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STUDIES ON THE NATURE OF NON-SPECIFIC STAINING IN NITRO-BLUE TETRAZOLIUM DETECTION OF DEHYDROGENASES IN POLY-ACRYLAMIDE GEL ELECTROPHORESIS ("NOTHING DEHYDROGE-NASE")

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SUMMARY

The cause of non-specific nitroblue tetrazolium staining of polyacrylamide gels in the absence of substrate following electrophoresis of human serum or tissue extracts was investigated. Discrete bands of tetrazolium staining ("nothing dehydrogenase") were related mainly to non-dialyzable elements of the sample and consisted of: (1) an NAD^+-dependent component which appeared to correspond to lactate dehydrogenase isozymes and was observable only with tissue extracts containing considerable lactate dehydrogenase activity but not with purified lactate dehydrogenase, suggesting a requirement for a complex reaction, perhaps involving interaction of lactate dehydrogenase with other macromolecule-containing systems; and (2) an NAD^+-independent component which was attributable to the non-NAD^+-dependent reducing capacity of non-dialyzable macromolecules (probably largely protein sulfhydryl groups) and, to a minor extent, to non-dialyzable molecules. Diffuse background staining was not dependent on the presence of electrophoresed sample and was greatly reduced by increasing the diameter of the staining tube.

INTRODUCTION

In visualization of pyridine nucleotide linked dehydrogenases in tissue section[1,2] or in various electrophoresis media[3–12] by reaction with tetrazolium compounds it has been observed that reaction may occur in the absence of substrate. It has long been noted by histochemical workers[1,2] that this background reaction may be related to the activity, termed "nothing dehydrogenase" in laboratory jargon, of dialyzed protein fractions which resulted in reduction of NAD^+ and $NADP^+$. This reaction was hypothesized to be caused by certain sulfhydryl groups[13], and to be related to earlier observations on the reducing capacity of well washed tissue residue[14].

The blank discrete staining noted in electrophoresis has been variously ascribed to the enzymic activity of lactate[6,8,11,12], alcohol[5], or glutamate and malate dehydrogenases[9], protein reducing capacity in which sulfhydryls are probably important[7], or

a combination of lactate dehydrogenase enzymic activity and non-enzymatic reduction of the tetrazolium compound[3]. The presumed lactate dehydrogenase enzymatic activity in turn has been ascribed to enzyme-bound substrate[3], lactate-like groups on the supporting media[12], or to the presence of lactate in the tissue extract (undialyzed)[6]. In some cases it was noted that no reaction in the absence of added substrate was observed in the absence of added NAD^+ (refs. 6 and 8) or in the presence of $NADP^+$ instead of NAD^+ (ref. 11), or that reaction consists of NAD^+-requiring and non-NAD^+-requiring portions[3,15].

In the course of study of malate dehydrogenase isoenzymes in human sera and tissues with polyacrylamide gel electrophoresis we have encountered staining of gels in the absence of substrate. This report is concerned with studies on the nature of the background staining and with diminution of some of it.

MATERIALS AND METHODS

Materials

Acrylamide, N,N'-methylenebisacrylamide and N,N,N',N'-tetramethylenediamine were obtained from Eastman Kodak (Rochester, N.Y., U.S.A.); ammonium persulfate, NaCl, sucrose, acetic acid, KNO_3 and potassium phosphate from Baker (Philadelphia, Pa., U.S.A.); L-cysteine, L-methionine, nitroblue tetrazolium, phenazine methosulfate, L-malic acid, L-lactic acid, α,α'-dipyridyl, *o*-phenanthroline, Tris base, sodium pyruvate and bovine muscle lactate dehydrogenase (type X, 800 units/mg) from Sigma (St. Louis, Mo., U.S.A.); NAD^+ and NADH from P. L. Biochemicals (Milwaukee, Wisc., U.S.A.); aniline blue black from Allied Chemical (Morris Township, N.J., U.S.A.); and sodium oxalate from Fisher Chemical (Springfield, N.J., U.S.A.).

Methods

Electrophoresis with polyacrylamide gels was performed basically as described by Davis[16] and Ornstein[17] using a Canalco Model 24 disc gel electrophoresis apparatus. Following preliminary electrophoresis of the separating gel (no sample or stacking gels were used) for 60 min to eliminate residual persulfate ions[18], 20–50 μl of a solution of sample mixed with 60% sucrose were layered on the gel and a current of 2.5 mA/tube was applied for 2.25–2.5 h at 2°.

Following electrophoresis, gels were stained in darkness (to prevent photochemical reduction of the nitroblue tetrazolium) in 16 × 100 mm test tubes (initially 10 × 75 mm; inner diameters were 14 and 8 mm, respectively) with a solution of the following composition: 0.88 m*M* nitroblue tetrazolium, 2.3 m*M* phenazine methosulfate, 0.47 m*M* NAD^+, 3.1 m*M* NaCl, substrate (31 m*M* malate, 3.1 m*M* lactate or 31 m*M* ethanol) and 77 m*M* Tris–HCl at the pH specified for each experiment.

After 2 h of darkness at 37°, the gels were removed, washed with distilled water followed by 7% acetic acid and stored in 10 × 75 mm test tubes in 7% acetic acid. The gels were scanned for optical density at 546 nm with a slit of 0.05 × 2.36 mm in a Gilford 240 spectrophotometer with a linear transport attachment, and the optical density recorded on a Heath Kit Model EUW-20A recorder. The anode was down and the cathode up in relation to the gels; the anode was to the right and the cathode to the left in relation to the absorbance tracings. The baseline was obtained with a stained gel containing no sample, except for study of diffuse background

staining in which case baseline absorbance was obtained with an unstained gel containing no sample.

Gels were stained for protein in 0.5% aniline blue black in 7% acetic acid for 15 h, followed by 5 h of electrophoretic destaining at 5 mA/tube.

The amount of diffuse background staining of the gels was greatly diminished by increasing the size of the test tubes used in staining from 10 × 75 mm to 16 × 100 mm, as determined by staining gels which did not contain sample with a staining solution containing NAD^+ and malate at pH 9.5. The larger size tubes were routinely used for the work here reported.

Normal human liver and heart high-speed supernatant (tissue obtained at autopsy from a young adult accident victim) were prepared by homogenization for about 30 min in a glass tissue grinder equipped with a PTFE pestle at 0° in 0.25 *M* sucrose–0.1 *M* potassium phosphate, pH 7.4 (preliminary 15 min centrifugation at 1200 *g* and 15,000 *g*; 2 h centrifugation at 120,000 *g* in a Spinco L2 ultracentrifuge and dialysis).

Dialysis was done at 2° against 0.1 *M* potassium phosphate (pH 7.4, 1 l/ml of sample) for 3 h and against 1 l of fresh buffer for an additional 15 h.

Protein was determined by the method of Lowry *et al.*[19].

RESULTS

Nitroblue tetrazolium staining at pH 8.5 for malate dehydrogenase activity of serum electrophoresed on polyacrylamide gel revealed two large peaks of activity (Fig. 1A; 1D-2). Control gels stained at pH 9.5 in the absence of malate (Fig. 1B-1,2) or both malate and NAD^+ (Fig. 1C; 1D-3,4) revealed a number of similar bands of staining, while tetrazolium reaction at pH 8.5 in the absence of NAD^+ resulted in significant, though lesser staining (Fig. 1B-3,4). The banding pattern observed in the absence of malate did not correspond to the distinct malate or lactate dehydrogenase banding patterns, and was even more intense at pH 9.5 with a serum containing little specific malate dehydrogenase activity (Fig. 1D-1,3) as compared to a serum containing high malate dehydrogenase activity (Fig. 1D-2,4). Only a slight reduction in staining intensity was found when dialyzed serum was used (Fig. 1A, B, C), indicating that most of the non-specific staining was related to non-dialyzable material in the serum sample. No non-specific banding pattern was obtained with polyacrylamide gel lacking serum or other extracts.

As suggested by the results of Fig. 1, the intensity of the non-specific staining reaction was directly proportional to the pH of the staining mixture when studied at pH 7.5, 8.0, 8.5 and 9.5 (Fig. 2). These results thus suggested that the non-specific staining patterns on polyacrylamide gel were related mainly to non-dialyzable components of the electrophoresed serum sample, were for the most part unrelated to the presence of malate or NAD^+, and did not appear to be due to an NAD^+-linked dehydrogenase.

If the non-specific banding reaction were due to enzymatic activity of a dehydrogenase(s), it would be expected (1) to be suppressed by inhibitors of the dehydrogenase involved and (2) to conform to the banding pattern for that dehydrogenase. Potent inhibitors of lactate (Figs. 3 and 5) and alcohol (Figs. 4 and 5) dehydrogenases (oxalate; α,α'-dipyridyl, *o*-phenanthraline and isobutyramide, respectively),

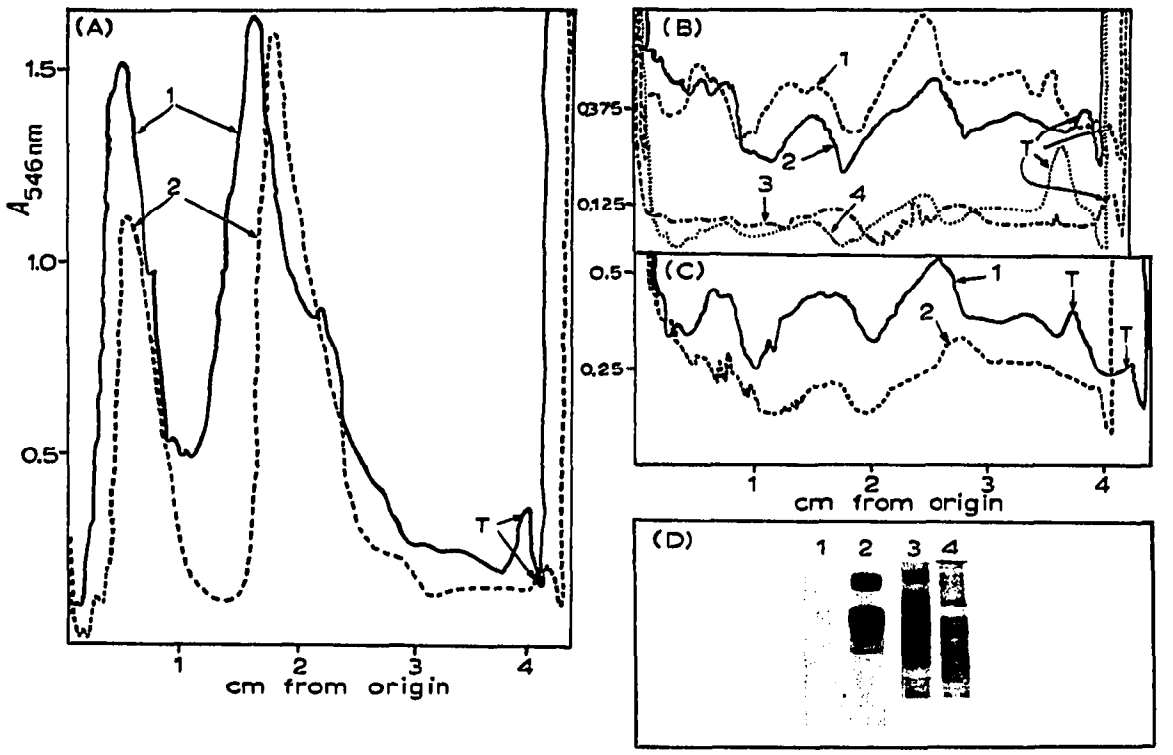

Fig. 1. Effect of malate, NAD^+, pH and dialysis of the serum sample on nitroblue tetrazolium staining of polyacrylamide gels. Gels contained 10 μl of electrophoresed human serum and were stained and scanned for absorbance as described in Materials and methods. T indicates peaks due to tracking dye. (A) Malate and NAD^+ present, pH 8.5; 1, undialyzed; 2, dialyzed. (B) 1 and 2, malate absent, pH 9.5; 3 and 4, NAD^+ absent, pH 8.5; 1 and 3, undialyzed serum; 2 and 4, dialyzed serum. (C) NAD^+ and malate absent, pH 9.5; 1, undialyzed; 2, dialyzed. (D) Polyacrylamide gel photographs; 1 and 3, and 2 and 4 contain the same serum; 1 and 2, malate and NAD^+ present at pH 8.5; 3 and 4, malate and NAD^+ absent, pH 9.5. 1 is at the same magnification as 2; 3 has the same magnification as 4.

which have been implicated as causing the non-specific tetrazolium staining[5,6,8,11,12] were ineffective in significantly altering the non-specific staining reaction observed with liver supernatant in the absence of substrate and NAD^+ or in the presence of substrate alone (Figs. 3B, 4 and 5-12–16, 6′, 7′, 10′, 11′) despite the marked inhibition of NAD^+-dependent lactate dehydrogenase (Fig. 3). Further, the positions of bands of non-specific staining did not correspond to malate (Figs. 1 and 5-12′) or lactate (Fig. 3) dehydrogenase bands and were not significantly altered by the presence or absence of lactate or ethanol (Fig. 5-12, 13, 10′, 11′).

However, in the presence of NAD^+ and the absence of substrate, polyacrylamide gels containing dialyzed and electrophoresed heart muscle and liver high-speed supernatant had, in addition to other tetrazolium staining, several bands which were in the same position as several lactate dehydrogenase bands and were for the most part similarly inhibited by 0.6–600 μM $AgNO_3$ and 1.5–31 mM oxalate (Fig. 5-1–11, 1′–5′). These bands did not correspond to malate dehydrogenase isozymes (Fig. 5-12′),

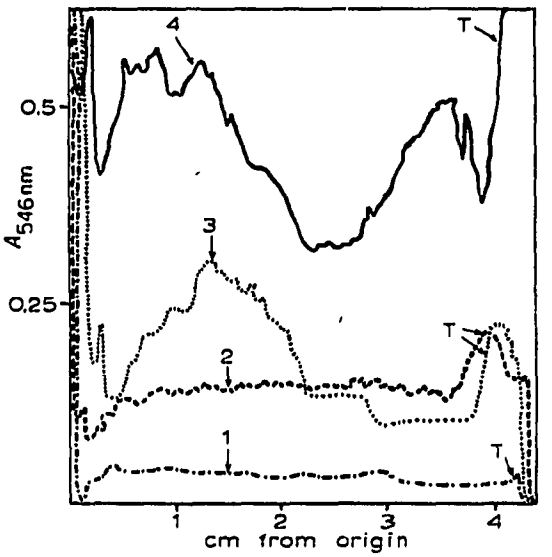

Fig. 2. Absorbance tracing showing the effect of the pH of the staining mixture on the non-specific staining of polyacrylamide gels containing electrophoresed serum as described in Materials and methods. NAD^+ and substrate were absent. pH: 7.5 (1), 8.0 (2), 8.5 (3), 9.5 (4). T indicates peak due to tracking dye.

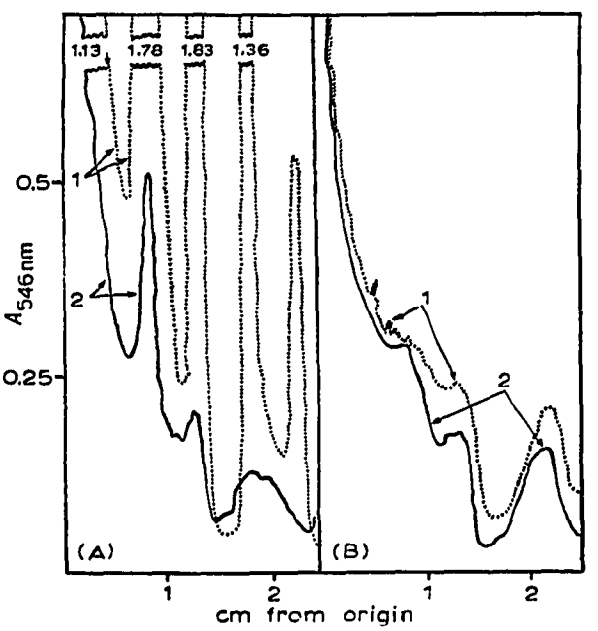

Fig. 3. Effect of the lactate dehydrogenase inhibitor oxalate on lactate dehydrogenase and non-specific nitroblue tetrazolium staining (pH 9.0) of polyacrylamide gels containing electrophoresed human liver supernatants (protein content, 280 μg) as described in Materials and methods. (A) Lactate and NAD^+ present; (B) lactate and NAD^+ absent; 1, oxalate (31 mM) absent; 2, oxalate present.

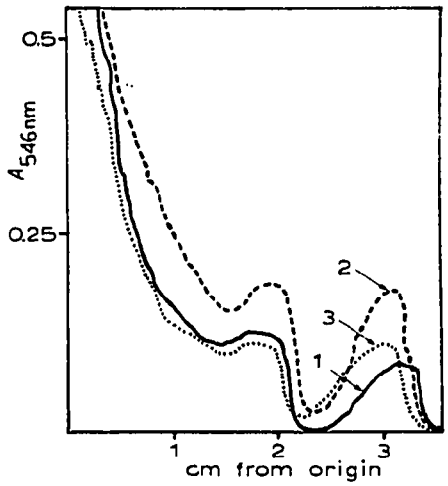

Fig. 4. Effect of alcohol dehydrogenase inhibitors on non-specific nitroblue tetrazolium staining of polyacrylamide gels containing electrophoresed human liver supernatant (protein content, 280 μg). The staining mixture at pH 9.0 lacked NAD^+ and substrate and contained: (1) no inhibitor, (2) 10 m*M* α,α'-dipyridyl, and (3) 5 m*M* *o*-phenanthroline.

were not affected by the presence or absence of ethanol (Fig. 5-4′, 8′, 9′) and were eliminated as readily observable bands by the absence of NAD^+ from the staining mixture (Fig. 5-12–16, 6′, 7′, 10′, 11′). They were apparently intensified rather than inhibited and somewhat altered in electrophoretic mobility by the alcohol dehydroge-

Fig. 5. Polyacrylamide gels containing human heart (1–16) or liver supernatant (1′–12′) (protein content, 396 μg and 270 μg, respectively). Electrophoresis and staining (at pH 9.0 unless otherwise specified) are described in Materials and methods. The dark region at the lower end of some of the gels is due to the tracking dye. Substrate, coenzyme and inhibitors were included in the staining mixtures as follows:

	Gel No.															
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*
Substrate*	1	1	1	1	1	1						1		1		1
NAD^+	+	+	+	+	+	+	+	+	+	+	+					
Inhibitor**		1	3	4	5	6		1	3	4	5			3	3	5

	Gel No.											
	1′	*2′*	*3′*	*4′*	*5′*	*6′*	*7′*	*8′*	*9′*	*10′*	*11′*	*12′*
Substrate*	1	1	1					2		2		3
NAD^+	+	+	+	+	+			+	+			+
Inhibitor**		1	2		1		3	7	7	7	7	

* (1) 3.1 m*M* lactate; (2) 31 m*M* ethanol; (3) 31 m*M* malate pH 7.5.

** (1) 1.5 m*M* oxalate; (2) 15 m*M* oxalate; (3) 31 m*M* oxalate; (4) 0.6 μM $AgNO_3$; (5) 60 μM $AgNO_3$; (6) 600 μM $AgNO_3$; (7) 31 m*M* isobutyramide.

nase inhibitor isobutyramide (31 m*M*) (Fig. 5-4′, 8′, 9′). Further, the intensity of these bands obtained in the absence of substrate was directly proportional to the lactate dehydrogenase activity of the sample. Thus, the intensity of the bands was greater in the case of heart muscle as compared to liver (Fig. 5-7, 4′) and such NAD^+-related bands were not observed with the sera studied. Further, the non-specific NAD^+-requiring bands which were visualized with heart and liver supernatant corresponded to the most intensely staining lactate dehydrogenase isozymes (Fig. 5-1,7: heart, the two most anodal bands; Fig. 5-1′, 4′: liver, the two most cathodal bands). Thus, there

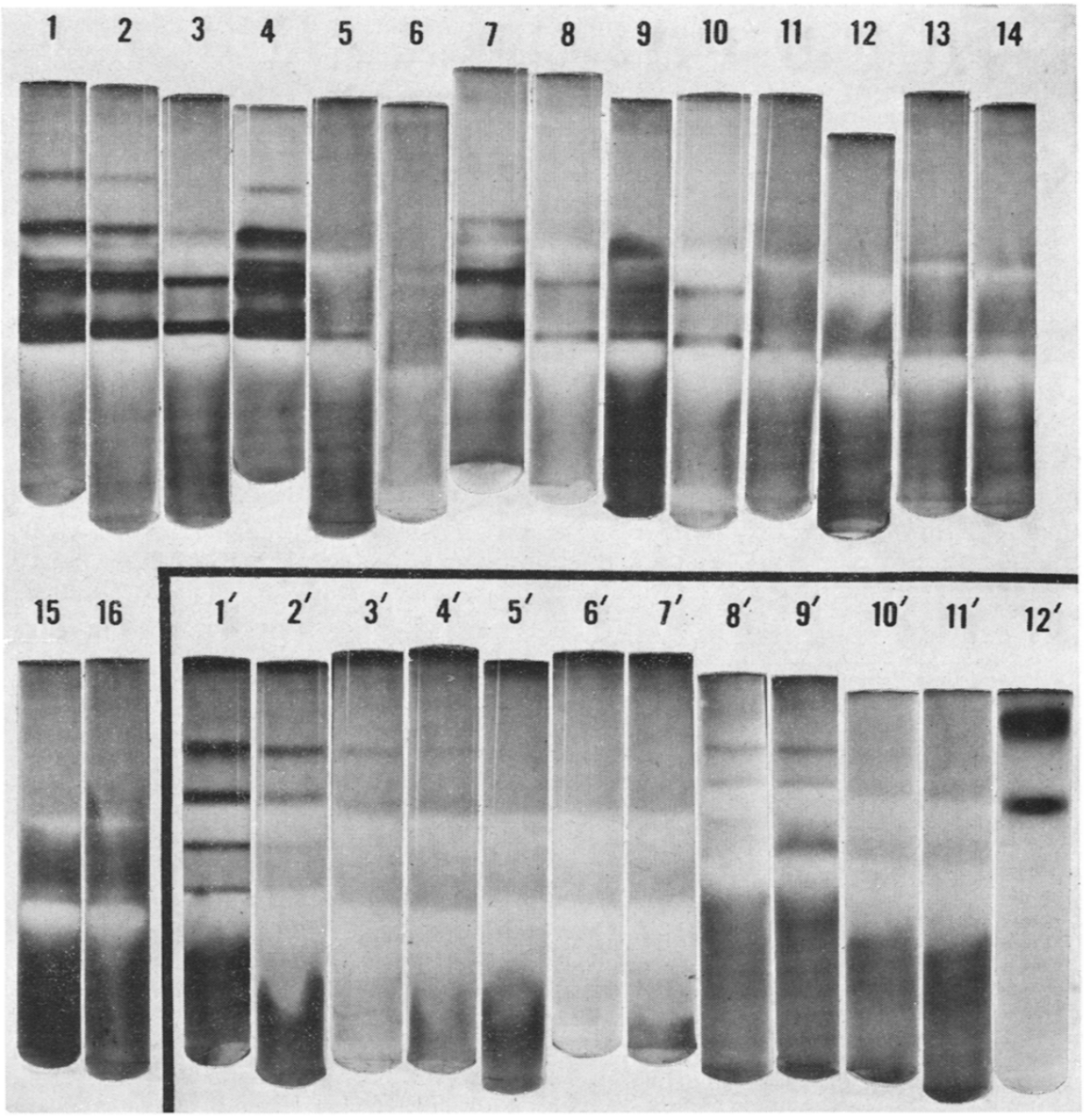

Fig. 5

appeared to be two types of tetrazolium staining in the absence of substrate related to the complex mixture of non-dialyzable macromolecules in tissue samples: (1) NAD^+-requiring, corresponding to discrete lactate dehydrogenase bands, inhibited by lactate dehydrogenase inhibitors and observed only with samples containing substantial lactate dehydrogenase activity, and (2) the majority of staining which did not require substrate or NAD^+, was not inhibited by lactate or alcohol dehydrogenase inhibitors and did not correspond to malate, lactate or alcohol dehydrogenases.

A similar experiment was performed with purified bovine muscle lactate dehydrogenase (10 or 20 μg/gel). Staining in the presence of lactate and NAD^+ resulted in the appearance of five bands: an intense cathodal band, followed by two bands of intermediate intensity, and two relatively faint anodal bands. No NAD^+-dependent band was observed in the absence of substrate. Two faint bands, apparently corresponding to bands 2 and 3 (from the cathodal end) were observed in the absence of lactate, NAD^+ or both. Aniline blue black stain for protein revealed only bands corresponding to the lactate dehybrogenase isozymes.

Interestingly, 0.6 μM $AgNO_3$ resulted in an increase in intensity of the lactate dehydrogenase bands of heart muscle supernatant (Fig. 5-1,4), suggesting enzyme activation by reaction of Ag^+ with highly reactive sulfhydryls, but in decreased intensity of staining in the absence of substrate (Fig. 5-7,10). Presumably, the activation, conformational or otherwise, occurs with respect to the substrate lactate but not with respect to the reaction in the absence of added substrate.

Electrophoresed bovine serum albumin, which did not contain alcohol or lactate dehydrogenase activity when tested spectrophotometrically at 340 nm with NAD^+ and alcohol or lactate, contained several bands when stained in the absence of NAD^+ and substrate. The intensity of staining was clearly diminished when the serum albumin had been previously reacted with iodoacetamide (Fig. 6), suggesting that sulfhydryl groups may be involved in the non-specific staining. Addition of the thiol-containing compound cysteine to staining solutions at pH 9.5 lacking substrate and NAD^+ produced the coloration characteristic of a staining reaction. Similarly,

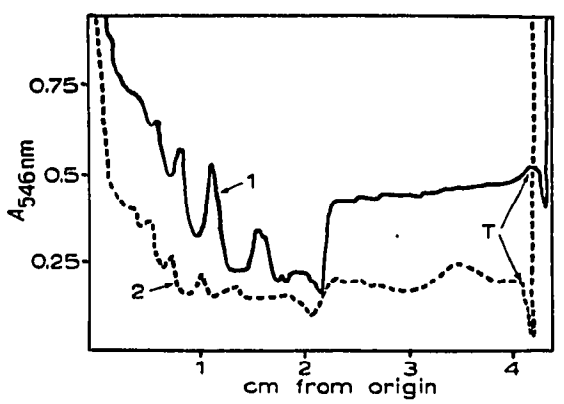

Fig. 6. Effect of iodoacetamide on the absorbance tracing of the non-specific nitroblue tetrazolium staining of polyacrylamide gels containing 10 μg of crystalline bovine serum albumin electrophoresed as described in Materials and methods. In 2, but not 1, the bovine serum albumin was first pretreated with 13 mM iodoacetamide prior to electrophoresis for 15 h at 25°.

polyacrylamide gels bearing electrophoresed L-cysteine were heavily stained by the staining solution. As little as 0.1 mg of L-methionine (chromatographically purified on Whatman No. 3MM paper in *n*-butanol–acetic acid–water, 12:3:5) per ml of lactate dehydrogenase staining solution greatly reduced the absorbance at 254 nm of the staining solution with or without lactate incubated for 2 h in the dark. However, gels containing electrophoresed L-methionine were stained bright blue with staining solution. Apparently interaction with the gel is required to produce color with methionine.

The protein banding pattern of electrophoresed serum did not conform to the non-specific staining pattern (Fig. 7). Some tissue protein bands corresponded to regions of minimal non-specific tetrazolium reaction, while other regions containing much less protein had significant non-specific staining, suggesting that the quality of protein was more important than its quantity in relation to the staining reaction.

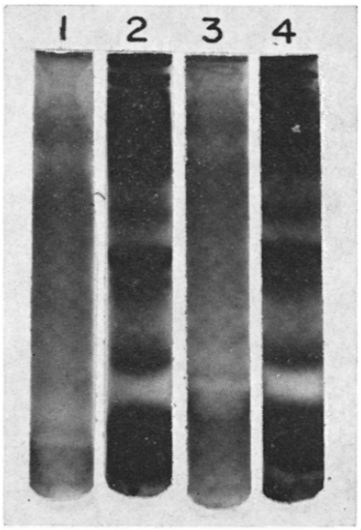

Fig. 7. Polyacrylamide gels containing 10 μl of electrophoresed human serum as described in Materials and methods. 1 and 3, nitroblue tetrazolium stain at pH 9.0 lacking NAD^+ and substrate; 2 and 4, aniline blue black stain for protein.

DISCUSSION

The results presented indicate that the appearance of bands in polyacrylamide gels by phenazine methosulfate–nitroblue tetrazolium staining reaction in the absence of substrate ("nothing dehydrogenase") is dependent on the presence of electrophoresed sample and is due to a number of factors. Most of the staining is due to non-dialyzable macromolecules, while a small portion is due to dialyzable material. The major portion of staining is not dependent on substrate or pyridine nucleotide. Several discrete bands which are dependent on the presence of NAD^+ in the absence of substrate may be seen with tissue samples containing substantial lactate dehydrogenase activity and appear to be due to lactate dehydrogenase isozymes on the basis of position, relative intensity as compared to the isozymes, and inhibitor study. On the

other hand, NAD^+-dependent bands were not observed with purified bovine muscle lactate dehydrogenase, suggesting that the presence of molecules in addition to lactate dehydrogenase is required or that the NAD^+-dependent bands are not lactate dehydrogenase but fortuitously have the electrophoretic mobility of lactate dehydrogenase isozymes. The former explanation is favored for the reasons already given.

A number of observations suggest that the tetrazolium staining independent of substrate or pyridine nucleotide may be due to the reductive capacity, particularly that due to certain sulfhydryl groups, of various protein fractions. Cysteine causes a strong tetrazolium reaction. Our results as well as those of others[1,2,7] indicate that covalent binding prior to electrophoresis of reactive groups of the tissue sample, particularly sulfhydryls, with compounds such as N-ethylmaleimide or iodoacetamide reduces or eliminates staining of bands in the absence of substrate. Also consistent with the role of thiol-reducing groups in the blank reaction is the observation in the present work, which is consistent with reports of histochemical work[1,2], that the intensity of the tetrazolium staining is directly proportional to pH in the range studied (7.5 to 9.5). The correlation of staining with pH is consistent with the reduction of an acceptor by sulfhydryls which are in turn oxidized to disulfides by a mechanism which involves an $R–S^-$ group and acceleration by alkali[20].

The NAD^+-dependent bands seen in the absence of substrate in the staining solution and ascribable to lactate dehydrogenase isozymes in conformity with the observations of Falkenberg *et al.*[12] may be due to enzymatic activity on the basis of suppression by lactate dehydrogenase inhibitors of the bands stained in the presence and absence of lactate, and the significant intensity of reaction suggesting the possibility of a catalytic rather than a stoichiometric reaction. The source of oxidizable substrate in the absence of added substrate may be lactate-like groups insolubly bound to the polyacrylamide gel[12].

It has recently been shown that lactate dehydrogenase may catalyze the oxidation of NADH by superoxide radicals generated by irradiation or xanthine oxidase reaction, and that this catalysis is inhibited by the lactate dehydrogenase inhibitor oxalate[21,22]. One may speculate that an alternative enzymic mechanism for non-specific staining might involve NAD^+ and lactate dehydrogenase in a complex reaction not involving lactate and resulting in reduction of phenazine methosulfate and nitroblue tetrazolium. The apparent requirement for molecules in addition to lactate dehydrogenase in such a mechanism might thus be related to a requirement for an additional reaction, such as, perhaps, the generation of free radicals, as has been shown to occur, for example, in the xanthine oxidase reaction[22]. Since dehydrogenases have some similarities in properties, it is conceivable that dehydrogenases other than lactate dehydrogenase might be involved in a similar reaction under appropriate conditions, including their concentration.

The possibility remains, however, though perhaps somewhat less likely, that the binding of the inhibitor oxalate to the lactate dehydrogenase molecule results in shielding of reactive groups, such as sulfhydryls at the binding site or at other sites, which are responsible for a non-enzymatic reaction. This possibility is lent support by the observation (Fig. 5) that 0.6 μM $AgNO_3$ activated the enzymatic reaction with lactate but suppressed the reaction observed in the absence of lactate. This result could be explained by reaction of certain lactate dehydrogenase sulfhydryls with Ag^+ rendering the enzyme more active in catalyzing the oxidation of lactate by NAD^+,

but leaving fewer enzyme sulfhydryls available for direct reduction of NAD^+ and subsequently of tetrazolium. Alternative explanations are (1) that $AgNO_3$-reacted enzyme was rendered more active toward a lactate substrate but less so with regard to the macromolecular polyacrylamide-bound substrate, presumably owing to access and fit of the substrate to the catalytic center; and (2) Ag^+ may inhibit the activity of another macromolecule which may be essential for the NAD^+-dependent reaction in the absence of substrate.

It is not clear why diffuse background staining in the absence of sample, substrate and pyridine nucleotide should be greatly diminished by use of test tubes having a wider diameter for staining of the polyacrylamide gels. It was not due to a relatively higher pH in the smaller tube (initial pH, 9.5; final pH in the 10 × 75 mm tube, 9.1; final pH in the 16 × 100 mm tube, 9.4). Perhaps it is related to increased ratio of volume of stain solution (and therefore total number of molecules) to glass and gel surface, or to dilution of possible chromogenic substances in the gel.

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