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HORMONE RESEARCH

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COMMITTEE ON ARRANGEMENTS

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PREFACE

Your editor has been asked from time to time to advance reasons for the consistent success of the Laurentian Hormone Conference. The data supporting the notion that these meetings are popular are incontrovertible. Year after year we fill to capacity the hotel at which the meetings are held, and year after year the requests for attendance exceed this capacity. A second, and more fundamental, attestation to the wide regard for these Conferences is the consistent and regularly increasing demand for the proceedings of the Conference published in "Recent Progress in Hormone Research." As a working hypothesis we therefore deduce that the full and timely presentation of advances in hormone research, along with the opportunity for full discussion, is the basic reason for the success of the Conference. Secondarily, we believe that the holding of the meetings in beautiful, natural surroundings with opportunity for recreation and informal congregation is opportunely conducive to the free flow of scientific information.

The Committee on Arrangements is responsible for deciding on the site of the meeting, arranging the scientific program, and issuing invitations to attend the meeting. Any qualified investigator in the field of hormones may, and indeed is, invited to request of the Committee an invitation to attend. Since the requests for such invitations tend to exceed the capacity of the hotel at which we meet, the Committee on Arrangements has enlisted the services of an anonymous Selection Committee to decide on invitation priority. To the nameless members of this Selection Committee, the Committee on Arrangements wishes to take this opportunity to express gratitude for their conscientious and wise assistance.

No membership fees are charged and the costs of the meeting are regularly subsidized by contributions from a group of companies especially interested in hormone research. We hereby gratefully acknowledge the sponsors of the 1962 meeting: Abbott Laboratories; Armour Pharmaceutical Co.; Ayerst Laboratories; Baxter Laboratories, Inc., Ciba Company Limited; Ciba Pharmaceutical Products, Inc.; Cutter Laboratories; Charles E. Frosst & Co.; General Mills, Inc.; Hoffmann-La Roche, Inc.; The Julian Laboratories; Lederle Laboratories Division, American Cyanamid Co.; Lilly Research Laboratories; Mattox and Moore, Inc.; Mead Johnson & Co.; Merck & Company, Inc., Merck, Sharp & Dohme; Wm. S. Merrell Co.; Nordic Biochemicals; Organics, Inc.; Organon, Inc.; Ortho Research Foundation; Parke, Davis & Company; Chas. Pfizer & Co.; Riker Laboratories; J. B. Roerig & Company; Schering, AG; Schering Corporation;

Searle Chemicals, Inc.; G. D. Searle & Co.; Smith Kline & French Laboratories; Smith, Miller & Patch, Inc.; E. R. Squibb & Sons of Canada; The Squibb Institute; Sterling-Winthrop Research Institute; Strasenburgh Laboratories; Syntex, S.A.; The Upjohn Company; Warner-Chilcott Laboratories; Warner-Lambert Research Institute; Wyeth Institute for Medical Research. Their generous support made it possible to have as our special guests from abroad Dr. K. A. Ferguson of the Commonwealth Scientific and Industrial Research Organization of Australia, and Dr. D. A. Denton of the University of Melbourne, Australia.

The scientific sessions of the meeting are held during the morning and at night, with the afternoons free for informal activities. At each session a member of the meeting takes the chair, and in 1962 we were fortunate in having as chairmen Drs. J. F. Tait, V. A. Drill, P. L. Munson, J. A. Leutscher, Jr., A. White, E. B. Astwood, R. W. Bates, and R. O. Greep.

Other members of the meeting have volunteered to help in certain recreational activities and we are indebted to Dr. C. H. Sullivan for a successful golf tournament, to Drs. G. A. Grant and J. C. Beck for various local arrangements and guidance. Arrangements at the hotel were capably handled by Mrs. Mary R. J. Robinson whose unfailing courtesy and kindness is most appreciated.

Acting as secretaries to the Conference and as special interpreters of the often involved and occasionally agitated discussions are Mrs. Jacqueline Foss and Mrs. Mina Rano. We are especially indebted to Mrs. L. P. Romanoff for the preparation of the subject index of this volume. We should like to express our appreciation to our publisher, Academic Press Inc., for its consistent maintenance of high standards of printing and publication.

The meat of the Conference is, of course, embodied in the papers contained in this volume. In ranging from methodology to discussions of glandular normalities and abnormalities, to cellular chemistry, and back to organismal adaptations, these papers may be taken to represent the wide scope of endocrinology. The marvelous regulatory systems developed with the hormones as effectors continue to amaze and delight the curious investigative mind. It is the purpose of our meetings and of this volume to inform and stimulate that investigative mind. Long may the purpose and the minds endure!

GREGORY PINCUS

Shrewsbury, Massachusetts
May 1963

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I. PHYSICAL METHODS IN HORMONE RESEARCH

The Characterization of Pituitary Hormones by Starch Gel Electrophoresis

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I. Introduction

We should distinguish between the characterization of proteins in a mixture and the characterization of purified proteins even though the same criterion may be used for both purposes. Complete characterization of the isolated protein—including a description of its amino acid sequence, the secondary and tertiary structure, and its physical, chemical, and biological properties—firmly establishes its uniqueness. The criteria that may be applied to the characterization of proteins in a mixture are much more limited; the characterization is thus less specific, although, in the examination of tissues, the pattern of proteins displayed by some separation procedures may confer a specificity absent in the characterization of an isolated protein.

The analytical ultracentrifuge and moving boundary electrophoresis apparatus have been used to characterize purified preparations of pituitary hormones and to provide criteria of purity of these preparations. Nevertheless these characterizations have not proved specific enough to follow the hormones through the fractionation procedure, and the isolation of protein hormones has depended largely on biological assay to guide it.

Zone electrophoresis especially on paper had an explosive growth after 1948, but its advantage over the Tiselius apparatus lay in the simplicity of the equipment and the complete separation of components rather than in any marked improvement in resolution. Relatively few reports appeared on its use in examining pituitary extracts. In our hands, paper electrophoresis was of limited usefulness for this purpose (71).

The greatly improved resolution afforded by starch gel as a medium for zone electrophoresis was demonstrated by Smithies (63), who with it provided the first clear demonstration of genetic polymorphism in serum proteins. It seemed that here was a procedure which might not only be used to guide the preparation of pituitary hormones, but also to detect possible changes in hormones during isolation. Species differences in protein hormones and intraspecific genetic polymorphism might also be detected. It

is perhaps customary to think of intraspecific genetic variation in endocrine activity in terms of the quantity of hormone secreted or reaching the target tissue, but the discovery of genetic polymorphism in an increasing number of plasma proteins suggests the possibility of an additional mechanism.

The large number of proteins present within a cell should warn us that a single electrophoretic separation of crude pituitary extracts is unlikely to be completely specific in the characterization of particular hormones. However, the specificity may be considerably increased by observing the differential effect of altered electrophoretic conditions on mobility. The effect of splitting off sialic acid from glycoproteins by prior incubation with sialidase (6, 54) and the striking action of 8 *M* urea and β-mercaptoethanol on the starch gel electrophoretic patterns of haptoglobin (66), α-2-macroglobulin (55), γ-globulin (57), and ceruloplasmin (56) are examples of further means of increasing specificity, though such procedures are more concerned with the investigation of the structure of the purified proteins. Further specificity may be attained by the use of specific staining reactions and biological or immunological tests on components separated by electrophoresis.

It is natural to seek to express the electrophoretic mobility of a particular protein in absolute terms after employing standard electrophoretic conditions. However, often conditions favorable to the best separation both in chromatography and zone electrophoresis are difficult to standardize exactly. The experience of biological assay with tests of potency and identity made with respect to standard preparations rather than in units of biological response suggests an alternative solution to this problem. It is to make an electrophoretic comparison of a protein mixture with appropriate standard protein preparations run alongside it—a comparison not possible in the boundary apparatus.

II. Technique of Starch Gel Electrophoresis

A. Preparation of the Gel

Smithies (63) described a Perspex apparatus for horizontal starch gel electrophoresis employing a gel thickness of 0.65 cm. Voltages of 6 volts/cm. for 6 hours caused albumin to move 5 cm. at pH 8.5 and room temperature. Subsequently, Smithies (64) described a vertical arrangement of the apparatus to eliminate electroconvection of proteins in the sample slot, allowing the proteins to be applied in free solution. Voltages of 5 volts/cm. for 16-20 hours gave improved separation.

In order to produce greater separation in a daytime run we have used average voltages of 20-30 volts/cm. for 5 hours and provided for cooling of the gel. Under such conditions a separation of up to 60 cm. has been produced

between the proteins in a mixture. The apparatus has been progressively modified, and the following description applies to that in current use.

Gels are cast on a sheet of glass 65 × 42 × 0.24 cm., the edges of the mold being formed by strips of Perspex 0.65 × 0.24 cm. in section stuck to the glass, forming a gel 0.24 cm. thick. Gels down to 0.08 cm. in thickness have been satisfactorily made but are more fragile in subsequent handling. The lateral edges to the mold are only lightly glued to the glass so that they may be subsequently removed.

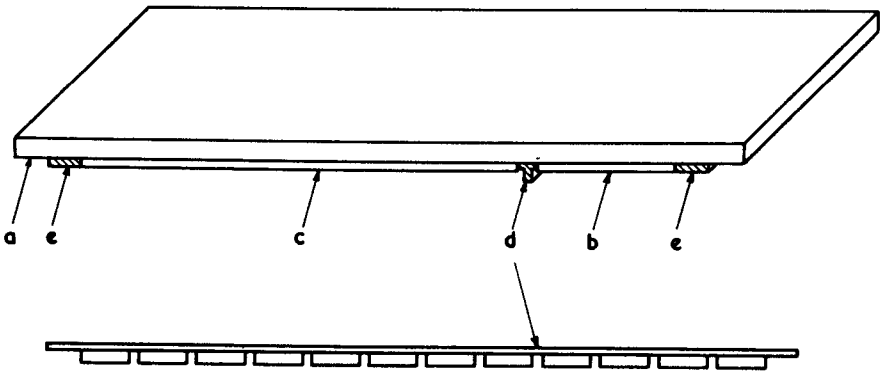

FIG. 1. Cover for molding starch gel: (*a*) plate glass sheet 70 × 42 × 0.64 cm.; (*b*) glass sheet 42 × 14.5 × 0.24 cm.; (*c*) glass sheet 42 × 45.5 × 0.24 cm.; (*d*) slot former, Perspex strip 42 × 0.64 × 0.16 cm. with 12 Perspex projections each 3.0 × 0.24 × 0.08 cm. separated from one another by 0.3 cm.; (*e*) Perspex strips 42 × 1.3 × 0.24 cm. The Perspex strips and slot former are joined along their length to the adjacent glass sheets by cellulose tape. This assembly is taped at the edges to the covering sheet of plate glass.

Partially hydrolyzed potato starch (Connaught Medical Research Laboratories, Toronto) is used. The suspension of starch in buffer (11-13 gm. per 100 ml. buffer) is mechanically stirred over a boiling water bath, vigorously until after the gelling point has been reached at about 65°C. and then more slowly to avoid incorporation of too much air in the gel. After stirring for about 45 minutes, the temperature reaching 90-95°C., the hot starch is degassed by connecting the flask to a water pump, shaking vigorously to prevent excessive loss due to frothing. The solution is now poured through coarse bolting silk onto the glass mold preheated with infrared lamps. The starch is immediately covered with two sections of preheated glass separated by the sample slot mold and kept flat by a sheet of plate glass with weights placed on it. The arrangement of the cover is illustrated in Fig. 1.

The position of the sample slots can be varied by altering the relative sizes of the two adjacent glass sheets (*b*) and (*c*) and sample slots of different thickness and width can also be made.

Within a few minutes of the starch being covered, the Perspex strips forming the long margins of the gel tray and only lightly stuck to it are prised away, thus allowing the cover glasses to move down as the gel contracts on cooling and during electrophoresis. After the gel has cooled, the plate glass (*a*), slot former (*d*) and Perspex strips (*e*) are removed, the latter exposing strips of gel for making contact with the electrode wicks. The exposed lateral edges of the gel are bound with cellulose tape which overlaps the lower and upper glass sheets.

B. Insertion of Protein Sample

In early experiments, protein samples were inserted into the gel on filter paper. This leads to streaking of some proteins, to adsorption of protein by the filter paper, and to separation of the gel at the sample slit when high voltage gradients are used, unless the gel is compressed after insertion of the sample.

Smithies (64) showed that protein solutions devoid of any stabilizing medium could be placed in slots if the gel was mounted vertically to prevent electrodecantation of proteins in the sample. We also now use a vertical arrangement but add to the protein solution an equal volume of starch gel homogenate containing equal parts of starch gel and the buffer with which the gel was made. The protein sample and homogenate are conveniently mixed by shaking in an agglutination tube prior to a measured portion being transferred to the sample slots with a micropipette. Slots of 0.08, 0.16, and 0.32 cm. thickness have been used containing samples of 0.06, 0.12, and 0.24 ml., respectively. The narrowest slots have been primarily used with freeze-dried protein samples, whereas the wider slots have been used for dilute protein solutions when required to avoid the need for concentration.

After filling, the sample slots and the adjacent gel between the cover glasses are covered with melted paraffin wax (45°C. m.p.).

C. Cooling the Gel

The glass tray containing the gel is mounted vertically against a copper plate (insulated with polyester film) through which water or water-alcohol solution is circulated by means of a pump and thermostat (Universal U8, Mechanik, Göttingen).

Studies of the effect of temperature on the relative mobility of different

proteins have been made by altering the temperature of the fluid circulated through the cooling plate. With increase in temperature there is a considerable increase in mobility, but the relative mobility of different proteins is also affected.

For most experiments the temperature of the cooling fluid has been set at 20°C., or tap water has been used instead of the circulating pump. With the discontinuous buffer system described below, the gel temperature is not uniform since this system generates a nonlinear voltage gradient.

D. Buffer System

Smithies (65) used sodium borate buffer and varied the concentration to give equally sharp front and back edges to the albumin zone in human serum. Concentrations of the order of 0.008 *M* sodium hydroxide and 0.02 *M* boric acid were used, giving a gel pH of 8.5.

Poulik (53) described a discontinuous system of buffers in which the gels are made with 0.076 *M* tris(hydroxymethyl)aminomethane (Tris), 0.005 *M* citric acid buffer solution, but the electrode vessels contain 0.3 *M* boric acid, 0.06 *M* sodium hydroxide solution. With this system Poulik obtained greatly improved separation of the proteins of diphtheria toxin preparations and showed that a zone of high voltage gradient migrated through the gel from the cathode. The average voltage gradient across the gel was 6 volts/cm.

We found that Poulik's system also gave improved separation of the proteins in pituitary extracts but tended to group many of the proteins together in a fast-moving zone immediately behind the front of the high voltage gradient zone. Experiments were carried out to moderate the effect of discontinuous system by using mixtures of Tris citrate and borate buffers in the gel with borate buffer in the electrode vessels. At the same time, higher voltages were made possible by cooling the gels and by using lithium hydroxide instead of sodium hydroxide.

It was found that an optimum distribution of components was obtained with a gel buffer comprising a mixture of 90% 0.0033 *M* citric acid, 0.016 *M* Tris and 10% 0.02 *M* lithium hydroxide, 0.076 *M* boric acid giving a pH at 20°C. of 8.1. The buffer in the electrode vessels was 0.1 *M* lithium hydroxide, 0.38 *M* boric acid, pH 8.5.

However, in order to alter the relative mobility of different proteins which tend to migrate together with this buffer system, different pH buffers have been employed and prepared simply by altering the concentration of Tris in the above mixture. These buffers will be referred to subsequently by the concentration of Tris used.

The relative mobility of different proteins has also been changed by using Tris citrate or lithium borate buffers alone in the gel to form completely discontinuous or continuous buffer systems, respectively. Other degrees of discontinuity than the system above have also been used.

The voltage gradient along the gel after three periods of electrophoresis with partially and completely discontinuous buffer systems is shown in Fig. 2. Measurements with a continuous system showed a linear voltage gradient along the gel which remains constant during the whole period of electrophoresis. The completely discontinuous system gives a nonlinear voltage gradient which changes during electrophoresis as an area of high voltage gradient spreads through the gel from cathode to anode, becoming progressively smaller as it extends. The partially discontinuous system gives rise to a similar, but less pronounced, moving nonlinear voltage gradient. The current flow with the continuous system remains constant throughout, but with the discontinuous and partially discontinuous system falls progressively.

These results indicate a decrease in the conductivity of the gel starting at the cathode and progressively extending along the gel with the discontinuous buffer systems. The extending high voltage gradient coincides with a lessened opacity of the gel which ends sharply in a front, which is the line reached by the borate ions moving from the cathode.

Since current flow is constant along the gel, the high voltage gradient also corresponds to a zone of higher temperature. The electrophoretic patterns obtained with continuous, completely and partially discontinuous buffer systems are illustrated in Fig. 9.

The resolution with the continuous buffer system is poorer for most of the proteins and the mobility is less despite the same average voltage gradient. However the voltage conditions and buffer composition were chosen to be comparable with the other buffer systems and are not necessarily optimum for a continuous system.

In the discontinuous systems, as may be seen in Fig. 2 the proteins are exposed to a higher voltage gradient than the mean for the greater part of the electrophoretic period, a condition that accounts for the greater mobility with these systems. The more pronounced peak in the voltage gradient of the completely discontinuous system increases the mobility of the most anionic proteins relative to the less charged proteins and concentrates them in a region behind the front. The partially discontinuous buffer system retains the sharpening effect produced as the anionic proteins are overtaken by the sudden increase in voltage gradient and provides a relatively constant gradient behind the front so that all the anionic pro-

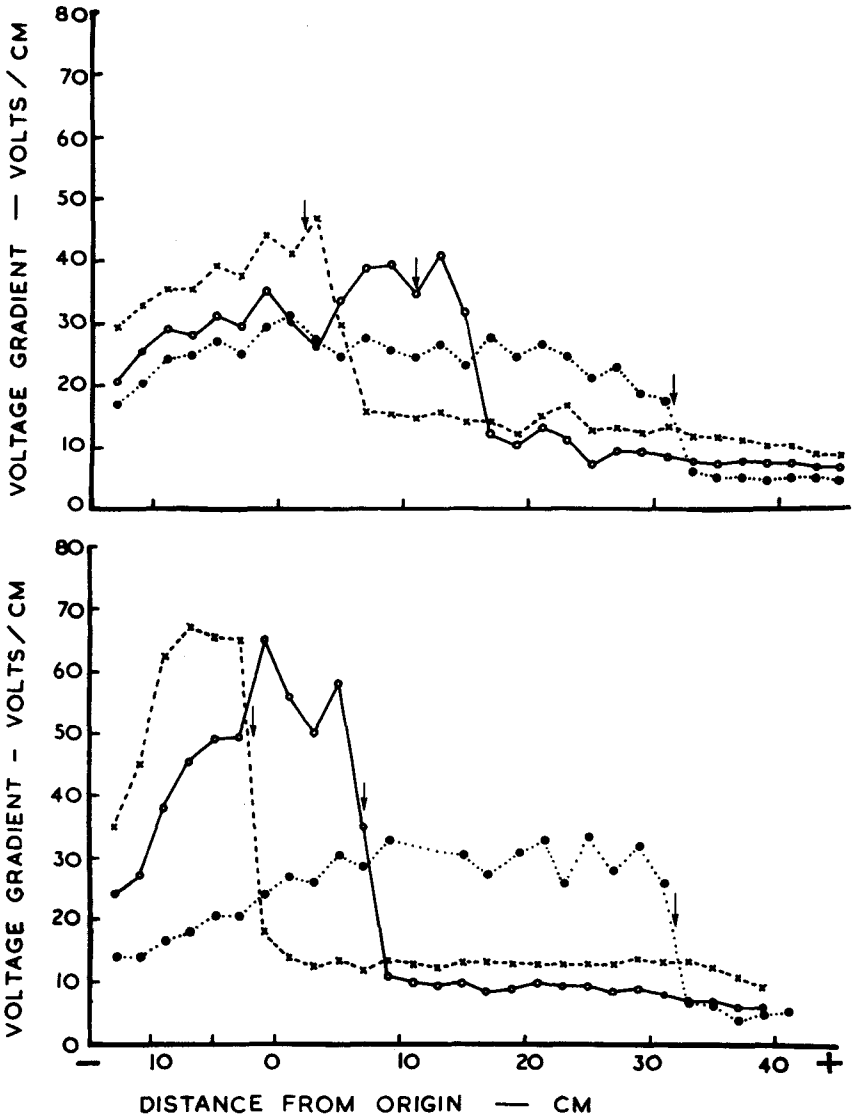

FIG. 2. Nonlinear voltage gradients along starch gel produced by completely and partially discontinuous buffer systems.

Upper diagram: Buffer system 90% 0.0033 *M* citric acid, 0.025 *M* Tris, 10% 0.02 *M* lithium hydroxide, 0.076 *M* boric acid in gel; 0.1 *M* lithium hydroxide, 0.38 *M* boric acid in electrode vessels. Voltage gradient, x-----x after 71 minutes; o——o after 108 minutes; ●·····● after 196 minutes. Borate front indicated by arrows.

Lower diagram: Buffer system 0.0033 *M* citric acid, 0.025 *M* Tris in gel; 0.1 *M* lithium hydroxide, 0.38 *M* boric acid in electrode vessels. Voltage gradient, x-----x after 48 minutes; o——o after 90 minutes; ●·····● after 287 minutes.

teins are well resolved and better distributed relative to their net charge.

A declining voltage gradient behind the front as seen with the completely discontinuous system would be expected to improve the resolution of the cationic proteins. However the resolution is not materially better than obtained with the other two systems.

In Fig. 9 it may be noted that the relative mobilities of albumin and the principal components of human growth hormone and sheep prolactin are reversed in the continuous and discontinuous systems. The nonlinear voltage gradient does not, therefore, only alter the spacing between the proteins of different mobility but also, in some cases, alters the order of mobilities. Intermediate effects were produced by different degrees of continuity of the buffer system.

The temperature and pH gradients produced by the discontinuous buffer systems may, at least in part, account for such reversals of mobility as well as supplementing the effect of the nonlinear voltage gradient on resolution.

Different relative mobilities may also be produced by changing the ionic strength of the buffer system, but for most purposes the ionic strength of 0.02 used appeared to be optimum.

E. Staining

Proteins and peptides in the gel have been stained by inverting the gel in a solution of 0.0125% nigrosin (water soluble, Gurr) and 0.0125% naphthalene black 12B (Imperial Chemical Industries) in methanol 50, acetic acid 10, water 40. After staining overnight, the gels are rinsed in several changes of solvent, preferably over 2 days. The dye solution is best used freshly prepared and not reused, to prevent the precipitation of nigrosin in the gel and darkening of the background.

F. Location of Components for Biological Assay

In early experiments with shorter length gels the gel was subdivided longitudinally between samples and transferred to a cutting block, where the edges were serrated by a template prior to slicing off the top section for staining. The components on the unstained lower section were located by the corresponding serrations.

The technique in current use is to cut the gel with a serrated template about one-third in from the edge of the sample and to stain this strip in order to locate the components on the unstained two-thirds.

For biological assays the segments of starch gel containing the components have been homogenized with saline, the standard solutions being

prepared with the same proportion of starch gel. In all assays carried out, the presence of starch gel has potentiated the hormone response and cut down the frequency of injections for some assays.

G. Measurement of the Concentration of Components

The affinity of different proteins for dyes varies. In consequence, the measurement of dye concentration by densitometric means, while valid for measuring different amounts of a particular protein, does not measure the relative amounts of different proteins. We have therefore made comparisons of concentration only between the same component in different preparations on the same gel. The techniques of biological assay are appropriate for such comparisons, the concentration of a component being expressed relative to the concentration in a standard preparation.

The stained gels have been photographed on 35 mm. film (Adox Dokupan) and the negatives scanned in a microdensitometer (Joyce Loebl) using a slit width of 20μ. The integral of the optical density curve for a component is proportional to the amount of the preparation applied to the gel (following the Beer-Lambert law) only for small concentrations. In such cases the technique of slope ratio assays may be used for analysis. At higher intensities of components, the optical density integral is linearly related to the log of the amount of preparation applied, and the usual method of log-dose response assays may be used for analysis.

It is clear from the demonstration of multiple active forms of most pituitary hormones discussed below, that the concentration of any component is not necessarily a measure of hormone activity. Such an objective cannot be achieved until all the active forms have been specified and measured.

III. The Detection of Heterogeneity in Protein Preparations by Starch Gel Electrophoresis

Colvin *et al.* (12), after a survey of investigations into the heterogeneity of purified proteins using a number of criteria, concluded that all present samples of any protein were heterogeneous. They distinguished heterogeneity arising from (a) distinctly different protein contaminants, (b) derivatives produced by the preparative procedure, and (c) normal variations between molecules of the protein within the cell. These authors concluded that the greater part of the observed heterogeneity in some proteins was in this third category. While more recent work on genetic polymorphism of plasma proteins demonstrates different discrete forms of the same protein, many would not accept their conclusion that a native protein does not