

rates developed without detectable radioactivity in assay of the saliva samples and during the first 10-minute urine sample in one subject, it might be speculated that this agent was initially attached to certain receptor sites to effect a change in functional activity. Subsequently, changes in the rates of excretion of this agent approximated the change in rates of fluid output in the non-hydrated subject. This time-intensity relationship between diuresis and furosemide-S<sup>35</sup> excretion was also demonstrated with the diuresis associated with mercaptopimerin-Hg<sup>203</sup> (12,13).

Since the renal clearance of furosemide-S<sup>35</sup> was greater than inulin, it appeared that the renal clearance of this diuretic must require tubular secretion as well as glomerular filtration. The mechanism whereby furosemide was excreted in excess of glomerular filtration presumably involved that utilized by hydrochlorothiazide (14).

*Summary.* Furosemide labeled with radioactive sulphur (S<sup>35</sup>) was given intravenously, intramuscularly or orally to adult subjects without any detectable cardiorenal disease. Peak plasma levels of radioactivity were found at 30 minutes and at 60 minutes after intramuscular and oral administration, respectively, of single doses. Approximately 80% of the dose given was recovered in the urine in 24 hours after intravenous or intramuscular injection of single doses. Following oral administration of single doses to 2 subjects, the 24-hour urinary recoveries were 26% and 54%, and fecal recovery was

2.1%. Comparison of rates of urinary excretion revealed that between 69% and 97% of the recovered activity was excreted in the first 4 hours after the drug was given, regardless of route of administration.

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## Differential Disc Electrophoresis of Serum Proteins.\*† (31392)

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The Ornstein-Davis polyacrylamide gel electrophoresis procedure permits the separation of extremely complex ionic mixtures. Human serum proteins, in particular, have been extensively examined by separating in the standard 7% gel(1) and more than 20

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protein components are demonstrated(1,2). Few reports exist on the electrophoretic examinations of animal sera, and in all cases, the standard gel was employed(3,4). However, the high degree of separation and resolution of human serum becomes misleading when employing the standard gel for the electrophoretic analyses of animal serum proteins or other complex mixtures.

Relatively little attention has been given to the use of different pore size gels for the analyses of whole serum: Slater(5) produced gels of constantly decreasing pore size in the direction of migration. The non-linear density gels varied in concentration from 4-22%. Fresh human serum separated into 30 or more protein bands, rat plasma into 25 protein bands and a 100,000  $\times$  g supernatant of rat liver homogenate into over 50 bands. Different pore size gels have been employed primarily for separation and isolation of specific protein molecules: Lytle(6) used disc gels of 15% to isolate human chorionic gonadotropin from urine; a 3.5% gel was employed by Narayan, Narayan and Kummeron(7) to separate human serum lipoproteins, and Price(8) used a 4.75% gel to separate human serum mucoids.

An adequate survey of different pore size gels is required to obtain maximum separation and resolution of a protein mixture. The ease with which the pore size of polyacrylamide gels can be varied is a striking attribute which offers a distinct advantage over other gel matrices.

The standard gel was found unsatisfactory for separation of bovine serum in this laboratory. This paper describes a simple procedure for better separation and resolution than that by the standard procedure. By separating in 2 different pore size gels, greater use of molecular sieving was obtained. We have tentatively named this procedure: "Differential disc electrophoresis." Convenient apparatus for staining and destaining these gels is also described.

*Materials and methods. Standard disc electrophoresis* was performed as described by Davis(1).

*Differential disc electrophoresis. Reagents.*

1. Acrylamide stock solution No. 1 for the

10%<sup>§</sup> working solution:

1. 40 g acrylamide  
0.4 g N, N' methylenebisacrylamide (BIS)

Dissolve and bring to 100 ml with distilled water

2. Acrylamide stock solution No. 11 for the 4.75%<sup>§</sup> working solution:

19 g acrylamide

0.4 g BIS

Dissolve and bring to 100 ml with distilled water

The stock and working solutions for the spacer-sample gel and electrolytic solutions are the same as described in the standard procedure(1).

Pyrex tubes (6 mm i.d.  $\times$  12 cm) are marked at 11 cm, 7.5 cm, 4 cm, and 2 cm starting from the base end. Prior to use, the tubes are cleaned in a detergent solution. The inner walls thoroughly cleaned with a cotton tipped applicator stick, rinsed thoroughly in tap water, then in distilled water and stored in a solution consisting of 1 part Kodak Photo Flo solution in 200 part distilled water. The tubes are drained and allowed to dry. Then they are inserted vertically into vaccine-bottle stoppers(1) and filled to the 7.5 cm mark with the 10% monomeric solution. The 4.75% monomeric solution is carefully added to the 4 cm mark, water layered and allowed to polymerize for 40 minutes. The water layer is shaken off and rinsed with spacer gel. Spacer gel is added to the 2 cm mark, water layered and photo-polymerized for 15 minutes. A 1 ml tuberculin syringe with a 25 gauge needle is convenient for water layering and for applying the spacer and sample gel solutions. Sample gel, prepared by mixing 8  $\mu$ l of fresh serum with 0.4 ml of spacer gel, is added and photo-polymerized for 30 minutes.

*Electrophoresis.* The upper buffer reservoir has a capacity for 8 tubes. Electrophoresis is started at 2 ma/tube until the marker dye reaches the separating gel (4 cm mark), approximately 20 minutes. The current is adjusted to 4 ma/tube until the

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<sup>§</sup> Total per cent (w/v) of monomer and comonomer.

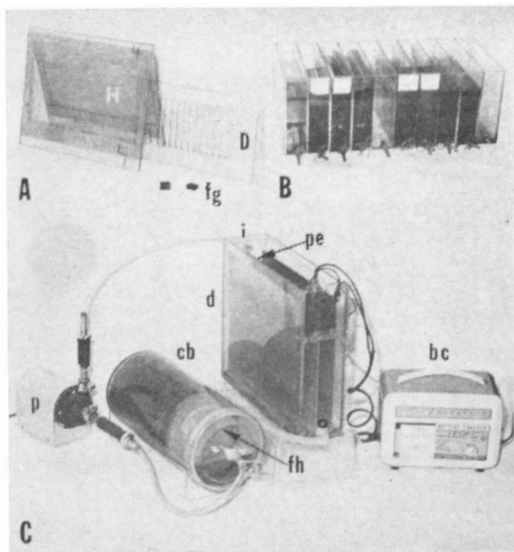


FIG. 1. Staining and destaining apparatus. A. Gel holder (H), disc gel adapter (D), and fiberglass clips (fc). Each frame of the gel holder (H) is made of  $\frac{1}{4}$  inch plexiglass and plastic screen mounted on one side. The internal dimensions are  $9\frac{11}{16} \times 6\frac{1}{4}$  in.; the outer dimensions are  $10\frac{3}{4} \times 9\frac{1}{4}$  in. The adapter (D) consists of 12 slots, each slot measures  $5\frac{1}{4} \times \frac{1}{4}$  in. spaced  $\frac{1}{4}$  inch apart. B. Staining tank with 10 compartments ( $27\frac{3}{4}$  in.  $\times$   $12\frac{1}{2}$  in.  $\times$   $10\frac{1}{2}$  in.). Each compartment holds 4 gel holders. C. Destaining assembly. The internal dimensions of the destainer (d) are  $11\frac{1}{2}$  in.  $\times$   $10\frac{1}{2}$  in.  $\times$   $2\frac{1}{2}$  in. The positive stainless steel electrode (pe) separates the two compartments and is positioned one inch from the 2 negative electrodes so that the cathodes are  $\frac{1}{2}$  inch and anode  $\frac{1}{4}$  inch from the gel holder. Current is provided by a 6-12 volt battery charger (bc). The destaining solution is continuously circulated through an activated charcoal bed (cb) by means of a circulation pump (p) inlet (i) and outlet (o). The filter ( $13 \times 5\frac{1}{2} \times \frac{1}{2}$  in.) has an 8 inch charcoal bed (cb) with a capacity for 2 lb granular charcoal. The charcoal is held back by two  $\frac{1}{2}$  inch thick glass wool filter pads which are held in place by a filter holder (fh) mounted on the back of the screw-on cover of the plexiglass cylinder. Equipment is currently available from Plastics Mfg. Inc., Lansing, Mich.

albumin reaches the 11 cm mark, approximately 1 1/2 hours.

Following electrophoresis, the gel columns are removed from the glass tubes after placing in ice water for 5 minutes and rimming with a finely tapered steel needle.

*Staining and destaining.* The gel columns are stained and destained with a modification of apparatus designed by the senior author for starch and acrylamide gel slabs (Fig. 1-A).

The disc gel adapter (D), which consists of 12 slots, is placed into the bottom half of the gel holder (H) and the gels inserted into the slots. The top half of the gel holder is then connected to the bottom half by 2 fiberglass clips (Fig. 1-A).

The gel holder is placed in the compartment of the staining tank (Fig. 1-B) containing 0.5% Amido Black 10 B in 5% acetic acid. The gels are stained for 30 minutes at room temperature.

The gel holder is removed from the stain, washed with running tap water to remove excess stain and placed into the destainer (Fig. 1-C), that was especially designed and coupled conveniently with the staining tank. It has two compartments so that 2 gel slabs or 24 disc gels can be destained at one time. Sixteen to 24 gels are destained in 30-45 minutes at 4 amps.

*Scanning.* Densitometric analyses of the disc gels was accomplished with the Chromoscan<sup>†</sup>, a double-beam recording and integrating densitometer (9). The gels were scanned by transmitted light.

*Results.* Fig. 2, 3, 4, 5 and 6 demonstrate the general type of results obtainable with this procedure.

Fig. 2 and 3 illustrate the striking increase in separation and resolution of human and bovine serum proteins. Human serum separated into 44-50 components compared to 22-28 by the standard procedure. With the standard system, bovine serum separated into 15-18 bands with poor resolution while separating into over 30 bands by the differential procedure. Because of the better resolution, the corresponding densitometric tracings (Fig. 4) markedly improved, especially the post albumin region of bovine serum.

A 4.75% gel in a standard tube (6.5 cm) afforded better separation than the standard procedure (7% gel), especially in human serum, which increased by 5-8 more bands. The number of bands, however, did not increase if 12 cm tubes were filled only with 7%, 4.75%, or 10% monomeric solution and electrophoresed under the same conditions

<sup>†</sup> Chromoscan (National Instrument Laboratories, Rockville, Md.)

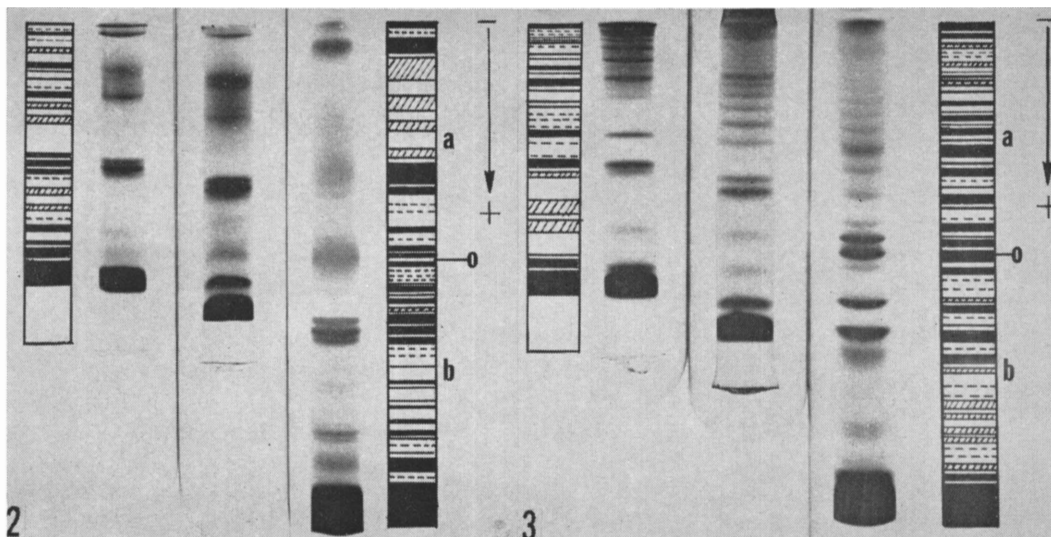


FIG. 2. Comparison of separation of bovine serum in standard 7% (left), 4.75% gel (middle), and differential gel (right). Cross hatched areas and dotted lines indicate lightly stained and fine bands, respectively. O = origin of 10% separation gel, a = 4.75 gel b = 10% gel.

FIG. 3. Comparison of separation of human serum in standard 7% gel (left), 4.75% gel (middle), and differential gel (right). Cross hatched areas and dotted lines indicate lightly stained and fine bands, respectively. O = origin of 10% separation gel, A = 4.75% gel B = 10% gel.

described for the differential system. Because of diffusion, the bands were extremely fuzzy.

Fig. 5 illustrates the variation in protein bands from animal to animal and demonstrates the reproducibility obtained from run to run as depicted by the distance the albumin band migrated.

The superiority of this system was clearly demonstrated with sera collected before inoculation (initial) and prior to autopsy (post inoculation) from a cow experimentally

infected with *Mycobacterium bovis* (Fig. 6). By the standard procedure, the bands were poorly resolved and only a decrease in concentration of the individual bands could be demonstrated in the post inoculation serum. However, the differential system revealed a number of bands not detected in the 7% gel, particularly in the initial serum. Moreover, both the separation and resolution were far better than in the standard gel.

Sera from a number of other species (sheep, rabbit, dog, horse, guinea pig, pig, mouse, chicken and duck) were separated by the differential system and the standard procedure. The increased separation of sheep serum was similar to that of cow serum, increasing from 19 to 31 bands while 5 to 8 more bands were separated in rabbit, horse, guinea pig and dog sera. This method did not improve the separation of mouse, dog, chicken or pig serum, although the resolutions were as good, or better, than that found in the standard gel. With all sera tested, a 4.75% gel by the standard procedure gave better separation, better resolution and more reproducible results than the 7% gel.

*Discussion.* The separation of human se-

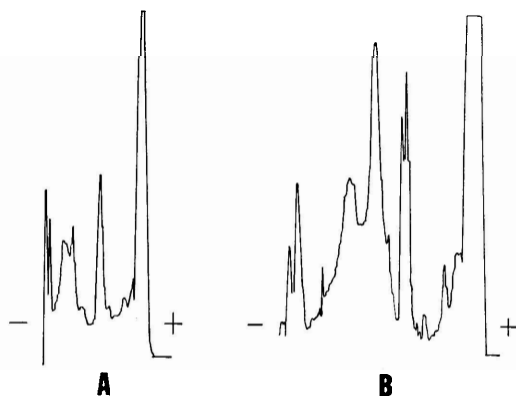


FIG. 4. Densitometric tracing of the bovine serum separated in 7% (A) and differential (B) gels illustrated in Fig. 2.

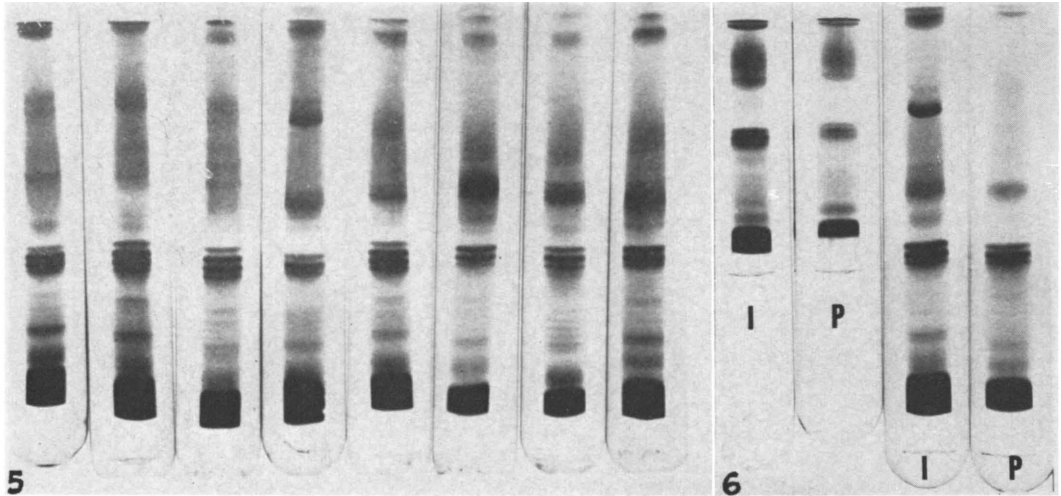


FIG. 5. Eight different bovine sera, each taken from an 8-column run with the differential system, illustrating variation in protein fractions in individual sera, and reproducibility of the distance migrated by the albumin band in a single run.

FIG. 6. Comparison of separation by the standard (left) and differential (right) systems of initial serum (I) and post inoculation serum (P) from a cow experimentally infected with *Mycobacterium bovis*.

rum by the standard procedure results in a large number of fine bands closely spaced, that migrate only a short distance into the separating gel (gamma globulin zone). When cow serum was electrophoresed in 7% gel, a wide, densely stained region, the "haze zone", appears a short distance from the origin. Leaving some of the protein at the point of origin is a common difficulty and crowds the slower moving components together(2).

The differential disc electrophoretic procedure allows the complex protein mixture (serum) to migrate first in a large pore gel (4.75%) which separates the large molecules from the smaller or faster moving components. These smaller molecules, apparently "trapped" by the larger components in the 7% gel subsequently separate in the small pore size gel (10%). Similarly, the large molecular weight components which were trapped in stiffer gels because of almost identical molecular dimensions but differed slightly in charge separate further in the large pore gel.

The length of the upper separating gel (4.75%) was a critical feature. If the distance of migration was too short, protein molecules would pile up at the origin of the 10% gel, which masked other bands; and the post

albumin bands were poorly resolved. A length of 3.5 cm was satisfactory for human and bovine serum proteins.

Because large quantities of sample could be applied, bands detected only by the differential procedure may not have been "new" bands, but because of the increase in concentration, they now could be detected. However, no new bands could be detected when the same sample size was applied to 12 cm tubes containing only 4.75 or 10% separating gel.

Possibly, too, some of the new bands might have been "created" due to splitting or denaturation of certain proteins. Hjerten, Jerstedt, and Tiselius(10) hypothesized that photo-polymerization of certain ionic mixtures could result in denaturation of the sample. However, when we compared photo-polymerized samples with samples that were added directly to the column(1), identical patterns developed. Therefore, photo-polymerization did not appear to alter the protein molecules in the sera tested.

Although, the separation of bovine serum was markedly improved, human serum gave the most striking results: In some cases, 100% more proteins could be detected. This increased resolving power becomes extremely

important when analyzing sera from diseased individuals. The reflection of disease in a tuberculous cow was best demonstrated by the new procedure while the standard procedure revealed little except a change in the concentration of bands.

The differential system yielded greater separation of serum proteins from a number of different animals. However, apparently this combination of pore size would not be suitable for all protein mixtures, since the separation of mouse, dog, chicken and pig serum proteins did not improve. Therefore, it is unlikely that any one combination of separating gels will adequately separate all the fractions of a complex mixture.

*Summary.* A polyacrylamide electrophoretic procedure, in which a complex ionic mixture migrates, through both a large pore and a small pore size gel on a single column is described. The procedure is simple, flexible and results in better separation and resolution of serum proteins. Human serum separated into 44-50 components; and bovine serum

into over 30 bands. An economical apparatus for the rapid staining and destaining of disc gels is described which eliminates handling of the gels during the staining and destaining processes.

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### Amino Acids and Ammonia of Fetal Calf Serum During Storage.\* (31393)

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The addition of fetal calf serum to tissue culture media to promote the growth of cells has received general acceptance since its introduction by Fisher(1). In spite of the widespread use of fetal calf serum in tissue culture, there is a lack of information on stability and changes that fetal calf serum may undergo during storage. Proteolysis, with liberation of amino acids, occurs in pooled human serum(2). This type of enzymatic reaction may also occur in fetal calf serum with loss of the glycoprotein, fetuin, which is believed to be responsible for much of the activity of fetal calf serum(1).

These considerations suggested a study of the stability of the growth-promoting properties of fetal calf serum and an analysis of the changes in amino acids during storage as an indication of proteolytic activity.

*Methods and materials.* Unfiltered, fetal calf serum was obtained from Reheis Chemical Co., Division of Armour Pharmaceutical.† The serum was divided into several portions, one of which was stored at  $-30^{\circ}$  to provide a control for the storage tests. The other portions were stored at  $5^{\circ}$  and  $37^{\circ}$ . Sterility tests were conducted on all of the serum samples during and after incubation at  $5^{\circ}$  and  $37^{\circ}$  by adding a 0.1 ml sample of serum to

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