Starch Gel Electrophoresis of Rat Serum Proteins

I. PROCEDURE AND DESIGNATION OF COMPONENTS*

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In 1955, Smithies (1) described a procedure for the separation of human serum proteins by horizontal zone electrophoresis in starch gels. The separation of zones was improved by a later modification to a vertical electrophoresis in which the sample was inserted in the gel, free from supporting medium (2). With these methods, it has been demonstrated that many more protein fractions can be separated from human sera than by any of the previously accepted electrophoretic procedures. The methods have been widely applied in the investigation of enzymatic preparations, biological preparations, and in the study of genetically controlled variations in serum proteins of the human and other animals. Smithies (3) has reviewed some of the many applications of starch gel electrophoresis, and the factors affecting protein migration in starch gels. The protein zones seen after electrophoresis of human sera have been identified by Smithies (3) and others in terms of their mobilities on filter paper and their specific properties.

Latner and Zaki (4) used starch gel electrophoresis in a comparison of animal sera, including that of the rat. Marked species differences were evident. In detailed studies of rat sera, at least fourteen protein zones can be distinguished. Before experimental and other factors affecting the relative intensity of these zones can be discussed, a suitable, descriptive designation of the zones must be formulated. The denomination used in the electrophoresis of human sera (3) is not suitable. The conventional designation of albumin and four globulin fractions is inadequate. Such a reference designation of rat serum proteins, separated by starch gel electrophoresis, is formulated in the present paper.

EXPERIMENTAL PROCEDURE

Materials and Methods

In the present studies, more than two hundred individual serum samples have been examined. Most of the rats used were of the Wistar strain from the Carworth Farms colony; a few animals of the same strain were obtained from the Woodlyn Farms colony. The pattern of serum proteins seemed to be similar in animals from either source. In all cases, blood was collected from the neck in petrolatum-coated tubes after stunning and decapitation. After clotting and centrifugation the serum was rapidly frozen and stored in sealed tubes at -10 to -15°C until examined electrophoretically. Preliminary investigation revealed that there was no change in the electrophoretic pattern of rat serum proteins stored under these conditions for several months. The rats were normally fasted for 18 hours before killing, although comparison of fasted and unfasted rat serum samples revealed no consistent differences. The rats used in these studies had been subjected to a variety of dietary and experimental histories; these will not be discussed here.

Vertical starch gel electrophoresis was carried out in equipment similar to that described by Smithies (2). With rat sera it was found that a sample slot approximately 0.4 mm thick was preferable to the 0.75-mm slot used by Smithies for human sera. Rat serum causes a breakdown of the starch gel at the point of entry, perhaps because of an amylase moving toward the anode. The use of the thin sample slot (and, hence, a smaller sample) minimizes the breakdown of the starch gel and also permits sharper resolution of the protein zones in the starch gel. The starch gels were made up with barbital-acetate buffer described above. Filter paper-starch gel electrophoresis, 6-μl aliquots of serum were placed on strips of Schleicher and Schuell 598-YD filter paper, 0.5 cm × 35 cm, and electrophoresis was carried out for 18 to 20 hours at a potential gradient of 3.5 volts per cm. The buffer used in most studies was composed of 0.048 M sodium acetate, 0.048 M sodium barbital, and 0.0075 M hydrochloric acid, pH 8.6. Under these conditions five protein zones could be distinguished in normal rat sera. The barbital buffer with added calcium of Laurell et al. (5) gave a wider separation of the protein zones, but seemed to offer little advantage, for our purposes, over the barbital-acetate buffer described above. It was used only to confirm the findings with the barbital acetate buffer. Filter paper strips were stained with Amido black 10B.

When samples were examined by means of two-dimensional filter paper-starch gel electrophoresis, 6-μl aliquots of serum were separated on filter paper as described above. Duplicate separations were carried out. One strip was stained to locate the protein zones. This portion of the unstained strip was cut out of the paper and inserted in a cut in the starch gel at right angles to the direction of the current flow (6). A small piece of filter paper wetted with the serum was also inserted next to the electrophoresis strip; this served as a reference pattern on the gel.

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For these studies it was possible to cast the gels in simple molds of approximately 12 x 15 x 0.6 cm in size. Electrophoresis was carried out in a horizontal manner (1) for 6 hours at a potential of 5 to 5.5 volts per cm; the gel composition was as described for horizontal gels.

After electrophoresis, the gels were sliced horizontally to give two halves 0.3 cm thick. Protein zones were detected by staining with saturated Amidoblack 10B in a dye solvent of 50:50:10 methanol-water-glacial acetic acid. Staining was carried out for 4 minutes, taking care to stain only the cut surface. A semitransparent gel was obtained by washing in a solution of 1% aqueous acetic acid recirculated through charcoal to remove the dye, followed by a period of 2 to 3 days of standing in the acetic acid to remove further dye from the gel. The gels were viewed by transmitted light. This procedure permitted the detection of protein zones not readily visible in the opaque gel, obtained by washing in the methanol dye solvent.

The presence of iron was detected by staining one-half of the gel for 15 minutes in an aqueous solution of 1% hydroxylamine hydrochloride, 2.7% sodium acetate (0.3H2O), 0.5% Nitroso R salt, and 1.5% glacial acetic acid. The stained gels were washed with the methanol dye solvent for 3 to 4 hours. Iron stained as a light green color.

Lipid material was detected by staining half of the gel, overnight, with a mixture of 1 part saturated oil red O or oil blue N in methanol and 1 part 20% aqueous trichloroacetic acid. The lipid-containing zones were seen as reddish or bluish bands on a white background.

Hemoglobin was detected by staining with a solution of 200 mg of benzidine (dissolved in minimal methanol) and 0.4 ml of 30% hydrogen peroxide in 200 ml of 0.2 M sodium acetate, 0.2 M acetic acid. After 1 to 2 hours of staining, followed by washing with water, hemoglobin was seen as a black zone.

In all cases where the detection of iron, lipid, or hemoglobin was carried out by staining procedures, the cut surface of one-half of the gel was stained for protein and the other cut surface was stained for the material in question. It was possible to make direct comparison of the protein and other zones and, hence, to locate the iron, lipid, or hemoglobin in terms of the protein zones.

It should be noted that gels stained with Amidoblack may be wrapped in moisture proof plastic and stored under refrigeration. If they are not frozen, they undergo very little change and may be kept for future reference. Gels treated with other stains are not as stable.

**Results and Discussion**

Preliminary studies revealed that the electrophoretic conditions and gel composition described for use with human sera (2), with the aforementioned reduction of sample size, gave a sharp resolution of the majority of rat serum protein zones.

The majority of samples were examined only by vertical starch gel electrophoresis. By this procedure, at least fourteen distinct zones could be detected. In horizontal gels, the protein zones were not as sharply resolved; however, it appeared that as many zones were distinguishable in horizontal gels as in vertical gels. This is not the case with human sera (2).

Fig. 1 is a composite drawing representing the location of protein zones after two-dimensional electrophoresis of rat sera. By means of the two-dimensional electrophoresis depicted, the relationship between the migration on filter paper and in starch gels can be observed. It should be noted that not all of these zones would be readily visible with a single serum sample, nor would their relative intensities always be the same.

A designation of the various protein zones is also shown in Fig. 1. This system of identification has been based on the accepted nomenclature of moving boundary and filter paper electrophoresis combined with the relative rates of migration in starch gels. It can be seen in Fig. 1 that more protein zones are detectable in two-dimension than in one-dimension gels. α1, α2, and β-globulins which move at the same rate in starch gels would appear as a single zone. In these cases, the designation is based on the component which usually stains with the greatest intensity. In addition to the zones depicted in Fig. 1, an additional zone, probably a lipoprotein, has been observed between the sample slot and the slow α2-globulin zone. This has been seen only in the sera of very young rats under the age of 2 weeks. Some of the characteristics of the various protein zones are described below.

**γ-Globulin**—This zone, adjacent to the sample slot and migrating toward the cathode, is diffuse. Often, one or more sharp narrow zones have been seen within the larger zone.

**Slow α2-Globulin**—In normal adult rats studied in this series, this zone was not detectable. It was seen only in pregnant, very young, and tumor-bearing rats (7, 8). A similar zone was
observed in two rat serum samples submitted from elsewhere; these animals were of unknown genetic background and had undergone a variety of experimental treatments.

**Slow α1-Globulin**—This intensely staining band which has been seen in every rat serum studied to date shows very little individual variation. Lipid material can be demonstrated in this zone. In the normal adult rat, it is the only zone that suggests specific lipid-binding. A more diffuse, slower migrating lipid-binding zone can be seen in very young rats. Slow α1-globulin is probably a lipoprotein. Biserte et al. (9) have described the presence of a lipoprotein of rat sera migrating in the area of α1-α2-globulin in agar gel electrophoresis.

It should be noted that, when serum samples were placed on filter paper before insertion in the starch gels, additional diffuse areas of stainable material appeared adjacent to both the slow α1- and slow α2-globulin zones, migrating a bit more slowly. This effect is depicted in the two-dimensional portion of Fig. 1 (zones b' and c'). These may represent proteins having slightly different properties than those remaining in the respective slow α1- and slow α2-globulin zones. They are not detectable when serum samples are inserted free from suspending medium. A similar effect has been noted in human sera (1).

**Post Transferrin**—This is an indistinct and variable zone migrating more slowly than transferrin II. It is named solely by location and slows no iron-binding property.

**Transferrins I and II**—These zones migrate as β-globulin on filter paper electrophoresis. The proteins of these zones specifically bind iron added to the serum before electrophoresis (0.6 mg of iron/100 ml, as ferrous ammonium sulphate) and the name transferrin has been applied in agreement with the nomenclature for human sera. In the human, this iron-binding protein is also known as siderophilin (10). No other zones show iron-binding properties. The relative intensities of these zones are variable, but both zones have always been present in adult sera. The presence of two β-globulins in rat sera has also been suggested by Biserte et al. (9).

**Fast α1-Globulin**—Although this zone is subject to variation in intensity, it is one of the most distinct zones seen in rat sera. Hemoglobin also migrates in this region, but there is no indication that this protein binds hemoglobin added to the serum before electrophoresis as do the haptoglobins of human sera (3).

**Post Albumins I—IV**—The post albumins are the most variable in intensity of any of the detected zones. They are not derived from the albumin fraction but rather from the α1- and α2-globulin fractions; the name is used only to designate location. Post albumins I and IV are normally the most distinct of this group of proteins. In some samples, post albumin IV appears to separate into two zones; under the conditions of this study, usually only one zone is detectable.

**Albumin**—This large, deeply staining zone is the fastest migrating zone on paper and is preceded by only one small zone in starch gels. A gradation in the intensity of staining suggests that there may be two protein zones migrating in close proximity to each other. Decreasing the staining time and, hence, the over all intensity of staining accentuates this gradation. The possibility of two albumin fractions has also been suggested by other workers (11, 12) using the Tiselius procedure.

**Prealbumin**—The rather indistinct zone preceding albumin in starch gels is subject to variation in intensity and is frequently difficult to demonstrate.

As already noted, more zones can be detected in two-dimensional gels than are apparent in normal vertical electrophoresis. These faint zones migrate at the same rate, in starch gels, as do stronger zones, and hence are masked in vertical electrophoresis. In a few sera, the post transferrin zone appears to be derived in part, at least, from a protein migrating as α1-globulin rather than α2-globulin on filter paper. “Fast α1-globulin” undoubtedly contains some protein migrating as β-globulin on filter paper. Similarly, “post albumin II” (α2) contains a small amount of an α1-globulin. It is possible that, under certain experimental conditions, the relative proportions in these mixed zones may undergo change.

**CONCLUSIONS**

Examination of rat serum and plasma by moving boundary, filter paper, and agar gel electrophoresis has suggested the presence of at least six and possibly seven demonstrable protein fractions. Early studies with Tiselius apparatus have given variable separation of the fractions. Thus, Moore et al. (13) and Moore (14), working at pH 7.4, detected only albumin, β-, and γ-globulin; at pH 8.5, one well defined α-globulin and a suggestion of a second α-globulin were described. Mackay (15), working at pH 8.0, and Deutsch and Goodloe (11), working at pH 8.6, were unable to demonstrate more than one α-globulin, although the patterns shown by Deutsch and Goodloe suggest the presence of an additional α-globulin. Li (16), working at pH 8.5, described a small peak between albumin and α-globulin which might constitute a second α-globulin. All of these workers, except Deutsch and Goodloe, described the presence of single zones for albumin, β-globulin, and γ-globulin. Deutsch and Goodloe (11) failed to resolve β-globulin; however, they did describe a separate component migrating ahead of albumin. Halliday and Kelwick (12), working at pH 8.0, described the presence of two albumin fractions and two α-globulin fractions. In a study of the effect of age, these workers noted that albumin fell to quite low relative concentrations in older rats, as did also the α-globulin, particularly α1-globulin.

Filter paper electrophoretic studies by Biserte et al. (9) revealed a single albumin, α1-, α2-, β-, and γ-globulin with veronal buffer; with Tris-ethylenediaminetetraacetate buffer their results suggested a third α-globulin. Electrophoresis of rat serum in agar gels (9) yielded evidence of a prealbumin, as well as one γ1-, two γ2-globulin fractions, and two β-globulin fractions.

In reported studies of rat serum proteins, various workers have described two albumin zones (12), three α-globulin zones (9), two β-globulin zones (9), and one γ-globulin zone. The methods used in these studies have included moving boundary, filter paper and agar gel electrophoresis; in addition, the buffer composition has varied. Not all of the above zones have been detected by a single procedure. In contrast, the studies with vertical starch gel electrophoresis suggest the presence of two albumin zones, ten α-globulin zones (5 designated α1 and 5 designated α2), two β-globulin zones (transferrins), and one or more γ-globulin zones. Most of these zones may be demonstrated in a single electrophoretic pattern. It is likely that, by varying the conditions of electrophoresis, further separation of some zones with combination of other zones could be obtained. In addition, two-dimensional filter paper-starch gel electrophoresis suggests the presence of even more zones.

In view of the complexity of this electrophoretic pattern, it is
apparent that a descriptive nomenclature for the zones is needed.
The pattern obtained with rat sera is quite different from that
obtained with human sera. Although it has been possible, in
filter paper and moving boundary electrophoresis, to use a
simple nomenclature applicable to all species (11), this does not
appear to be suitable for starch gel electrophoresis. The nomen-
clature devised by Smithies (3) and others for human serum
protein patterns is not applicable to the rat. It is, however,
helpful to retain a relationship to the existing and widely recog-
nized designation of albumin, α1-, α2-, β-, and γ-globulin for
purposes of comparison. For these reasons, a designation based
on the existing nomenclature and the relative mobilities of the
protein zones was devised (Fig. 1). As the properties of these
various protein zones are elucidated, a more accurate nomen-
clature (e.g., transferrin) can be substituted.

Smithies (3) has stressed the great influence of molecular
weight and configuration in the control of human serum protein
migration in starch gels. Proteins migrating through the col-
loidal structure of the starch gel are impeded in proportion to
molecular size in starch gels. Confirmation of this hypothesis
for rat serum proteins will have to wait until the various proteins
have been separated and their molecular sizes determined.

Many of these zones are subject to variation in intensity
under a variety of experimental conditions. Factors influencing
the relative intensities of the protein zones will be the subject
of future papers.

SUMMARY

A vertical starch gel electrophoresis method of Smithies has
been modified for the examination of rat sera. By this proce-
dure fourteen distinct protein zones can be identified. Two-
dimensional filter paper-starch gel electrophoresis gives evidence
of at least three more zones. A suggested designation of these
zones, referred to the accepted nomenclature of albumin, α1-, α2-, β-, and γ-globulin, is described.

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Addendum—During the publication of this paper, Espinosa (1)
published the results of examination of rat serum proteins by
starch gel electrophoresis. He described the presence of 17 zones
in two-dimensional electrophoresis, using Tris-EDTA buffer for
the filter paper electrophoresis. The zone designated “slow α-
globulin” was not observed by Espinosa in normal adult male
rats.

REFERENCE

Starch Gel Electrophoresis of Rat Serum Proteins: I. PROCEDURE AND DESIGNATION OF COMPONENTS
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