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Received August 30, 1960. P.S.E.B.M., 1960, v105.

Starch Gel Electrophoresis of Lactic Dehydrogenase from Rat Kidney.* (26153)

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Markert and Moller(1) and Tsao(2) have reported methods for localization of dehydrogenase activity after starch gel electrophoresis. Markert and Moller employed neotetrazolium as the terminal electron acceptor in a medium containing substrate, cofactor, methylene blue, diaphorase and hydrazine while Tsao employed the same tetrazolium, omitted diaphorase and replaced hydrazine with cyanide. Use of neotetrazolium necessitated the application of the incubating medium in the form of an agar(1) or starch gel(2) overlay to obtain anaerobic conditions and also required long periods of incubation.

This report describes an improved method for demonstration of lactic dehydrogenase (LDH) after starch gel electrophoresis based on the use of the more sensitive tetrazolium, Nitro BT, and substitution of phenazine methosulfate (PMS) for exogenous diaphorase. In addition, the standard incubating medium was varied in an attempt to characterize more closely the LDH fractions obtained from homogenates of kidneys of adult rats. Also, a technic is described for demonstration of diphosphopyridine nucleotide (DPN) diaphorase after starch gel electrophoresis.

Methods. Whole rat kidneys, homogenized in an equal volume of water, were frozen and thawed several times and centrifuged to remove tissue debris. A portion of the supernatant from each kidney was placed on a filter paper strip, inserted into a starch column, and subjected to electrophoresis for 6 hours(3,4).

Following electrophoresis, each column was sliced into 5 strips. One strip was incubated in the standard medium containing sodium lactate, 0.1M; DPN, 0.3 mg/ml; PMS, 20 μg/ml; Nitro Bt, 0.5 mg/ml; potassium cyanide, 0.1M; and phosphate buffer, 0.25M (pH 7.4) at 37° for one hour. In initial determinations, exogenous diaphorase was employed in the standard medium but this was subsequently replaced by PMS. Of the remaining strips, one each was incubated in a modification of the standard medium in which either DPN, PMS, or both DPN and PMS were omitted. DPN diaphorase activity was revealed by incubating a strip in a medium containing reduced DPN, 1.0 mg/ml; Nitro BT, 0.5 mg/ml; and phosphate buffer, 0.5M (pH 7.4). Malic dehydrogenase activity was demonstrated by substituting sodium malate for sodium lactate in the standard medium.

Results. Five distinct bands exhibited LDH activity when the column was incubated in the standard substrate (Fig. 1). Four of the bands migrated toward the anode while one band was on the cathodal side of the origin. When the column was incubated in the medium from which DPN had been omitted, bands 1, 4, and 5 could not be demonstrated, while either band 2 or band 3 was enzymatically active. In the absence of

* This work was supported in part by Univ. of Michigan Cancer Research Inst.

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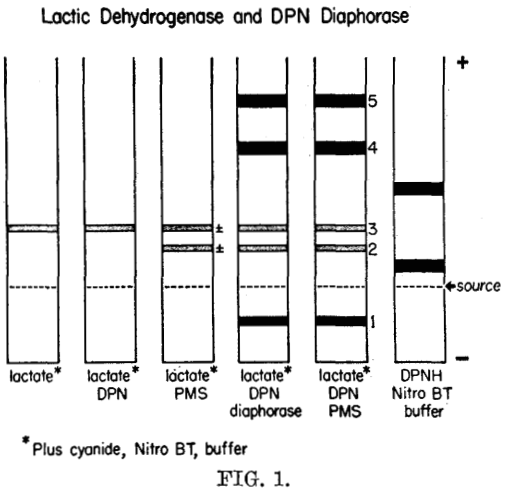


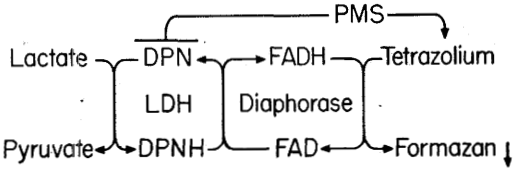
FIG. 1.

PMS or both DPN and PMS only band 3 could be demonstrated. Separation of bands 2 and 3 was often incomplete. The migratory rates of these bands is so nearly similar that a sufficiently long period of electrophoresis is required for their resolution. LDH fractions in homogenates of rat heart were similar to those of rat kidney except that all required DPN and PMS in the incubating medium.

DPN diaphorase activity, as demonstrated with exogenous DPNH, was not present in any of the LDH fractions but was revealed in 2 additional bands (Fig. 1). These occurred as a slow moving band between the origin and band 2 of LDH and a more rapidly moving band situated between bands 3 and 4 of LDH. The diaphorase bands also contained malic dehydrogenase.

Discussion. The present method offers distinct advantages over those previously employed(1,2). The use of Nitro BT affords a greatly reduced incubation time and obviates the necessity for anaerobiasis. Thus the present system does not require use of an agar or starch gel overlay. Cyanide was used as a carboxylic acid trap since hydrazine nonspecifically reduces Nitro BT under the conditions employed. However, cyanide was not essential for the reaction to proceed but did facilitate the reaction rate.

Use of phenazine methosulfate was suggested by the work of Singer and Kearney (5) and Farber and Bueding(6). Singer and Kearney(5) proposed that the site of action of PMS in transfer of electrons to an artificial acceptor is near or likely at the level of the dehydrogenase. The present work would indicate the site of action is the dehydrogenase since it was found that PMS and diaphorase were interchangeable as requirements for demonstration of certain of the LDH bands. The reaction sequence is assumed to be as follows:



The electrophoretic heterogeneity of tissue LDH has been clearly substantiated by the work of Wieland(7), Markert and Moller (1), Tsao(2), and the present report. The presence of 5 LDH fractions in rat kidney homogenates is in agreement with the findings of Markert and Moller(1) in adult porcine kidney with the exception of one band which migrated toward the cathode.

Certain of the LDH fractions of rat kidney homogenates were found to differ in cofactor requirement. Bands 1, 4, and 5 always required DPN and either exogenous diaphorase or PMS. Band 2 always required addition of diaphorase or PMS but variably required DPN. Band 3 did not need added diaphorase and also exhibited variation in its requirement for exogenous DPN. Since bands 2 and 3 varied inversely in their requirements for exogenous DPN this would suggest that endogenous DPN was present in either of the 2 bands, while endogenous diaphorase was present only in band 3.

Occurrence of bound DPN in various enzyme preparations has been reported by several workers(8,9,10). A less probable explanation would be the existence of a non-DPN linked enzyme. The failure to demonstrate endogenous diaphorase activity in LDH band 3 with reduced DPN as substrate, may be due to presence of a segment of the respiratory chain which is only capable of oxidizing DPNH generated *via* the dehydrogenase. The alternate possibility that this

fraction does not function through a diaphorase would seem unlikely. Green(11) has demonstrated that the respiratory chain of beef heart mitochondria tends to fragment into several particles each of which contains several characteristic enzymes. In view of these findings it seems possible that certain of the LDH fractions, as demonstrated in starch gel, may consist of enzyme units. This is further suggested by the finding that the major diaphorase bands also contain malic dehydrogenase.

It is apparent that additional explanations for the electrophoretic heterogeneity of LDH must be considered before accepting the postulation that this heterogeneity represents different molecular species(1). Such possibilities as sulfhydryl or other protein interaction (12) or coupling of the dehydrogenase to various components of the respiratory chain cannot be excluded.

Summary. 1. An improved method for demonstration of lactic dehydrogenase after starch gel electrophoresis is described. This method employs a more sensitive tetrazolium and phenazine methosulfate. 2. Rat kidney contained 5 lactic dehydrogenase fractions which varied in cofactor requirements. DPN diaphorase was not demonstrable in any of these fractions, when reduced DPN was employed as the substrate, but was demonstrated in 2 additional bands. 3. The possibility is discussed that at least certain of the lactic dehydrogenase fractions from rat kidney exhibit electrophoretic heterogenity because of their association with various components of the respiratory system.

We are deeply indebted to Mrs. Julia Ternak for technical assistance.

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Received August 31, 1960. P.S.E.B.M., 1960, v105.

Neuromuscular Blocking Properties of Various Polypeptide Antibiotics.† (26154)

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The neuromuscular blocking action of several antibiotics has been reported. Pittinger and associates(1,2) demonstrated the neuromuscular blocking action of neomycin sulfate and the enhancement of this effect by ether anesthesia. Brazil and Corrado(3) reported the "curariform" action of streptomycin. Timmerman and co-workers(4) reported neuromuscular blockade following administration of dihydrostreptomycin sulfate, polymyxin B sulfate and kanamycin sulfate. This report describes the neuromuscular blocking action of several polypeptide antibiotics and the effect of neostigmine methylsulfate and calcium chloride on it. Various constituents of the polypeptides were also evaluated for neuromuscular blocking activity.

† This work supported in part by a grant from the U.S.P.H.S. (RG-B-1396).