EFFECTS OF FIXATION AND SUBSTRATE PROTECTION ON THE ISOENZYMES OF ASPARTATE AMINOTRANSFERASE STUDIED IN A QUANTITATIVE CYTOCHEMICAL MODEL SYSTEM

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ABSTRACT

The cytochemical technique of Lee and Torack for the demonstration of aspartate aminotransferase activity was tested on a model system consisting of either total liver homogenate or the mitochondrial or soluble cytoplasmic fraction, incorporated in polyacrylamide film. After incubation of portions of film in a medium of α-ketoglutarate, L-aspartate, and lead nitrate, the lead oxaloacetate formed was converted to lead sulfide. The absorbance determined at 520 nm in a film spectrophotometer and expressed in terms of unit weight of film provided a measure of the contained enzymatic activity, and was directly proportional to the concentration of chemically determined oxaloacetate in the film. Both mitochondrial and "soluble" isozymes of aspartate aminotransferase reacted with the cytochemical media to a quantitatively similar degree, but were considerably inactivated after 15 min of treatment with 1% glutaraldehyde or 3.7% formaldehyde in imidazole buffer, the rate of inactivation being greater for the soluble isozyme. Application of the principle of substrate protection delayed inactivation. Thus, for both isozymes the rate of inactivation decreased if ketoglutarate was added to the fixative. Similarly, it was shown that the optimal incubation medium for the demonstration of the soluble isozyme must contain 4 mM of α-ketoglutarate and 20 mM of L-aspartate. Under these conditions the turnover-number for the cytochemical system is 70% of the value obtained from biochemical estimations. Cytochemical K_m values differed for each isozyme and were in accord with values determined by biochemical techniques, indicating that the model system can be used as a link between biochemical and cytochemical data in enzymatic studies.

INTRODUCTION

Aspartate aminotransferase (glutamate oxalacetate transaminase; E.C. 2.6.1.1.) occupies a central position in amino acid metabolism. The products of its catalysis are not only involved in transamination reactions but also participate in the Krebs cycle, urea and ammonia formation, pyrimidine as well as carbohydrate and fatty acid metabolism, and in the early stages of the synthesis of sialic and muramic acid components (1, 2, 6, 17, 29). The enzyme is widely distributed in mammalian tissues (6), especially heart, skeletal muscle, liver, and kidney. Biochemical, electrophoretic, chromatographic, immunologic, and cell fractionation studies have demonstrated the existence

of at least two isoenzymes (2, 3, 4, 5, 11, 12, 13, 15, 26, 27, 28, 34). One isoenzyme is associated with mitochondria while the other is found in the "soluble" fraction of the cytoplasm. The terms "mitochondrial" and "soluble" will be used to distinguish these two isozymes.

A histochemical method for the demonstration of this enzyme has recently been introduced by Lee and Torack (19, 20, 21, 22, 23, 24) and is based on the precipitation of oxaloacetate as a lead salt at an alkaline pH. With this technique, aspartate aminotransferase activity has been demonstrated both histologically and ultrastructurally. The ultrastructural studies have demonstrated that the mitochondrial isozyme can be detected by this technique (21, 22).

In the present study the behavior of this histochemical reaction was examined in a model system consisting of rat or mouse liver homogenate incorporated in thin polyacrylamide films. After incubation of the film in the cytochemical medium, the absorbance due to the final reaction product (which remains within the polyacrylamide mesh) is estimated at a suitable wavelength and can be used as a measure of enzymatic activity in the film if expressed in terms of unit weight of the dried film. The efficacy of such model systems as aids in the study of cytochemical techniques has already been demonstrated (7, 8, 9, 10, 31). They provide means of quantitatively assessing the efficiency of cytochemical reactions for both light microscopic and ultrastructural studies under conditions which closely simulate those present when tissue studies are performed. It was established that, with the available cytochemical technique, the soluble isozyme of aspartate aminotransferase can be demonstrated and conditions favoring its demonstration can be defined.

The degree of enzymatic denaturation induced by glutaraldehyde or formaldehyde fixation was determined for both isozymes. On the assumption that addition of substrate or a reversible inhibitor of the enzyme to the fixative protects the active site during the process of fixation, substrate was added to the fixatives used. This decreased the rate of inactivation.

The information obtained was applied to the fixation and incubation techniques utilized in subsequent ultrastructural studies (30). This enabled an extension of the biochemical and cytochemical studies of Lee and Torack on rat liver (22, 23) and rat heart (20), in which primarily the mitochondrial isozyme was demonstrated.

MATERIALS AND METHODS

Preparation of Polyacrylamide Films Containing Mouse or Rat Liver Homogenate

Mouse or rat liver was homogenized in six times its volume of 0.05 M imidazole (Koch-Light Laboratories Ltd., Colnbrook, Buckinghamshire, England) buffer (pH 7.3) at 0°C in a motor-driven Potter-Elvehjem homogenizer. Wedge-shaped polyacrylamide films were prepared, according to the method of van Duijn et al. (8, 9), from a mixture containing 6.6 ml of monomer solution and 3.3 ml of mouse liver homogenate. To achieve reproducible results, thorough mixing of homogenate and the acrylamide mixture is necessary. At least 15 min of constant agitation, avoiding the formation of air bubbles, is advisable. Polymerization, performed in a cold room at 4°C to minimize heat denaturation of the enzyme was initiated by thorough mixing with 0.1 ml of 5% ammonium persulfate per ml of polymerizing mixture and was allowed to proceed for an hour at 4°C. The films produced were washed three times in cold distilled water, each wash lasting 10 min.

Fixation Techniques

Sample parts of polyacrylamide films containing liver homogenate were fixed in a 1% solution of glutaraldehyde (Fluka AG, Buchs, Switzerland) or 3.7% formaldehyde (E. Merck AG, Darmstadt, Germany) in 0.05 M imidazole buffer at pH 7.2 for 5, 10, and 15 min at 4°C. In some experiments substrate was added to the fixative. The substrates added were either D- or L-aspartic acids (266 mg/100 ml of fixative), α-ketoglutaric acid (117 mg/100 ml of fixative), or a combination of both aspartic and α-ketoglutaric acids. In one series of experiments pyridoxal phosphate (Koch-Light Lab., England) was also added to the fixative (5 mg/100 ml), and, in another, Lubrol (Imperial Chemical Industries Ltd., London S.W. 1, England) in distilled water was used at a concentration of 0.1% to pretreat the acrylamide films prior to incubation in an attempt to increase enzymatic activity (33).

Biochemical Estimations

Aspartate aminotransferase activity was estimated in total liver homogenates and homogenate fractions as well as in the films by the method of Karmen (18). Lubrol was used as recommended by Schnaitman and Greenawalt (33). For accurate estimations of enzymatic activity in polyacrylamide films containing liver homogenates, vigorous shaking becomes necessary to ensure adequate diffusion of the biochemical medium during incubation (8). Oxaloacetate estimations were also performed on the films after incubation

in the cytochemical medium. The precipitated lead oxaloacetate was dissolved in nitric acid, and the oxaloacetate content was determined by a modification of the technique of Reitman and Frankel (32).

Cytochemical Procedure for the Demonstration of Aspartate Aminotransferase Activity

The method developed by Lee and Torack (19, 21, 22, 23, 24) was used in most of the experimental procedures. The incubation medium consisted of 266 mg of L-aspartic acid (Koch-Light Lab., England) and 58 mg of α-ketoglutaric acid (Koch-Light Lab., England) dissolved in 10 ml of distilled water to which 25 ml of 0.2 M imidazole is added. After adjusting the pH to 7.5 with 0.2 N NaOH, the volume was adjusted to 50 ml. Just before use, an equal volume of a 12 mM solution of lead nitrate was added. The medium could be regularly produced free of any precipitate if carbon dioxide–free water was used in the preparation of all reagents, and if the lead nitrate solution was added dropwise from a burette whose tip was immersed in the solution containing the other components of the incubation medium, the resultant solution being constantly agitated with a magnetic stirrer (7). Incubation times varied from 15 to 60 min, depending on the experimental conditions, and were performed at room temperature (20°–22°C) with constant agitation. After incubation, acrylamide films were washed three times in fresh imidazole buffer (pH 7.2) and then immersed in freshly prepared 2% sodium sulfide solution in distilled water for 2 min. After three washes (each for 1 min) with distilled water, the acrylamide films were measured for absorbance in a film spectrophotometer or were stored for several days in 0.05% sodium sulfide at 4°C before measuring (7).

In another series of experiments, incubation media were used in which either the concentration of L-aspartic acid or ketoglutaric acid was varied. Media containing a low concentration of aspartate were difficult to prepare because of precipitation of lead salts.

Spectrophotometric Measurements of Acrylamide Films

The techniques previously described by van der Ploeg and van Duijn (9, 31) were utilized. Because of light scatter produced by the particulate nature of both the homogenate and the final reaction product (PbS), opal glass was used in the cuvette so that the "totally diffuse density" was measured. The absorbance of the acrylamide films was measured with incident light of 520 nm wavelength.

Fractionation Studies

Rat liver was homogenized in 0.25 M sucrose, and the mitochondrial and supernatant fractions were separated according to the method of Hogeboom (14). These two cytoplasmic fractions were incorporated separately in polyacrylamide, and cytochemical reaction was applied on parts of the resulting films that were either unfixed or fixed in media which did or did not include substrate. In this way the two isozymes were tested separately. The fixatives contained varying amounts of α-ketoglutarate, and the incubation media were of varying substrate composition.

Controls

Control experiments were produced by substituting D-aspartic acid for L-aspartic acid in the incubation medium. The former amino acid is not deaminated by the enzyme.

RESULTS

Polyacrylamide Films Containing Whole Liver Homogenates

Polymerization at low temperatures yields films in which about 50% of the enzymatic activity present in the original liver homogenate can be detected biochemically. Absorbance spectra of films containing the final reaction product (lead sulfide) after incubation in the cytochemical medium for aspartate aminotransferase are shown in Fig. 1. Since no distinct maximum was present in the spectrum, the absorbance of the reaction product at a wavelength of 520 nm was arbitrarily chosen for all further measurements of absorbance in polyacrylamide films on which the histochemical reaction was performed.

In wedge-shaped films, a direct linear relationship was found between the absorbance measured at 520 nm and the weight of a constant area of film (Fig. 2). This indicates a direct proportionality between the amount of reaction product (PbS) and the enzymatic activity in any given portion of film. The absorbance per unit weight of film can then be calculated from these data by the method of least squares (35). When this value is calculated for films incubated for increasing periods of time (15, 30, and 45 min), again a direct linear relationship without intercept is obtained (Fig. 3), revealing a direct proportionality between reaction product and incubation time. The value of the absorbance per mg after subtraction of the value

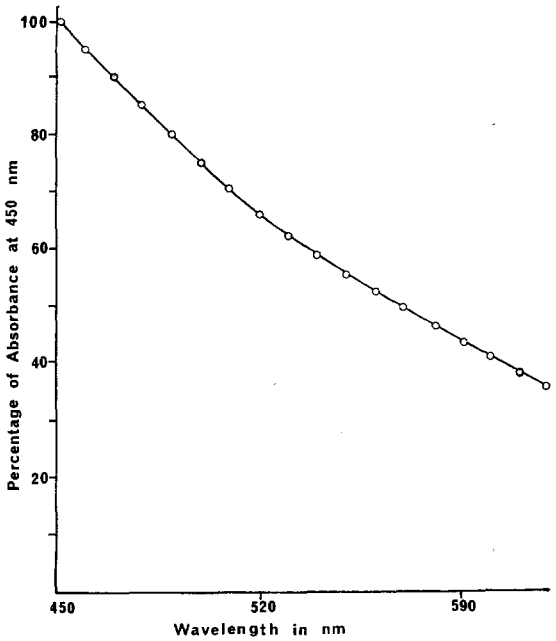

FIGURE 1 Absorbance spectrum of brown lead sulfide—the final reaction product of the cytochemical reaction. The absorbance at 450 nm was arbitrarily chosen as 100%.

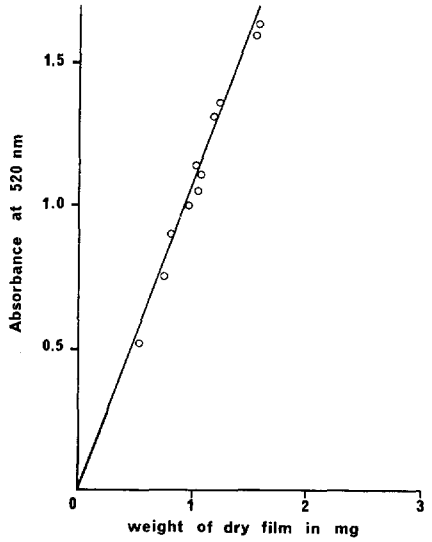

FIGURE 2 The relationship of absorbance at 520 nm to the weight of dry film, after performance of the cytochemical reaction. Wedge-shaped films of polyacrylamide were used in which liver homogenate was incorporated.

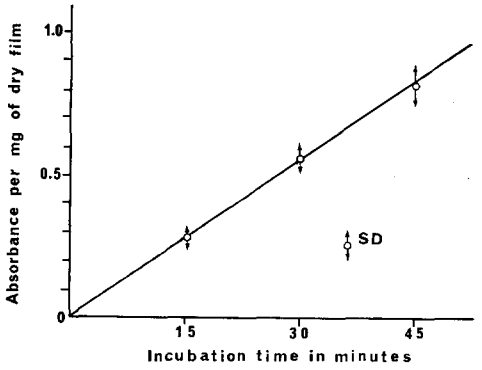

FIGURE 3 A linear relationship exists between absorbance per mg and incubation time. In this and in all subsequent graphs, the value of the absorbance per mg is that calculated by the method of least squares from 7 to 10 individual estimations on wedge-shaped polyacrylamide films containing liver homogenates.

of the control was compared to the biochemically estimated enzymatic acitvity expressed in milliunits per mg of the same batch of film. It was calculated that, for the system used, an absorbance of 0.60 ± 6% per mg dry weight resulted after 30 min of incubation in the cytochemical medium (at 20°–22°C) per milliunit of biochemically determined enzymatic activity in sample parts of the films.

A direct linear relationship is also found when the absorbance per mg of the brown lead sulfide (after correction for the value of the control) is compared to the amount of oxaloacetate determined biochemically on sample parts of the same batch of film (Fig. 4). This clearly establishes the correlation between absorbance produced by the brown PbS and the amount of reaction product estimated directly as oxaloacetate. From these data the amount of oxaloacetate produced per minute in the cytochemical medium can be directly compared with the calculated amount produced per minute during incubation in the biochemical medium. It is found that the oxaloacetate formed per minute during incubation in the cytochemical medium is approximately 70% of that produced during incubation in the biochemical medium in the same length of time. Differences may be due to variation in temperature, substrate concentration, pH, or the presence of lead.

Glutaraldehyde diluted to 1% in imidazole buffer (pH 7.2, 0.05 M) leads to rapid inactivation of the enzyme in the homogenate within the first 5 min of introduction. In this short length of time,

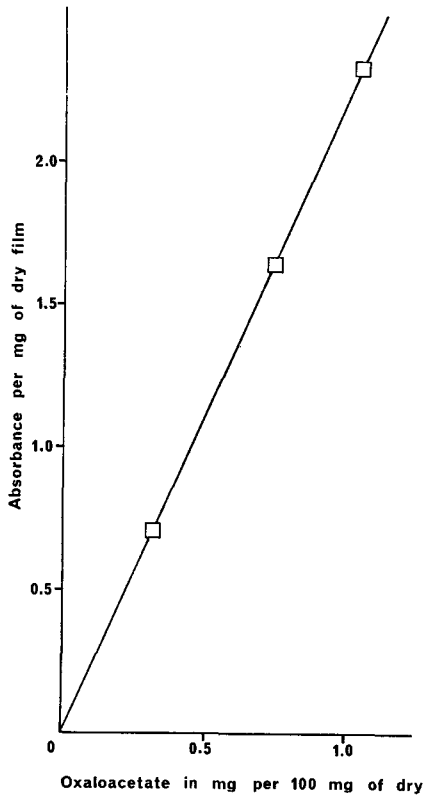

FIGURE 4 The absorbance per mg of dry film (less the value of the D-aspartic acid control) correlates with chemical estimations of oxaloacetate concentration (performed by dissolving the precipitated lead salts in acid) in the same batch of film, after incubation in the cytochemical medium for 15, 30, and 45 min.

approximately 70% of the histochemically demonstrable enzyme is inactivated (Fig. 5). If substrate is incorporated in the fixative solution, the rate of inactivation is slower and, even after 15 min of fixation, some 45% of enzymatic activity is histochemically detectable. This type of substrate protection is maximal when both aspartate and ketoglutarate are incorporated in the fixative. Ketoglutarate acts somewhat more effectively than either D- or L-aspartate, both of which produce a similar degree of enzyme protection. Pyridoxal phosphate in the concentration used produced no appreciable degree of protection.

Formaldehyde at a concentration of 3.7% proved to be a milder inactivating agent. Only 40% of the histochemically demonstrable enzyme is denaturated in the first 5 min, and, even after 30 min, 25% of the enzymatic activity remains (Fig. 6). Again, the presence of α-ketoglutarate in the fixative delays enzyme inactivation. However, this effect is less pronounced after a fixation period of 30 min.

In an attempt to increase enzymatic activity, the detergent Lubrol was used in a series of experiments on unfixed portions of polyacrylamide film containing liver homogenate. The film was pretreated for 10 min with the Lubrol solution and then the histochemical reaction was applied. No significant change in enzymatic activity could be detected between the treated and the untreated controls.

Polyacrylamide Films Containing Mitochondrial and Supernatant Fractions of Rat Liver

INCORPORATION INTO POLYACRYLAMIDE FILMS induced inactivation of part of the enzymatic activity in the homogenates during the polymerization. Of the original mitochondrial isozyme, 47% could be biochemically detected after polymerization, while 54% of the soluble isozyme remained active.

THE EFFECT OF SUCROSE: In order to exclude the sucrose solution used in the centrifugation procedure as a possible means of enzyme inactivation, polyacrylamide films containing liver homogenate were pretreated with 0.25 M sucrose and then incubated in the cytochemical substrate medium; the results were compared with those of untreated controls. There was no significant difference between the results in the two groups.

DEMONSTRATION OF BOTH ISOZYMES BY THE CYTOCHEMICAL TECHNIQUES: Aspartate aminotransferase activity was detected with the cytochemical medium not only in films containing the mitochondrial fraction but also in those in which the supernatant portion was incorporated, indicating that the cytochemical reaction can demonstrate the soluble isozyme. Absorbance values after incubation in the cytochemical medium of the same batch of films on which biochemical estimations were performed provided data for calculating the relationship of absorbance to biochemically determined enzymatic activity. The values obtained for the soluble and mitochondrial isozymes were an absorbance of 0.55 $\pm$ 7% and 0.61 $\pm$ 6%, respectively, after 30 min incubation in the cytochemical medium (at 20°–22°C) per milliunit of biochemically determined

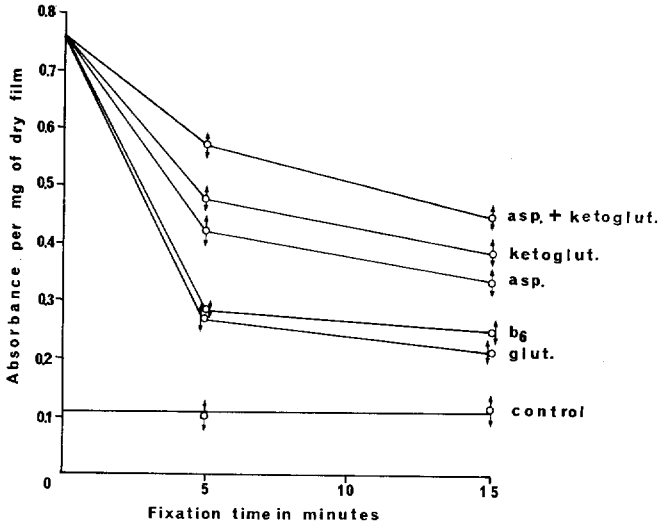

FIGURE 5 The effect of 1% glutaraldehyde in 0.05 M imidazole buffer on enzymatic activity expressed as absorbance per mg is shown. Addition of aspartate (asp.) and ketoglutarate (ketoglut.) lessen the degree of inactivation when added to the fixative, but pyridoxal phosphate (b_6) has no effect.

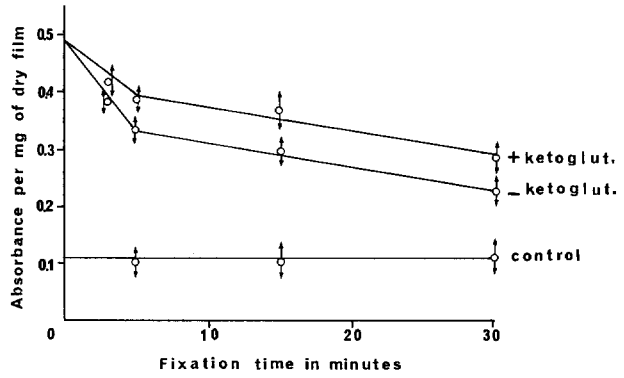

FIGURE 6 Enzymatic inactivation induced by 3.7% formaldehyde in 0.05 M imidazole buffer. The addition of ketoglutarate (ketoglut.) to the fixative delays inactivation.

enzymatic activity. These values indicate that in the unfixed condition the soluble isozyme can be demonstrated with the same degree of sensitivity as the mitochondrial isozyme by means of the cytochemical method.

THE EFFECTS OF FIXATION AND SUBSTRATE PROTECTION: The effect of glutaraldehyde fixation on the two cytoplasmic fractions differed. Enzymatic activity of the supernatant was lost at a quicker rate than the enzymatic activity of the mitochondrial fraction (Fig. 7). Substrate protection with α-ketoglutaric acid reduced the degree of enzymatic inactivation in both mitochondrial fraction- and supernatant fraction-containing polyacrylamide films. The degree of protection offered by α-ketoglutaric acid was more pronounced in films containing the supernatant fraction. This was evident even after 15 min of fixation when 35% of the enzymatic activity was still present in films which were protected by substrate as compared to 10% in those which were not (Fig. 8). In all cases the 100% value is that of the unfixed group of polyacrylamide films containing the appropriate fraction, after subtraction of the value of control films incubated in D-aspartic acid–containing media.

The mitochondrial isozyme was inactivated at a slower rate, especially in the early stages of fixa-

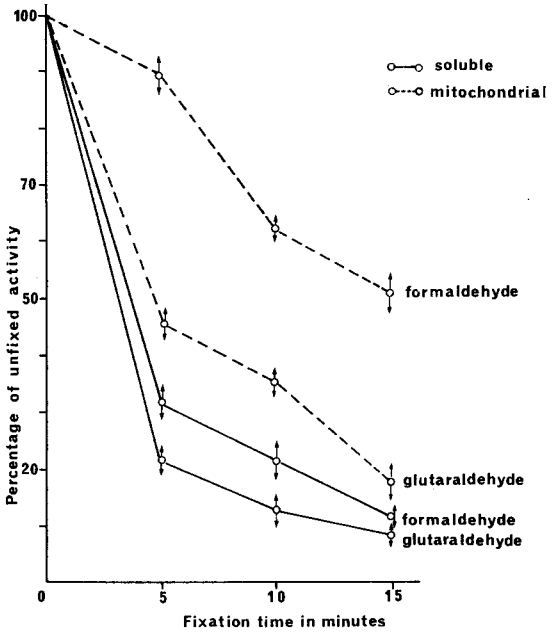

FIGURE 7 The rate of inactivation of the soluble and mitochondrial isozyme of aspartate aminotransferase by 1% glutaraldehyde and 3.7% formaldehyde in 0.05 M imidazole buffer is shown. The 100% value is the absorbance per mg of unfixed batches of film (calculated by the method of least squares) from which the value of D-aspartic acid controls has been subtracted. The soluble isozyme is inactivated at a quicker rate.

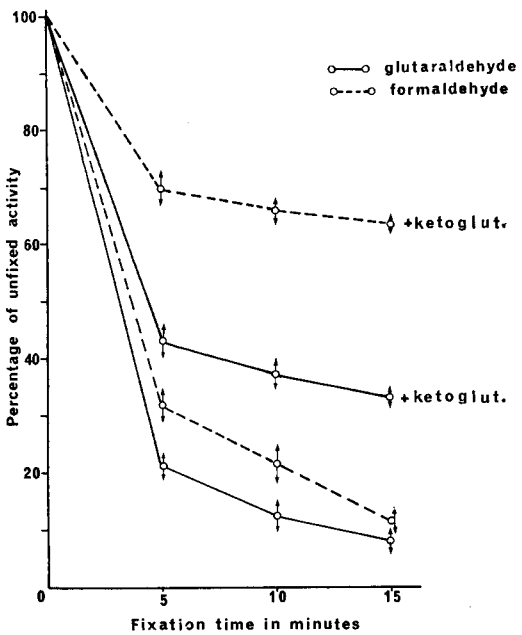

FIGURE 8 The effects of 3.7% formaldehyde and 1% glutaraldehyde in 0.05 M imidazole buffer on the activity of the soluble isozyme of aspartate aminotransferase. The "protection" afforded by ketoglutarate (ketoglut.) is evident.

tion. Within the first 5 min of fixation with 1% glutaraldehyde, 45% of mitochondrial aminotransferase activity can be detected as compared with 20% in similarly treated polyacrylamide films containing the soluble isozyme. Again, the presence of ketoglutarate decreases the rate of enzymatic inactivation by the fixative (Fig. 9).

Formaldehyde in the concentration used also induced rapid inactivation of the soluble isozyme and, at the end of 15 min of fixation, its effects are comparable to those of glutaraldehyde (Fig. 8). However, the degree of protection afforded by ketoglutarate is more pronounced when formaldehyde is used as the fixative agent than when glutaraldehyde is used (Fig. 8). Approximately 65% of the soluble enzymatic activity can still be retained after formaldehyde fixation in the presence of substrate, compared to 35% when glutaraldehyde is substituted as the fixing agent. In both cases, only 10% of enzymatic activity remains if ketoglutarate is omitted from the fixative solution. On the other hand, the mitochondrial isozyme is less rapidly inactivated by formaldehyde than by glutaraldehyde fixation (Fig. 9). However, a greater degree of substrate protection can be obtained if glutaraldehyde is permitted to act in the presence of ketoglutarate. Thus, after 15 min of fixation a residual enzymatic activity of approximately 65% is obtained irrespective of whether glutaraldehyde or formaldehyde is used, provided ketoglutarate is present during the fixation process.

THE EFFECT OF VARYING CONCENTRATIONS OF KETOGLUTARATE IN THE FIXATIVE: In this series of experiments, the degree of protection afforded by varying concentrations of α-ketoglutaric acid during the process of glutaraldehyde fixation of polyacrylamide films containing either the soluble or mitochondrial isozymes was assessed. The concentrations of ketoglutarate selected were 2 mM, 4 mM, 8 mM, and 16 mM, and a fixation time of 10 min was chosen. In contrast to the situation in which ketoglutaric acid was omitted from the fixative, all concentrations of ketoglutarate chosen provided some de-

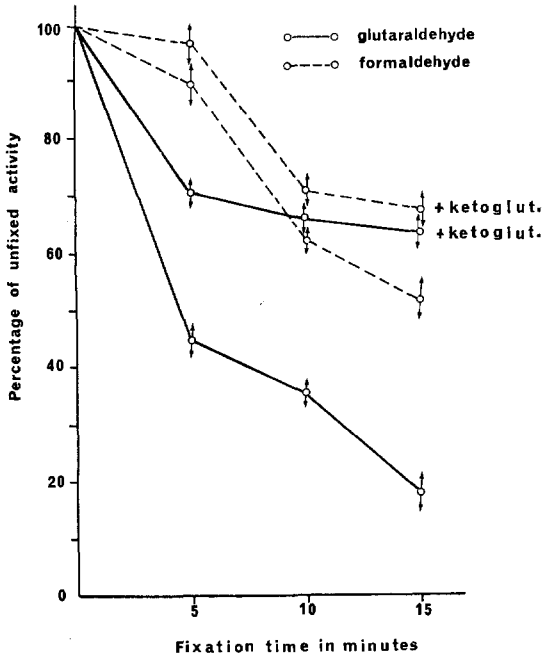

FIGURE 9 The effect of 3.7% formaldehyde and 1% glutaraldehyde in 0.05 M imidazole buffer on the "mitochondrial" isozyme of aspartate aminotransferase. Ketoglutarate (ketoglut.) when present in the fixative decreases inactivation.

gree of protection (Fig. 10). The protection was most pronounced for both the soluble and mitochondrial isozyme when the concentration of ketoglutarate was adjusted at 8 mM. When the concentration of ketoglutarate reached 16 mM, the degree of enzymatic protection was lowered. This was more obvious in films containing the soluble isozyme. The degree of protection with increasing concentrations of ketoglutarate is higher with the soluble than with the mitochondrial isozyme.

THE EFFECT OF VARIATIONS IN SUBSTRATE CONCENTRATIONS IN THE INCUBATION MEDIUM: Two groups of incubation media were prepared. In the first the concentration of aspartate was maintained at a constant level (20 mM) while the concentration of ketoglutarate was varied (1 mM, 2 mM, 4 mM, and 8 mM). The results obtained with polyacrylamide films containing either the soluble or the mitochondrial isozyme are given in Fig. 11.

When the concentration of α-ketoglutarate in the incubation medium was 4 mM, the amount of reaction product resulting from soluble isozyme activity reached a maximum. The amount of reaction product dropped slightly when the concentration of ketoglutarate was adjusted to 8 mM. The mitochondrial isozyme behaved somewhat differently and the amount of reaction product increased with the increasing concentration of ketoglutarate in the range tested. A Lineweaver and Burk plot (25) was then constructed from the values obtained (Fig. 12), and the Michaelis constants of the two isozymes were estimated.

In this type of graph, the inverse values of the absorbance per mg of polyacrylamide films containing either the mitochondrial or the soluble fraction after 30 min of incubation (after subtraction of the value of the D-aspartic acid control) were plotted against the inverse values of the ketoglutarate concentration. From the point of intercept the approximate K_m (ketoglutarate) values for the soluble and mitochondrial isozymes were graphically estimated to be 0.8 mM and 2.3 mM, respectively.

In the second series of experiments the concentration of the aspartate was varied (5 mM, 10 mM, 20 mM, and 27.5 mM), while the concentration of ketoglutarate was maintained at 4 mM. The media

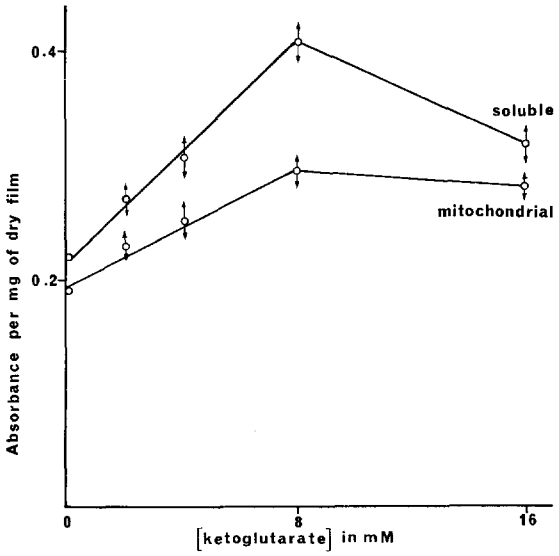

FIGURE 10 The degree of "enzymatic" protection afforded by increasing concentrations of ketoglutarate when 1% glutaraldehyde is used as the fixative is shown for both the soluble and the mitochondrial isozyme. The optimum concentration is 8 mM. The fixation period in all cases was 10 min.

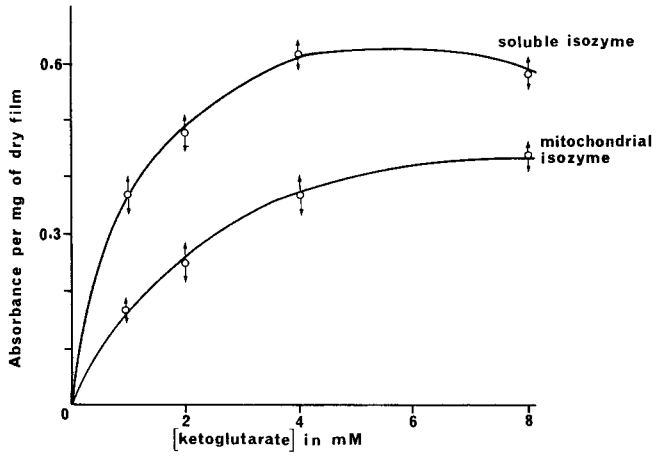

FIGURE 11 Incubation media containing varying concentrations of ketoglutarate influence the demonstrable activity of the two isozymes. Aspartate concentration was maintained at 20 mM and the incubation period was 30 min. (The value of the D-aspartic acid control has been subtracted from each group.) A ketoglutarate concentration of 4 mM is optimal for the soluble isozyme, but a somewhat higher concentration is more suitable for the mitochondrial isozyme.

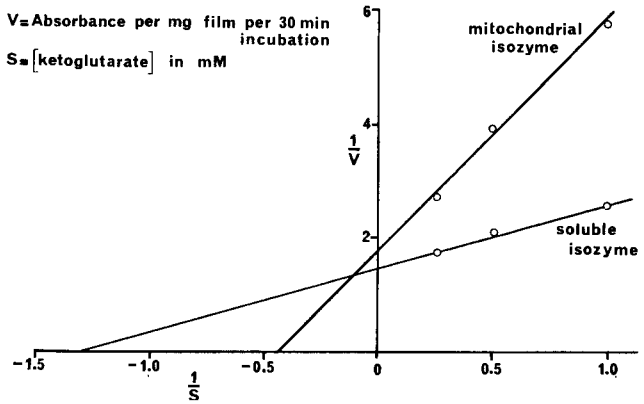

FIGURE 12 A Lineweaver and Burk plot for the estimation of the Michaelis constant (K_m—ketoglutarate) for the two isozymes. From the points of intercept the K_m can be estimated. The K_m (ketoglutarate) of the soluble isozyme differs from that of the mitochondrial isozyme.

containing the low concentrations of aspartate were difficult to prepare because of precipitation of lead salts, necessitating the use of a lower concentration of lead nitrate. Thus the results are difficult to compare with those in which the concentration of lead nitrate was higher. Nevertheless, it appears that for both soluble and mitochondrial isozyme and maximum amount of reaction product is reached when the aspartate concentration is adjusted at 20 mM. Higher concentrations of aspartate diminish the amount of reaction product formed (Fig. 13).

DISCUSSION

The model system used in these studies offers a practical means of testing a histochemical reaction both qualitatively and quantitatively. It provides a good guide for determining not only the optimum composition of the reaction medium but also the effect of fixation and a means of determining the best combination of acceptable tissue fixation with adequate enzymatic preservation. The latter becomes critical in any ultrastructural study. The present results show that the kinetics of the cytochemical system in which the precipitated lead

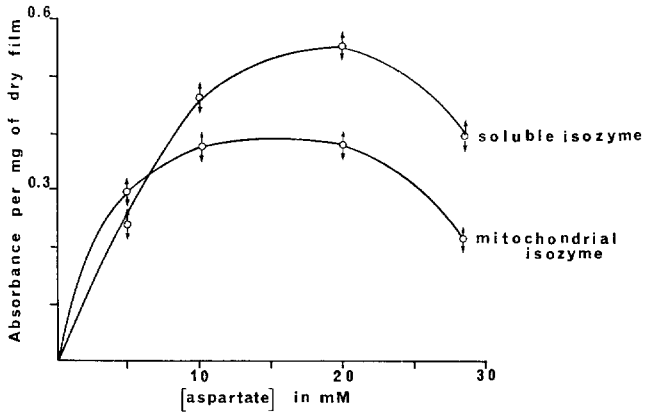

FIGURE 13 The effect on the rate of oxaloacetate formation in the cytochemical system with varying concentrations of aspartate. Ketoglutarate concentration was maintained at 4 mM and the incubation period was 30 min. (The value of the D-aspartic acid control has been subtracted from each group.) An aspartate concentration of 20 mM is optimal for both isozymes.

salt of oxaloacetic acid is measured after conversion to PbS are similar to those of a biochemical enzyme reaction carried out in solution. The amount of reaction product increased linearly with time. The enzyme was inhibited by D-aspartic acid. Biochemical studies on the effects of lead and fixatives have been carried out by Lee and Torack on purified pig heart enzyme and rat heart tissue (20) sections. Their findings, probably related to the mitochondrial isoenzyme, in general, could be confirmed on the polyacrylamide-incorporated homogenates, though in our system we found more inactivation by lead. In biochemical studies on suspensions of rat hepatic mitochondria, Lee and Torack (22) observed enzyme inactivation in sucrose solutions. This we could not confirm. Activation of the enzyme by 6 mM lead also was not observed in our system. These different results probably are due to the differences in methods used. The present system has the advantage that it adheres more closely to the actual conditions operating when tissue blocks are fixed and incubated in actual electron microscopical cytochemical studies. The results, therefore, can be correlated with more confidence with the electron micrograph images finally obtained. The use of polyacrylamide films made it possible to obtain data on the degree of inactivation by glutaraldehyde and formaldehyde for each of the isozymes separately. For both isoenzymes, glutaraldehyde proved a stronger inactivating agent than formaldehyde. This may be due to its ability to react more strongly with amino groups (16).

It is not unexpected that the enzymatic activity in the cytoplasmic supernatant was found to be more prone to inactivation by glutaraldehyde and formaldehyde than is the mitochondrial enzyme. It is not unreasonable to assume that molecules of aspartate aminotransferase buried in the remnants of the lipoprotein layers of the mitochondrial substructure could be less liable to the effects of glutaraldehyde. On the other hand, it may reflect differences in the physicochemical properties of the two isozyme molecules which have also been shown to possess different chemical, electrophoretic, chromatographic, and immunological properties (2, 3, 4, 5, 11, 12, 13, 26, 27, 28, 34).

In an attempt to increase fixation time, thus aiming eventually at a better degree of preservation, substrate was introduced into the fixative, on the supposition that it would preferentially attach to the active site of the enzyme and prevent its interaction with glutaraldehyde. In the presence of ketoglutarate in the fixative, the rate of inactivation of the soluble isozyme by either glutaraldehyde or formaldehyde was retarded, permitting the demonstration of the isoenzyme after fixation times which were compatible with acceptable morphology (30). The degree of protection afforded by ketoglutarate is greater when formaldehyde is used as the fixative than when glutaraldehyde is used. The reason for this is not known. It is interesting to note that ketoglutarate with the longer carbon chain has a somewhat greater degree of protection than L- or D-aspartic acids, which possess a shorter carbon chain. The combination

of both α-ketoglutaric acid and aspartic acid has almost an additive effect on enzyme protection.

When solutions of fixative and aspartic acid were prepared a yellow coloration appeared within minutes, indicating that a chemical change had occurred. Since glutaraldehyde can react with amino groups, part of the protection offered by aspartate may only be apparent and be due to interaction of its amino group with glutaraldehyde (16), thus decreasing the effective concentration of the fixative. On this premise, aspartic acid was therefore not used as a means of substrate protection in subsequent experiments and the ultrastructural studies which followed (30). It is interesting to note, in relation to the substrate protection observed, that in biochemical studies aspartate was found to increase the heat lability of the enzyme, whereas ketoglutarate decreased it (17).

Addition of pyridoxal phosphate (which acts as a coenzyme in the transamination process) to the fixative failed to protect the enzymatic site. Attempts to enhance enzymatic activity with detergents such as Lubrol also proved negative. Possibly the polymerization and washing procedures had already removed enzymatic latency.

Films containing either the mitochondrial or the soluble fractions of hepatocytic cytoplasm and on which the histochemical reaction was applied demonstrated that the cytochemical technique of Lee and Torack (19, 21, 22, 23, 24) was sensitive for both the mitochondrial and the soluble isozyme to almost the same degree of efficiency. This is shown by the close agreement of the ratios of absorbance in the cytochemical system to the biochemically estimated enzymatic activity in the films, for the two isozymes.

The mitochondrial isozyme is also more quickly inactivated by glutaraldehyde than by formaldehyde. On the other hand, the degree of protection afforded by ketoglutarate is greater when glutaraldehyde acts as the fixing agent. Such observations may again reflect physicochemical differences between the two types of isozyme molecules.

Enzymatic protection for both the soluble and mitochondrial isozyme reaches a maximum when the concentration of ketoglutarate in the fixative is adjusted at 8 mM. Beyond that, the degree of protection falls, possibly due to the formation of an irreversible aminotransferase-ketoglutarate complex (13). The degree of protection afforded by increasing concentrations of ketoglutarate is greater in the case of the soluble isozyme and may be due to the greater affinity of the soluble isozyme for ketoglutarate (2, 12).

Variations in the substrate composition of the incubation medium have shown that the optimal medium for the demonstration of the soluble isozyme should contain 4 mM of α-ketoglutarate and 20 mM of aspartate. Higher concentrations of either component proved to be suboptimal. The reason for the suppression by higher concentrations of α-ketoglutarate may be due to the formation of an irreversible enzyme-ketoglutarate complex (13). Higher concentrations of aspartate suppress the formation of reaction product of both the soluble and the mitochondrial isozyme. The reason is not clear but may be related either to enzymatic suppression or to increased chelation of lead ion, rendering the ion unavailable for the precipitation of the final product.

The role of chelation of lead ions by aspartic acid may be complex. It may, on the one hand, decrease the concentration of free lead ions, which may be advantageous for enzyme action. On the other hand, sufficient lead ion should be available for a good capture reaction. The results with the polyacrylamide system, however, indicate that the cytochemical medium as described by Lee and Torack works with a turnover-number only 30% below that of the biochemical Karmen medium (18).

The K_m values for ketoglutarate obtained for both the soluble and mitochondrial isozyme by the histochemical technique differ significantly from each other and are in the range of values expected from biochemical estimations (2, 4). This also demonstrates that the substrates in the cytochemical medium did not function differently when compared with the biochemical system. The differences in the cytochemically and biochemically determined actual values may be accounted for by the presence of lead in the incubation medium as well as by the differences in aspartate concentration, pH, and temperature. The K_m values obtained supply further proof of the presence of the isozyme under test by providing an obviously different K_m for each of the two isozymes. The model system offers a means of obtaining quantitative information on the effects of a variety of manipulations on a given method. In this way, small degrees of improvement can be assessed which collectively may result in an appreciable change. Variations in incubation tem-

perature, and pH and lead ion concentration may also be tested on the present experimental system.

Model studies with substrate protection on separate isoenzymes, as described, may find application for other enzymes as well. Enzyme-containing preparations incorporated in polyacrylamide films can be fixed and incubated like tissue blocks, and at the same time analyzed independently by biochemical methods. This provides the necessary link between cytochemical and biochemical studies. The present investigation can be considered further proof of the versatility of this approach.

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