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STUDY OF SERUM CHOLINESTERASE VARIANTS BY MEANS OF ONE- AND TWO-DIMENSIONAL ELECTROPHORESIS IN DENSITY GRADIENT POLYACRYLAMIDE

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SUMMARY

Techniques are described for the electrophoresis of the isoenzymes of cholinesterase present in human serum using density gradient polyacrylamide. Application of the method to samples taken from patients homozygous for the abnormal, fluoride-resistant and silent genes has resulted in patterns recognisably different from those of normal individuals.

It is suggested that application of density gradient electrophoresis to serum from patients shown to be sensitive to muscle relaxants of the succinyl dicholine type, and yet normal by existing biochemical criteria, may prove to be useful in identifying new inherited enzyme abnormalities.

INTRODUCTION

Considerable advances have taken place in the electrophoretic separation of the serum isoenzymes of cholinesterase (acylcholine acyl-hydrolase EC 3.1.1.8.) in the twenty years since Pinter [1] succeeded in identifying three bands of enzyme activity located in α_2—β region. Improvements in electrophoretic and staining techniques enabled the enzyme to be separated into six or seven fractions [2], while the introduction of two-dimensional electrophoresis, coupling filter paper with starch gel, resulted in four areas of activity being detected in fresh serum in addition to the non-specific aryl esterase associated with albumin [3]. In serum subjected to storage two additional species could be recognised, while in a proportion of sera a fifth major band was observed [4], which is now thought to derive from a second locus. Sera found to possess this enzyme are referred to as C5 positive.

By use of polyacrylamide Juul [5] was able to increase the number of bands

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resolved to 12, while Nagy et al. [6] showed a similar number of electrophoretically distinct isoenzymes by use of density gradient polyacrylamide.

Several explanations can be put forward to explain the large number of bands of activity which are recognized including the possibility that each is a distinct molecular species [7]. Lamotta et al. [8] suggested that they represented a number of polymers of the same protein molecule, a theory which they supported on the basis of work incorporating ultracentrifugation. Previously Hess et al. [7] had considered the possibility that one enzyme molecule might be attached to a number of different carrier proteins. Although they discounted this theory, in view of the increasing data now available on the association of cholinesterase with lipoproteins [9, 10], it is a suggestion which might usefully be reconsidered.

With the exception of the C_5 band, relatively little attention has been paid to the separation of variants of cholinesterase by electrophoretic techniques. It is the purpose of this paper to describe differences in electrophoretic patterns, achieved by one- and two-dimensional electrophoresis in gradient polyacrylamide gel, of serum taken from patients homo- and heterozygous for the abnormal and fluoride-resistant genes.

MATERIALS

Bytyrylthiocholine iodide, α-naphthyl acetate and eserine sulphate were obtained from Sigma (London, Great Britain); fast red TR salt from Raymond A. Lamb (London, Great Britain) and PAA 4/30 polyacrylamide density gradient gel slabs from Pharmacia Fine Chemicals (London, Great Britain). All other chemicals were of analytical grade.

The following two buffer solutions have been used. Electrophoresis buffer, pH 8.35: 88.7 m*M* tris (hydroxymethyl)aminomethane—81.5 m*M* boric acid—2.5 m*M* disodium EDTA. Gel casting buffer, pH 8.35: 2.7 m*M* disodium EDTA—16.2 m*M* boric acid—18.9 m*M* ammonium sulphate—3.1 m*M* sodium azide.

METHODS

Sera were typed on the basis of dibucaine and fluoride inhibition as measured by the techniques of Kalow and Genest [11], and Harris and Whittaker [12].

Electrophoresis was performed using a Pharmacia GE-4 electrophoresis apparatus. The apparatus consists of two tanks, the lower one into which the buffer is placed being connected via a centrifugal pump to the upper vessel which supports the gel slabs and rods and the electrode mountings. In addition, a cooling platten is incorporated which ensures that the temperature of the buffer is kept constant during prolonged electrophoresis.

Polyacrylamide density gradient slabs were used for single dimensional electrophoresis and for the second dimension in two-dimensional electrophoresis. These slabs had a gel concentration at the origin of 4% increasing through the slab to a final concentration of 30%. For two-dimensional electrophoresis, the first dimension was carried out in 60 mm × 2.7 mm diameter polyacrylamide gel rods consisting of 7.5% polyacrylamide cast in the appropriate buffer.

Sera under study were diluted 1:1 with 5% sucrose containing bromophenol blue marker dye, and 10 μl of this solution were layered on top of the gel rods or into the special applicator above the PAA 4/30 gel slabs, positioned in the apparatus. In the case of the gel rods, the apparatus was connected to a power source, the current set to 21 mA and the electrophoresis continued until the marker dye approached the bottom of the gel. Gels were then removed either for staining or for layering across the gradient gel slab prior to electrophoresis in the second dimension.

For the PAA 4/30 gel slabs it was found that the best separation was achieved if the manufacturer's recommendation of a short period of electrophoretic pre-equilibration was carried out prior to loading the samples or gel rod. Electrophoresis was then performed at a current of 30 mA until the marker dye had entered the gel, following which the circulating pump was turned on, the current increased to 50 mA and the electrophoresis continued for at least 15 h. After this time the gels were removed and sliced for staining.

Total esterase activity was demonstrated using the staining procedure of Stern and Lewis [13] in which the gels were incubated for 45 min in a solution of α-naphthyl acetate and fast red TR salt in 0.2 *M* phosphate buffer pH 6.0, prior to fixing in a solution of methanol—water—acetic acid (50:50:10).

Staining for cholinesterase utilised the method of Karnosky and Roots [14] in which a 4-h incubation was carried out in a solution of butyryl thiocholine in phosphate buffer pH 6.0 containing copper sulphate and potassium ferricyanide.

RESULTS

The results of one-dimensional electrophoresis of serum taken from homozygotes for the normal, abnormal, fluoride and silent genes are shown in Fig. 1, staining having been performed with α-naphthyl acetate. Normal serum usually shows ten bands of activity in addition to the band due to non-specific aryl esterase. An eleventh band occasionally appears and may result from enzyme breakdown during storage. The bands due to cholinesterase are reduced to five in the case of patients homozygous for the abnormal and fluoride-resistant genes, and to one band only in the patient homozygous for the silent gene. In all cases the most marked differences are apparent in the bands showing intermediate mobility although in the case of the E_sE_s genotype slow running bands are also absent. Two-dimensional electrophoresis of normal serum is shown in Fig. 2 when no less than 22 areas of activity can be recognized. These are reduced to 12 in serum from the individual possessing only abnormal enzyme (Fig. 3), 10 for the E_fE_f genotype (Fig. 4) and 1 in serum from the E_sE_s homozygote (Fig. 5). Serum from heterozygotes for the normal enzyme with any of the three other variants cannot be distinguished with certainty from the normal pattern, but the electropherogram obtained with serum from an E_sE_f heterozygote is shown in Fig. 6 and this has clear differences from the other electrophoretic patterns obtained.

In all illustrations the position occupied by aryl esterase is shown as revealed by staining with α-naphthyl acetate. Staining with butyryl thiocholine

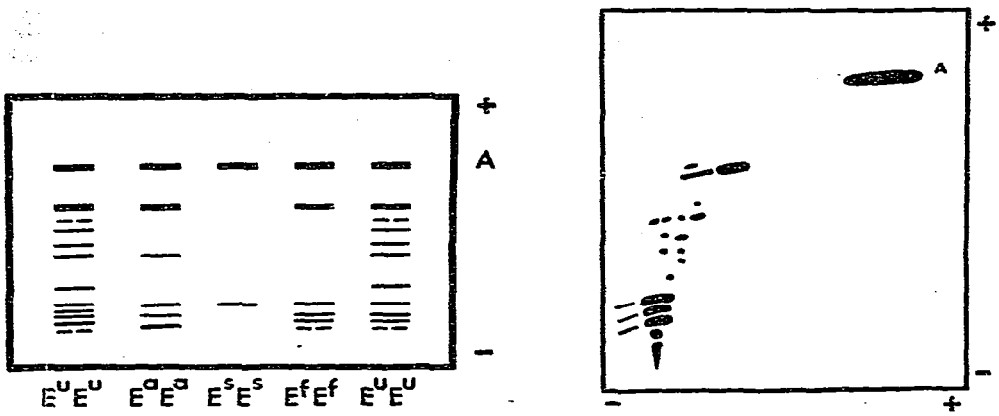

Fig. 1. One-dimensional electrophoretic patterns in gradient polyacrylamide gel of cholinesterase from individuals homozygous for the normal (E_uE_u), abnormal (E_aE_a), fluoride-resistant (E_fE_f) and silent genes (E_sE_s). Two normal sera are illustrated, one showing ten and one eleven bands. Activity due to both cholinesterase and aryl esterase is shown, band A representing the non-specific aryl esterase associated with albumin. Broken lines indicate areas of weak activity only.

Fig. 2. Two-dimensional electropherogram of cholinesterase isoenzymes from normal serum. Electrophoresis in the first dimension has incorporated constant density polyacrylamide and in the second dimension gradient gel polyacrylamide. Staining is for total esterase activity, band A representing the non-specific aryl esterase associated with albumin.

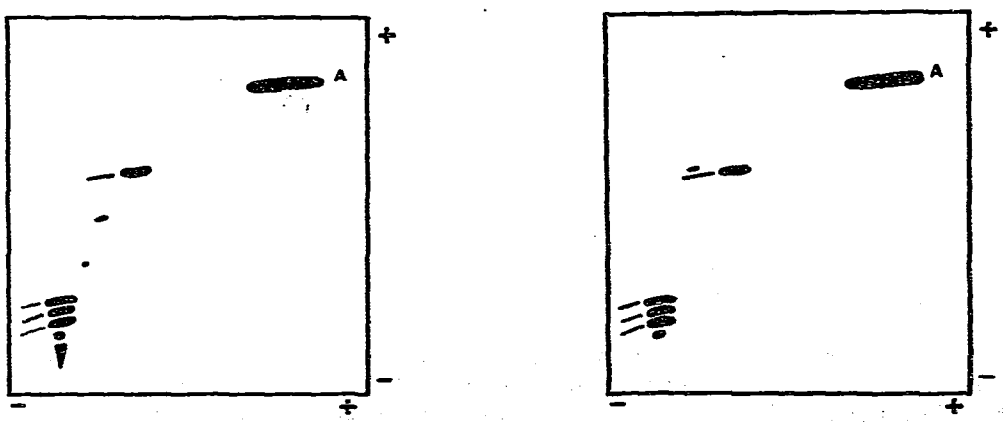

Fig. 3. Two-dimensional electropherogram of cholinesterase isoenzymes from the serum of an individual homozygous for the abnormal gene. Staining is for total esterase activity, band A representing the non-specific aryl esterase associated with albumin.

Fig. 4. Two-dimensional electropherogram of cholinesterase isoenzymes present in the serum of an individual homozygous for the fluoride-resistant gene. Staining is for total esterase activity, band A representing the non-specific aryl esterase associated with albumin.

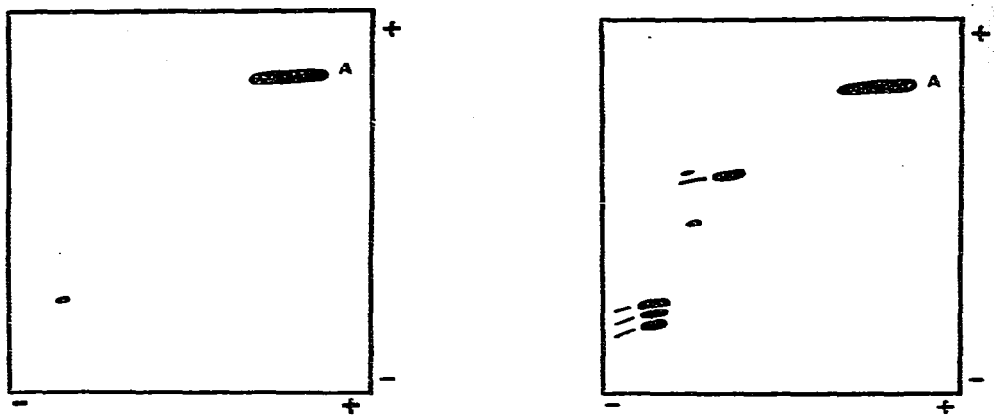

Fig. 5. Two-dimensional electropherogram of serum from an individual homozygous for the silent gene for cholinesterase. Staining is for total esterase.

Fig. 6. Two-dimensional electropherogram of serum from an individual heterozygous for the abnormal and fluoride-resistant genes for serum cholinesterase. Staining is for total esterase activity, band A representing the non-specific aryl esterase associated with albumin.

eliminates this band while incorporation of eserine sulphate into the α-naphthyl acetate results in patterns of activity indistinguishable from that given by serum from a homozygote for the silent gene, confirming the presence of a second serum enzyme with non-specific esterase activity. No alteration in electrophoretic pattern was observed following the inclusion of eserine into the staining reagent for serum from the homozygote for the silent gene, indicating in this patient, at least, a total absence of cholinesterase activity.

DISCUSSION

The biochemical identification of individuals showing abnormal sensitivity to short acting muscle relaxants of the succinyl dicholine type has until now depended entirely upon measurement of enzyme inhibition by means of dibucaine and fluoride. Unfortunately up to 50% of patients assessed as succinyl dicholine sensitive by clinical criteria have been shown to possess normal enzyme on the basis of biochemical findings. While some of these patients have doubtless been wrongly assessed clinically, in view of the many other causes of prolonged respiratory paralysis following anaesthesia [15], it is equally clear that some do not fall into this category and other abnormal biochemical variants of the enzyme undoubtedly exist. It is possible that investigations based upon the use of inhibitors may eventually resolve some of these problems, but it is equally possible that the application of different tools including electrophoresis may help to identify sensitive patients. Before this can be done, methods must be available for the separation of the different enzyme types likely to be present in serum with a high order of resolution and reproducibility in order that dif-

ferences in patterns can be recognised and interpreted. Until now techniques have not been good enough to be able to show differences between established genotypes, even less enzyme forms as yet undescribed. The technique described here enables marked differences between normal and abnormal enzyme forms to be distinguished and as a result may represent a useful tool for the study of succinyl dicholine sensitive patients.

Data at present available are insufficient to enable definite conclusions to be made concerning the interpretation of the two-dimensional electropherograms, but tentative hypotheses can be postulated.

A striking observation on all sera showing significant enzyme activity is the separation achieved during gradient polyacrylamide electrophoresis of the bands which are slowest moving during electrophoresis in the first dimension. In all cases a uniform separation has been achieved into either three of four bands. This indicates the presence of enzyme molecules having the same charge density but different molecular sizes and hence different molecular weights. In view of the uniformity of separation it is interesting to speculate that these bands indicate the presence of polymers of a basic enzyme subunit. Further information may come from experiments designed to reduce these enzymes to a single band of monomer.

Little information can yet be derived from the other areas of activity which have been revealed. However, the diffuse enzyme band shown to have slowest mobility in the second dimension in specimens from E_u and E_a homozygotes can perhaps be explained on the basis of an association of cholinesterase with lipoprotein in large molecular weight particles which have partially broken down during storage.

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