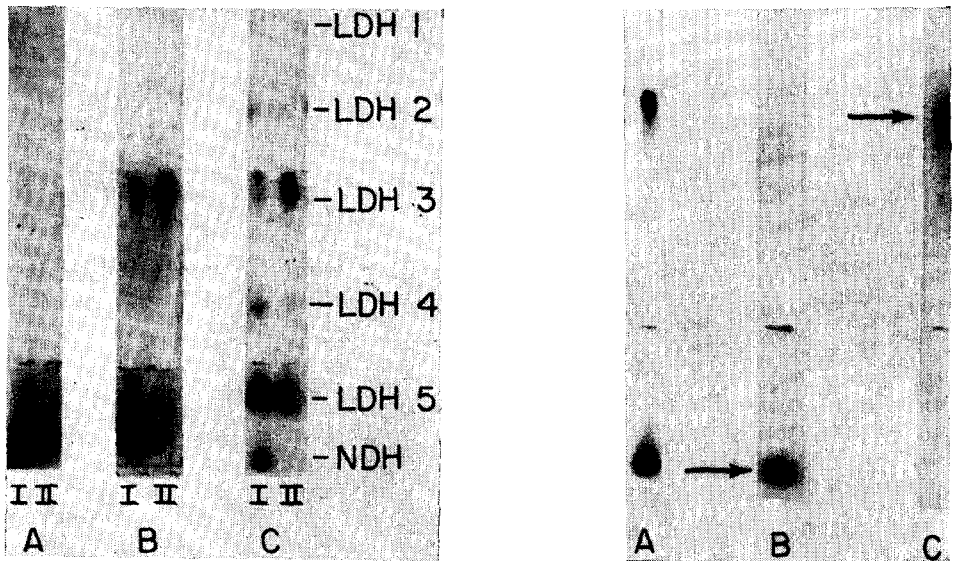

Fig. 1. Electrophoresis of human liver homogenates. Sample I was fresh while Sample II had been stored 18 days after homogenization. Gel A was developed without added substrate, glutamate was used in B, and both glutamate and lactate were used in C. Migration towards the anode is upward, throughout. LDH, lactate dehydrogenase.

Fig. 2. Electrophoresis of the isolated NDH band. All gels were developed with glutamate. Gel A shows unfractionated liver. Gels B and C show the isolated band, analyzed immediately after isolation in B, and 1 day later in C.

lactate was used in 1C. In all cases, the fresh liver homogenate exhibited an intense, far-cathodal band, while the older preparation exhibited a much less intense one. The older liver sample, however, exhibited a considerably more intense anodal glutamate dehydrogenase band than the fresh one.

This inverse relationship has been observed consistently with a number of different liver homogenates. In order to test the possibility that the glutamic band

is derived from the NDH, electrophoresis was performed on the purified NDH band (see EXPERIMENTAL). When analyzed immediately after isolation, all the activity observed was in the original, cathodal position. When another portion of the same isolated band was analyzed one day later, most of the activity was in the anodal glutamate dehydrogenase position and required glutamate for activity. These results, shown in Fig. 2, confirm that the glutamate dehydrogenase band is derived from the NDH.

Effect of allosteric reagents upon the conversion of nothing to glutamate dehydrogenase

Certain allosteric effectors, such as GTP combined with NADH, have been shown to induce a dissociation of glutamate dehydrogenase subunits (and to decrease the glutamate dehydrogenase activity, while increasing its alanine dehydrogenase activity). Other allosteric regulators, such as L-leucine, cause an aggregation of glutamate dehydrogenase subunits (and increase glutamate dehydrogenase activity, while inhibiting the alanine dehydrogenase activity[4,5]). Fig. 3 shows the results obtained when either leucine or GTP with NADH were incorporated into the starch
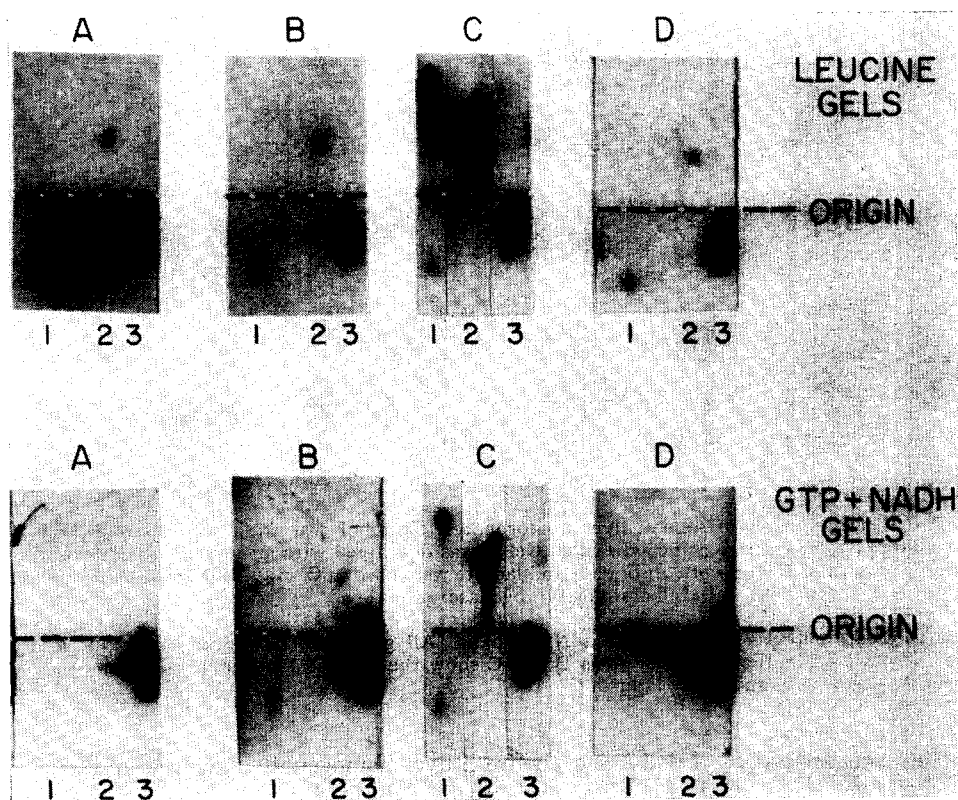

Fig. 3. Effects of allosteric regulators upon electrophoretic patterns. The top row of gels had leucine in them, while the bottom row contained GTP with NADH. The substrates used were alcohol in A, alanine in B, and glutamate in C. No substrate was added in D. The samples applied were (left to right): (1) human liver, (2) purified horse liver glutamate dehydrogenase, and (3) purified horse liver alcohol dehydrogenase.

gels. Human liver and commercial preparations of purified horse liver glutamate dehydrogenase and alcohol dehydrogenase were used.

With the leucine gels, activity was exhibited without the addition of substrate by the NDH in human liver, by all the bands of alcohol dehydrogenase, and by a slower migrating anodal band of glutamate dehydrogenase. Alcohol resulted in a greater intensity of the human liver NDH band than the other substrates.

When GTP and NADH were present in the gel, the liver NDH and slower glutamate dehydrogenase bands only exhibited significant activity when glutamate or alanine was used as substrate. When no effector was added, the results were intermediate between those of the gels with leucine, and with GTP and NADH. The increased intensity of the human liver NDH band resulting from the leucine, and the inhibition caused by the GTP and NADH has been consistently observed with other samples. The NDH band was also found to be inhibited more or less completely by dialysis for 16 h at 3° against 0.1 M phosphate buffer (pH 7.0) satd. with NaCl, or by freezing and thawing in the same phosphate buffer with 0.5 M NaCl. (In the case of lactate dehydrogenase, these conditions have been shown to result in hybridization, with, presumably, dissociation of the subunits[10,11]).

Since the NDH activity is inhibited under conditions which may lead to subunit dissociation, it appeared possible that its activity could be due to bound substrate, which is released during dissociation. Consequently, the liver NDH was isolated by electrophoresis and analyzed for glutamic acid. The glutamic acid in the NDH band, and in control sections taken half way between the NDH and the site of application, were each determined in triplicate. The results shown in Table I, indicated that glutamate is present in the NDH band.

TABLE I

CONCENTRATIONS OF GLUTAMATE IN ISOLATED NDH BAND

Expt. No.	Glutamate concn. ($\mu g/ml$)	
	NDH band	Control band
1	11.7	0.0
2	10.0	0.0
3	16.6	1.7

The effect of starch gel upon the detection of the NDH band

Fig. 4 presents the results obtained when the concentration of the starch was increased from 11.8 g per 100 ml, (which was generally used), to 15 g per 100 ml. The intensity of the NDH band is greater at the higher starch concentration. This is seen most clearly by a comparison of the gels with GTP and NADH, at the two starch-gel concentrations (Fig. 4). When the electrophoresis was conducted in agarose, the NDH band was not detected, even with leucine in the agarose. The detection thus appears to depend upon the starch or its gel structure.

The migration of molecules with molecular weights close to 10^6 (such as α_2-macroglobulin or γ_M-globulin) is generally retarded in starch gels more than lower molecular weight substances[12]. Since leucine promotes the aggregation of glutamate
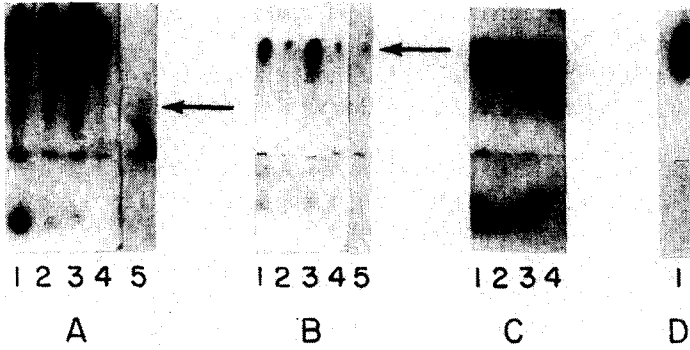
Fig. 4. Effect of eliminating the starch, or of increasing its concn. Glutamate was used as substrate. In A and B, the starch-gel concn. was 11.8 g per 100 ml, while in C it was 15.0 g per 100 ml. In D, the electrophoresis was conducted in 0.4 % agarose, without any starch. Leucine was used in A and D, and GTP with NADH were used in B and C. The samples applied were: (1) fresh human liver; (2) fresh liver diluted 1 to 4; (3) a human liver homogenate 20 days old; (4) human heart 20 days old; and (5) fresh human serum (bands indicated by arrows).

dehydrogenase (which has a molecular weight of about 10^6, as the polymer), while GTP with NADH promotes its dissociation into monomers (with molecular weights of about 250 000)[4,5], it would be anticipated that the migration of glutamate dehydro-
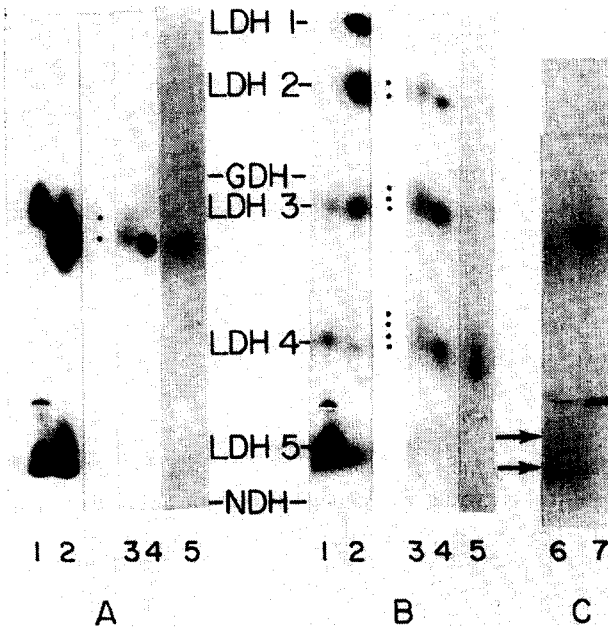

Fig. 5. Electrophoretic bands of malate dehydrogenase. The samples used were: (1) liver; (2) heart; (3) testis treated with formaldehyde; (4) untreated testis; and (5) sperm. Malate dehydrogenase bands are shown in A, while lactate dehydrogenase bands of the same samples are presented, for comparison, in B. C shows (6) the malate dehydrogenase bands, detected after electrophoresis of isolated NDH from liver, and (7) a sample of supernatant malate dehydrogenase, isolated from heart. The gel in C contained GTP with NADH. (Subbands produced by formaldehyde are indicated by dots). LDH, lactate dehydrogenase and GDH, glutamate dehydrogenase.

genase would exhibit a greater retardation in the leucine than in GTP–NADH gels. The anodal glutamate dehydrogenase bands show a retardation or 'tailing' from the origins in the leucine gel which is largely eliminated in the GTP–NADH gels, (Fig. 4). This effect is especially pronounced with serum, in which the glutamate dehydrogenase activity is considerably less than in liver. The tailing or retardation was not observed when the electrophoresis had been conducted in agarose, containing leucine.

Conversion of NDH to malate dehydrogenase

The malate dehydrogenase patterns of human heart, liver, testis, and a preparation of washed and sonicated sperm are presented in Fig. 5. The supernatant (anodal)[13] bands of liver malate dehydrogenase migrate more rapidly than those of the other tissues, when the gel contains either leucine or no allosteric effector. Dialysis, or the use of GTP–NADH in the gels abolished this difference. The testis sample is shown with and without an exposure to formaldehyde, which, in the case of lactate dehydrogenase, has been demonstrated to result in the splitting of individual isozymes into subbands[14]. A similar formation of subbands can be seen to result with the supernatant malate dehydrogenase. The only band exhibited by the sperm preparation migrated at about the same rate as the most intense band of the other samples.

Fig. 5 also presents the results obtained when the purified liver NDH band had been stained after electrophoresis for malate dehydrogenase activity. They indicate that mitochondrial malate dehydrogenase bands are formed from NDH. These results were found only when GTP with NADH was in the starch gel.

DISCUSSION

The migration of proteins in starch gels has generally been found to involve a retardation which increases with the protein molecular size[12]. The tailing or retardation of migration of glutamate dehydrogenase in starch gels containing leucine, and the elimination of this retardation in gels with GTP and NADH provides independent evidence consistent with the conformational changes believed to be caused by these agents[4,5]. The ability to correlate electrophoretic patterns with the aggregation or dissociates of glutamate dehydrogenase subunits has the advantage that crude or unfractionated tissue homogenates can be studied.

Malate dehydrogenase has also been shown to consist of subunits which can undergo conformational changes[13]. The various mitochondrial bands all appear to have the same catalytic properties and amino acid composition[15]. The interconversion of the different mitochondrial bands, which can be caused by reversible acid dissociation or iodination, suggests that they may be different conformational forms of the same isozyme[15]. The effects of GTP and NADH, and of formaldehyde, upon the migration of the supernatant bands (shown in Fig. 5) do not prove, but are consistent with the possibility, that the supernatant bands also differ in conformation rather than in primary structure. The supernatant malate dehydrogenase band in sperm migrates at about the same rate as bands in other tissues, in contrast to the sperm's lactate dehydrogenase band, which has a distinctive mobility[16]. (Little glutamate dehydrogenase activity was detected in the sperm).

The most apparent explanation for enzyme bands which exhibit activity without the addition of substrate would appear to be: (1) the buffer contains the substrate

as a contaminant, (2) the enzyme utilizes the buffer as a substrate, or (3) some of the substrate is bound to the enzyme. Since SHAW AND KOEN[2] were able to detect the NDH band in both Tris and phosphate buffer, the first two possibilities are unlikely. We also have detected NDH activity in veronal and glycine buffers.

Lactate dehydrogenase activity has frequently been detected in the absence of added substrate[2,17,18]. The studies of GRAYMORE[17], and of ROBBINS[18] both suggest that the binding of lactate to lactate dehydrogenase is the most probable explanation. Alcohol dehydrogenase may also migrate in association with substrate, since it is generally difficult to free the enzyme from traces of alcohol. The commercial horse liver alcohol dehydrogenase, used above, contained 0.6 mg of alcohol per mg of the enzyme, although it had been crystallized and was listed as 'substantially ethanol free.'

The following observations, described above, are consistent with the possibility that the activity of the cathodal NDH band, without added substrate, is due to substrate which is bound to it, but released when GTP and NADH are in the gel: (1) determinations of glutamic acid indicate that it is present in the NDH band; (2) the activities without substrate of both NDH and an anodal band of commercially purified glutamate dehydrogenase are inhibited by GTP and NADH, which are known to dissociate glutamate dehydrogenase. The requirement of GTP with NADH in the gel, for the apparent formation of mitochondrial malate dehydrogenase from NDH, is also consistent with a dissociation effect by these agents.

It appears that the NDH may be related to a number of different dehydrogenases. Besides alcohol, glutamate and malate dehydrogenase, it has been reported that the malate dehydrogenase molecule has aspartic dehydrogenase activity[19], and that glutamate dehydrogenase has alanine dehydrogenase activity[4,5]. The NDH may also involve the steroid activity described by PEITRUSZKO et al.[3], since the most cathodal alcohol dehydrogenase band, in which most of the steroid activity resides, is apparently the same as the NDH band in crude liver homogenates[2]. PEITRUSZKO et al. found that the steroid activity spontaneously decreases during storage, as does that of the human liver NDH (Fig. 1).

The conversion of the NDH into different dehydrogenases could be related to the dissociation of enzymes which had been bound together as a complex, to various conformational changes which may alter the enzyme activity in different ways, or to a combination of both. It is of interest in this connection that FONDY et al.[20] have shown that the peptides containing the active sites, for a number of pyridine nucleotide dehydrogenases, have similar amino acid sequences and essential thiol groups. It has been reported by HOUSSAIS[21], however, that under certain conditions, lactate dehydrogenase isozyme 5 (the most cathodal band) can liberate isozymes 3 and 4. The H subunits in isozymes 3 and 4 are not present in pure isozyme 5. The effects of GTP with NADH in the starch gels, presented above, suggest that dissociations are involved in the NDH conversions.

Although the nature and significance of the above relationships present intriguing questions, further conclusions about them would appear to be speculative at the present time.

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