Identification of Catalase Following Electrophoresis on Acrylamide Gels

By ALEXANDER BAUMGARTEN

ACRYLAMIDE GELS have been used with advantage for the electrophoretic separation of plasma proteins and hemoglobins because they give sharper bands and improved resolution of components as compared with starch gels, while their transparency facilitates photometric scanning. Also, the method of preparation is easier and they can be stored for long periods before use.

Recently, the author has developed a micro method of serum protein electrophoresis suitable for rapid typing of haptoglobins. This technic has also been used in investigating the electrophoretic mobility of hemoglobins and in some cases it was found advantageous to intensify the color of the hemoglobin band by using the staining procedure previously adopted for haptoglobins. In every case where this was done, a narrow, completely clear zone was seen, approximately midway between the hemoglobin A band and the origin, whereas the remainder of the gel assumed a reddish tint. It is the purpose of this paper to describe investigations into activity associated with the clear zone and to present a method for identifying catalase in acrylamide gels.

The method of Hale and Renwick depends on the binding of iodine by amylose and is therefore directly applicable only to starch gels, while the method of Uriel, which depends on the evolution of bubbles of oxygen gas from the region containing catalase, does not provide a permanent record, does not define sharply the boundaries of the region, and is unsuitable for examining a large number of samples.

MATERIALS AND METHODS

Preparation of Acrylamide Gels

The technic for preparing acrylamide gels described previously was used with some modifications. Briefly, a stock solution of 30 per cent w/v of acrylamide (supplied as chemical grout, AM9, by Cyanamid Co., Australia) in distilled water is prepared. Also, a solution of the following composition is prepared shortly before use:

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium persulfate</td>
<td>0.5 Gm.</td>
</tr>
<tr>
<td>Potassium ferricyanide</td>
<td>5.0 mg.</td>
</tr>
<tr>
<td>Distilled water</td>
<td>25.0 ml.</td>
</tr>
</tbody>
</table>

Immediately before use, the following solution is prepared:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stock acrylamide solution</td>
<td>25.0 ml.</td>
</tr>
<tr>
<td>Ammonium persulfate</td>
<td>25.0 ml.</td>
</tr>
<tr>
<td>DMAPN (β-dimethylaminopropionitrile)</td>
<td>0.5 ml.</td>
</tr>
<tr>
<td>Distilled water</td>
<td>100.0 ml.</td>
</tr>
</tbody>
</table>

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IDENTIFICATION OF CATALASE

This solution is poured into a flat methacrylate ("lucite") tray whose sides project 1.5 mm. above the level of the microscope slides lying on the bottom of the tray, and a lucite cover is slid over the solution. After gelling has occurred the slides are cut out and washed with a tris-citric acid buffer, modified from that of Poulik and containing 9.2 Cm. tris(2-amino-α( hydroxymethyl)-1:3; propanediol) and 1.0 Gm. citric acid per liter. They are kept in the buffer, at 4 C., until required for use.

Staining Solution

The staining solution as previously described is a modification of that suggested by Owen et al. for staining haptoglobins in starch gels and is of the following composition:

- Saturated solution of o-dianisidine in ethanol
- Ethanol
- Acetate buffer, pH 4.6, 2.7 M
- Distilled water

Following electrophoresis, the acrylamide gels are soaked in the solution for 10 minutes or longer and then 5 ml. of 30 per cent w/v hydrogen peroxide are added. The gels are removed after 10 minutes and soaked in water.

Starch Paste

A solution of potassium iodide in distilled water is prepared containing 0.1 Gm. potassium iodide in 10 ml. water. Enough hydrolyzed starch is then added to 1 ml. of the solution to form a moderately thick paste.

Dilute solution of hydrogen peroxide is prepared by adding 5 ml. of 30 per cent w/v hydrogen peroxide to 95 ml. distilled water.

Preparation of hemolysates. This was accomplished by thrice repeated freezing and thawing to break the red cells. Following centrifugation and separation the hemolysates were diluted 1 in 10 with distilled water.

Hemoglobin-containing sera were prepared by adding one drop of the hemolysate from a known normal blood donor to 0.2 ml. of serum.

Conditions of electrophoresis were the same as those previously described using a discontinuous tris-borate system and conducting electrophoresis for 30 minutes at 20 V/cm. Dimensions of the microscope slides and of the gels used were either 75 x 25 cm. or 75 x 50 cm. The gels were connected to the buffer tanks by filter paper wicks soaked in borate buffer. Slits for the samples were cut in the gels approximately 15 mm. from the cathodal end of each gel. Samples for insertion were prepared by cutting 1 x 5 mm. strips of Whatman chromatography paper No. 1 or of cellulose acetate membrane, immersing these strips in the material to be subjected to electrophoresis, and lightly blotting them before insertion.

RESULTS

Identification of Catalase Activity

Hemolysates of red cells obtained from 25 normal blood donors, and six sera free of hemoglobin, two of each of the three haptoglobin types (1-1, 1-2 and 2-2), were individually examined for the presence of catalase activity in the following manner.

At the completion of electrophoretic separation, each gel was placed for 2 minutes in the dilute solution of hydrogen peroxide and, with the hemolysate samples, evolution of oxygen bubbles was observed from two zones: slightly over the hemoglobin band, and intensely from a zone approximately midway between the hemoglobin band and the origin. The zone from which rapid evolution of oxygen occurred was considered to contain catalase in accord
with the method of Uriel for the identification of catalase following electrophoresis in agar gels and its level was marked by a V-shaped cut in the side of the gel. The upper and lower limits of this zone were then identified by using the blue color developed by the amylose-iodine complex. For this purpose, the gel was washed for 30 seconds in running water and then lightly blotted to remove excess hydrogen peroxide from the surface. Potassium iodide containing starch paste was then rapidly and evenly spread in a thin layer over the surface of the gel. The paste quickly assumed a blue color with the exception of a single white zone. Cuts were made with a scalpel blade at the edges of this zone and the gel was again washed in running water till all paste was removed. The gel was then placed in the staining fluid and stained as described for haptoglobins. In each case the gel assumed a reddish tinge with the exception of a single clear zone whose level corresponded to the V-shaped mark in the edge of the gel and whose upper and lower borders closely corresponded to the horizontal cuts marking the limits of the white zone revealed by starch paste.

In no case was any activity observed when the sample consisted of normal serum. The catalase activity was therefore confined to the hemolysates and absent from normal sera.

An attempt was then made to determine whether the catalase containing zone corresponded to a definite protein band. For this purpose two gels pre-stained with 0-dianisidine and containing three samples of hemolysates each were stained with an 0.2 per cent w/v solution of nigrosin in 2 per cent v/v acetic acid in water. The clear zone was still visible and there was no specifically stained protein band corresponding to this zone.

**Electrophoretic Mobility of Catalase**

To determine whether the electrophoretic mobility of catalase was subject to individual variation the following experiment was performed.

Six slits were made in each 75 x 50 mm. gel and samples of hemolysates of red cells from 25 normal blood donors were introduced in such fashion that the last sample in the one gel was from the same donor as the first sample in the next gel. In this manner, direct comparison of all samples was possible. Following electrophoretic separation and staining it was found that every clear zone occupied the same relative position from the origin. The mobility of catalase was thus found to have been the same in each of 25 hemolysates examined.

To localize the position of the catalase containing band further in hemolysate containing sera, nine sera containing hemolysate were prepared, three sera of each haptoglobin type. Following electrophoretic separation and staining, a clear zone was visible in all cases. This zone was seen immediately behind the red band representing hemoglobin-haptoglobin type 1-1 complex (see figure 1).

**DISCUSSION**

The prompt appearance of numerous bubbles in a limited zone of the gel following its immersion in dilute hydrogen peroxide after the electrophoretic
Fig. 1.—Left: serum of haptoglobin type 1–2 + hemolysate. Right: hemolysate only. Bands: 1, hemoglobin A; 2, hemoglobin A₂; 3, hemoglobin-haptoglobin 1 complex; 4, catalase; 5 & 6, high molecular weight hemoglobin-haptoglobin complexes. (Direct print using a contrast type enlarger, magnification x4.)

Separation of red cell hemolysates may be considered as strong evidence of catalytic activity such as is usually associated with catalase. However, this effect is still too diffuse, and too transient for acceptable analysis. The rapid blue coloration of potassium iodide containing starch paste in all areas of the gel except that in which the oxygen bubbles were originally seen to form, provides further evidence of the rapid and complete decomposition of hydrogen peroxide in one zone of the gel. Since it is an adaptation of the method already established for the identification of catalase following electrophoretic separation in starch gels, it appears safe to assume the presence of catalase in that zone. The considerable catalytic effect in this zone is again demonstrated by its remaining clear, owing to the decomposition of its contained hydrogen peroxide when the remainder of the gel is discolored by oxidized O-dianisidine. The proposition that the zone contains catalase is strengthened by its independence from the peroxidase activities of the haptoglobin complexes, and by the absence of demonstrable nigrosin staining in the zone which indicates low protein concentration there. The erythrocyte origin of the activity is shown by its absence from hemoglobin-free serum.

The appearance of the clear zone following O-dianisidine staining of the gel represents a quick and convenient method of identifying catalase following electrophoresis of hemolysates. Since the original technic is particularly suited to the investigation of hemoglobin mobility and the use of O-dianisidine...
staining enhances the color of the hemoglobin, it is suggested that this technic should be adopted in hemoglobin surveys to take advantage of catalase identification. Thus, cases of acatalassemia could be readily identified, and it is possible that catalase isozymes may be found. Moreover, this technic should prove useful in the investigation of other tissue homogenates containing catalase activity, particularly in view of the small amounts of homogenate required for electrophoretic separation by this method. It is of further interest to note the possibility of using this technic on leukocyte homogenates from cases of acute leukemia where Kidson has reported the existence of two enzymes with catalase activity.

**Summary**

During the performance of haptoglobin typing by a new technic described by the author, a clear zone was observed in the acrylamide gels used for electrophoresis. Investigations to determine the identity of this zone have shown it to be associated with catalase activity. Consequently, a new technic is presented for identifying catalase following electrophoretic separation of hemolysates. Twenty-five hemolysates from normal human subjects were investigated by this method and were found to contain catalase activity which had the same electrophoretic mobility in all cases. Six normal human sera did not contain this activity under the conditions employed. The catalase activity was found to migrate immediately behind the haptoglobin type 1-1 band.

**Sumario in Interlingua**

In le curso del typification haptoglobinic secundo un nove technica (que le autor describe), un zona clar esseva observate in le gels acrylamidic usate pro le electrophorese. Investigationes visante a determinar le identitate de iste zona ha monstrate que illo es associate con le activitate de catalase. Consequentemente, un nove technica es presentate pro identificar catalase post le separation electrophoretic de hemolysatos. Vinti-cinque hemolysatos ab normal subjectos human esseva investigate per iste methodo, e il esseva trovate que illos contineva un principio de activitate catalasic que habeva le mesme mobilitate electrophoretic in omne le casos. Sex normal seros human non contineva iste activitate sub le conditiones empleate. Esseva trovate que le activitate catalasic migrava immediatemente in retro del banda de haptoglobina tipo 1-1.

**REFERENCES**

5. Curtain, C. C.: Separation of haemoglobins Lepore and H from A and


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