"Starch-Gel Electrophoretic Study of Acid Casein, Rennin and Alkaline Milk Phosphomonoesterase. The use of Indigenous Starches (Potato and Cereal) in Electrophoresis as an Alternative to Imported Hydrolysed Potato Starch and the Action of Rennin on Casein".

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K.R. Aiyar

Massey University College of Manawatu

Palmerston North

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SUMMARY

1. The method of Horizontal Starch-Gel Electrophoresis for the study of casein and purified enzymes was satisfactorily developed. Its application to the study of casein and casein hydrolysates has been discussed, with particular reference to polymer separation and nomenclature.

2. The possibility of purified rennin existing in two polymeric forms has been shown.

3. The use of indigenous starches (potato and cereal) did not give promising results in electrophoresis. Possible reasons for the failure to obtain a suitable hydrolysed potato starch, comparable in properties to the imported hydrolysed starch, have been discussed.

4. Purified rennin appeared to have phosphatase activity on casein, the liberation of the orthophosphate being accompanied by the exposure on the casein molecule of arginine guanido groups, giving an apparent ultimate ratio of moles phosphorus released to moles arginine exposed of 1:2. Rennin did not possess phosphatase activity on synthetic substrates containing an -O-P- bond.

5. The possibility of phosphorus linking two arginine residues through their guanido groups on the casein molecule (i.e. an -N-P-N- bond) has been discussed.

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PART I

CHAPTER I

REVIEW OF LITERATURE ON STARCH-GEL ELECTROPHORESIS* AND SOME OF THE RELATED ELECTROPHORETIC METHODS.

Section I:

Principles of Starch-Gel Electrophoresis

In any electrophoretic method the mobility of an ion is directly proportional to the net charge carried by that ion. In a buffered system the charge on a protein molecule is dependent on the pH of the system and the protein type, as well as the type of buffer used (1). Buffer ions may modify the charge on the protein molecule through combination with it and, hence, the latter need not necessarily have the same net charge in two different buffer systems of the same pH (1,2,19).

Smithies (3) was the first to develop the method of Horizontal Starch-Gel Electrophoresis in his study of bovine blood proteins. The raw material consisted of a high grade potato starch which had been partially hydrolysed by a 1:100 concentrated hydrochloric acid acetone mixture at a temperature of 38.5°C for a suitable pre-determined time (3,4). For each new batch of potato starch pilot scale experiments must be run to determine the optimum hydrolysis time. Partial hydrolysis of the starch is done evidently to reduce the viscosity of the starch solution (37,40) to an optimum level.

* Unless otherwise stated, Starch-Gel Electrophoresis means Hydrolysed Starch-Gel Electrophoresis as per the method of Smithes (3,5,) as distinct from Unhydrolysed Starch-Gel Electrophoresis in the method of Bernfeld and Nisselbaum (26).

Poulik and Smithies [4] state that"the hydrolysis time is based on the mechanical properties of gel, obtained with a hydrolysed starch concentration of 14 g per 100 mls. of a buffer solution". The buffer used commonly was 0.02 to 0.03 M sodium borate [3].

A suitable quantity of the hydrolysed starch was heated in a suspension in the buffer and the viscous de-aerated solution [1,3] was then poured into suitable perspex or plastic trays [5] for setting into a gel. At the appropriate time protein solutions were applied at the slots in the gel and current applied at a suitable voltage. At the end of electrophoresis, the gel was sliced and the protein zones made visible by staining. Details of the process are given by Smithies [1,3], and Poulik and Smithies [4].

Some Modifications of the Method

The method originally worked out by Smithies [3] was Horizontal Starch-Gel Electrophoresis; the process being carried out with the gel tray placed horizontal on the buffer compartments. The horizontal method, however, suffered from a process of 'electro-decantation', [1,5] which tended to occur in the protein solution in the slots of the gel during a run.

In Smithies' modified procedure [5] of Vertical Starch-Gel Electrophoresis, the negative face of the slots was made horizontal to ground level; the faster moving proteins, though causing a density gradient, moved into the face of the gel, the force of gravity, and electrophoresis working in the same direction. Theoretically, therefore, no 'electro-decantation' would be expected.

'Electro-decantation' in Horizontal Starch-Gel Electrophoresis was prevented if the sample, under analysis, was applied in the gel as a saturated

strip of filter paper [1] or, alternatively, the sample's viscosity was increased by the addition of starch grains before applying the solution in the slots [1]. Either method, while reducing greatly or eliminating 'electro-decantation', did so at the cost of decreased resolution of the protein mixture; the first possibly by adsorption of some proteins on to the filter paper itself or through poor contact as a result of the paper's rough surface, and the second by irregular packing in the gel slots [1,5].

The generally accepted technique in Horizontal Starch-Gel Electrophoresis is to incorporate starch to the extent of 1% in the sample [7], although Pert et al. [6] mentioned that the actual quantity of starch was not critical.

Wake and Baldwin [7] used a Horizontal Starch-Gel Electrophoretic apparatus, similar to that of Smithies [3], but to facilitate slicing, the 6 mm tray sides were divided into three 2 mm deep parts each - the bottom 2 mm section being cemented to the tray, whereas the top two 2 mm sections were removable. All three sections were in position when the starch solution was poured into the tray. For slicing, each of the top two sections were removed in turn.

However, the most radical change from Smithies' original apparatus for Horizontal Starch-Gel Electrophoresis [3] is due to Cunningham and Magnusson [8]. These workers have described a plexiglass frame, made in two identical U-shaped parts which fit on top of each other. The U-shaped frame was placed in a transparent dialysing tubing. Hydrolysed starch solution in a buffer was poured in after clamping the tubing at one end. The gel set around

the filter papers placed at either end of the tubing and these served eventually as contacts in the actual electrophoresis. It is claimed by the authors that the apparatus described was simple, compared to Smithies' apparatus, and prevented evaporation and undue temperature changes in the gel during electrophoresis.

The simplicity of their apparatus is accepted, but the authors did not give any evidence on the separation of proteins in this apparatus, and it is doubtful whether a major advance has been made on the other problems mentioned above i.e. evaporation and temperature changes. Smithies' technique already keeps evaporation to a minimum. Heat conduction may be faster perhaps through the tubing than with the perspex.

Minor modifications have been suggested in the staining and washing procedures.

For staining, Smithies [3] in his study of serum proteins used a saturated solution of Amido Black 10B in methanol + distilled water + glacial acetic acid in the ratio 50:50:10. The same solvent mixture (without the dye) was used in the washing of the stained gels. The gel sections were held in the dye solution for half a minute.

Elton and Ewart [9], in their study of cereal proteins, used a one-minute staining time. Rubinstein et al. [10], in a quantitative study of the measurement of proteins in Starch-Gel Electrophoresis found that bovine serum albumin uptake of Napthalene Black 10B dye (Amido Black 10B) was the maximum after four hours in the staining bath.

It appears that, with staining, it is imperative to keep the gel section as long as is practicable in the staining bath, to allow the dye to penetrate through the gel for proper staining of the separated protein zones. The concentration of the dye in the solvent does not appear to be nearly so critical as the time allowed for the dye to gain access to the protein zones within the gel. This would be particularly important for detecting the minor components in a protein mixture.

Several workers have attempted to modify or add to the washing procedure to make the stained gel more transparent for easier detection of minor protein components, and for more dependable photometric studies of the protein zones using transmitted light.

Thus, Wake and Baldwin [7] incorporated glycerol to the extent of 10% in the washing solvents. When washing was completed, the gel was transferred to a glass plate and covered over by a fine textured filter-paper, which had been soaked in the washing solvent. The whole was left to dry overnight at room temperature and the filter-paper was peeled off in water next day. An investigation showed that a marked contraction and reduction in the thickness of the gel occurred by this method, which did allow the easier detection of minor components, as claimed by the authors. Glycerol prevented the gel from going brittle and, from experience, gels so treated kept flexible for six months at least, so long as they were not subjected to a temperature higher than room temperature and were kept closed in a box or a file.

Apart from keeping the gel moist, glycerol had another function - that of increasing the transparency of the gel. Gratzer and Beaven [11] observed that clear gels could be obtained by "matching the refractive index

of the starch with a liquid which replaces the water in the continuous phase". They tried morpholine (N_D^{20} = 1.455), but this was "disagreeable to work with", apart from producing unfavourable effects on the gel texture and protein stability. The use of glycerol (N_D^{20} = 1.473) was more successful. A gel stained in Amido Black could be made partially transparent by immersion in glycerol [11]. The stained and washed gels could also be heated in glycerol [15] to get them transparent.

Fine and Waszczenko-Z [12] claimed to have obtained a completely transparent gel by applying a 1.5% solution of agar to both surfaces of a thin gel. The exposed agar surface was then covered with Whatman No. 1 filter-paper for even drying and absorption of non-volatile mineral salts. The gel was finally dried in the oven at 37°C for 48 hours.

Vesselinovitch [13] boiled a stained and washed gel in 10% acetic acid solution, and claimed to have made the gel clearer and the detection of minor components easier. This method was investigated and it certainly appeared to clear the gel, but it cleared the minor protein zones off the gel too, and the claim that these were more easy to detect certainly was not substantiated - not in the case of casein components anyway. Also, the gel became brittle and difficult to handle.

Smithies [1] mentioned that, after the stained gel was washed in the usual methanol:water:acetic acid solvent (50:50:10), it could be further dehydrated in a second solvent containing more methanol, i.e. (70:30:10), and finally immersed in benzyl alcohol. Although complete transparency was achieved, a number of difficulties were also encountered [1], namely, the gels became

rigid and brittle, and "benzyl alcohol was a powerful solvent for many lacquers and varnishes generally used in electronic equipment". Damage to the optical instrument was, therefore, possible. Groulade et al. [14] suggested two possible treatments to improve on the fragility and opalescence of gels, which both involved the use of an infra red lamp.

Of the modifications given above, Wake and Baldwin's [7] method seemed most suitable and was used. It appeared the simplest and the gels kept for six months at least.

Fine and Waszczenko-Z's agar method was not tried, mainly because of the time factor involved. Other workers [10] have used this method.

Section II:

Photography of the Stained Gels

Since the protein zones tend to lose some of their colour over a period, workers in this field have attempted to photograph the stained and washed gel, as soon as possible after processing to maintain a permanent record.

Smithies [1] suggested the use of a Kodak Microfile 35 mm. film with a Wratten A red filter and Tungsten lamp illumination. He also suggested various developer formulas and recommended certain paper types for final enlargments. Wake and Baldwin [7] used a Kodak contrast Process Pan film and a Wratten G filter.

Photographs of Starch-Gels, shown in this thesis, were taken in an Alpa reflex camera, using Ilford Super Pan, 25 A.S.A. extra fine grain film. A red filter was used to obtain a good contrast on the photographs.

Transmitted tungsten lamp illumination through a yellow plastic box was used, and the gels were placed on the plastic box and cellotaped at the corners. Developing and printing was done by a Palmerston North photo studio. No details are available, except that it was carried out as usual as for commercial photographs on ordinary photographic paper.

Johns [16] described a method for obtaining rapid contact prints of stained gels. However, the contact prints were not suitable for reproduction

Section III:

Brief Description of Various Electrophoretic Methods and their Comparison with Starch-Gel Electrophoresis.

(i) Definition of Free-Electrophoresis and Zone-Electrophoresis:

By Free-Electrophoresis, we refer to a process occurring in dilute solutions with the resultant partial separation only of the different fractions, e.g. Moving-Boundary Electrophoresis in the Tiselius apparatus [2].

In Zone-Electrophoresis, a solid material is used as a supporting medium which allows the separation of a mixture into distinct zones. Apart from this, the zones may be made visible to the eye by staining methods, e.g. Filter-Paper Electrophoresis, Gelatin, Agar and Starch-Gel Electrophoresis, etc.

(ii) Moving-Boundary Electrophoresis:

A fine account in the theory and practice of Moving-Boundary Electrophoresis has been given by Longsworth [2].

The process is carried out in a U-shaped channel containing a buffer and a low concentration of a protein mixture. On the passage of current, the proteins move in a direction dependent on the pH of the system. A partial separation of the proteins occurs if convection currents are avoided,and special optical methods are used for the recording of the refractive index or its gradient along the arm of the U-tube, which distinguishes the overlapping boundaries of the partially separated proteins. In any arm of the U-tube, the lighter solution is always above the denser one at each boundary [2], at the end of the process, and this stabilization through gravity is characteristic of this method.

The Tiselius apparatus [17] and the related optical scanning devices are a complex and costly outfit, and complete separations of a protein mixture has not been achieved yet. The apparatus, in its most modified version, has not solved the problem of convection currents which tends to occur in the U-tube.

Recovery of the separated proteins is a difficult process [2]. The left arm of the U-tube is open to the atmosphere and it is possible to insert a capillary tube attached to a syringe and withdraw the protein layers at the boundaries indicated by an optical device. The only components which can possibly be taken out pure are the fastest components on the left arm and the slowest in the right arm in very small quantities. Often the latter cannot be obtained pure, due to the spreading of the descending boundaries [2].

Longsworth [2], in his conclusion, maintains that, in spite of the complexity of the apparatus and its limitations, it still remains the mildest of procedures for analysing protein mixtures and for preparation of purified proteins. The process can be done at low temperatures and the proteins do not,

at any stage, come into contact with a non-aqueous phase, which may give rise to irreversible changes.

(iii) Filter-Paper Electrophoresis

The process of Filter-Paper Electrophoresis occurs in moist chambers [19]; the filter-paper (generally Whatman No.1) being placed horizontally within the chamber with its ends dipped into the buffer compartments of the electrode vessels. Current is passed for the desired time at the desired voltage after a micro-quantity of sample is applied at one end. At the end of the process the paper strip is stained and, after washing, the stained protein zones are measured by either the elution method (elution of stained zones and measuring optical density of the solution), the light reflection method, or by direct photometry. In the latter method, the paper is made transparent by various chemicals [19] such as glycerol, or a mixture of paraffin oil, and α-bromonaphthalene, etc. The use of these solvents was to get the refractive index of the liquid phase in the paper to approximate that of the cotton fibres of the paper, thereby increasing transparency [19].

The technique has many modified versions and descriptions of the simplest apparatus [20] to the somewhat more costly apparatus [19] have been given.

Paper Electrophoresis is a Zone Electrophoretic method. Its advantage over Free-Electrophoresis is that the solid support medium (i.e. filter-paper) stabilizes the migration and the process is not affected by convection due to heat. The equipment is less costly and simpler than in Free-Boundary Electrophoresis.

The process, however, suffers from certain disadvantages. It is possible, as in Free-Boundary Electrophoresis, for electroosmosis to

occur. The fibre surface of the paper tends to acquire a negative charge [19] with respect to the buffer and the latter, being the moving phase and positively charged with respect to the paper, tends to flow towards the cathode and affects migration of the components.

The passage of current heats the paper and increases evaporation. Consequently, the ionic strength of the buffer tends to vary, which will again affect migration [19]. Evaporation is reduced by using 5% - 15% glycerin or propylene glycol in the buffer [21].

For good separation of components, it is imperative that there should be no 'physical sorption' or 'chemical sorption' on the paper of the components [19]. The latter defect may be due to impurities in the filter paper, mainly the ash content. Attempts to modify the surface of the paper to reduce electroosmosis and adsorption have been made, and Wunderley [19] has cited some references on the subject.

To quote Wunderley [19] "staining and the removal of excess dye connected with it constitutes the most difficult step in the technique of Paper Electrophoresis with regard to reproducibility". The intensity of colouration is not proportional to the concentration of the substrate. Also, there is no evidence to suggest that two different proteins at the same concentration would have the same colour intensity which makes quantitative evaluation difficult.

Wunderley concludes that paper electrophoresis under standardized conditions, and particularly with automatic technique, has gained a reliability of reproduction. Protein zones can be made visible to the eye by staining, and separation into discrete zones is a great advantage over purely boundary separation, as in Free-Boundary Electrophoresis. However, the difficulty of

preparative procedures still remains, since the sample applied to the paper is in very small quantities.

(iv) Starch-Grain Electrophoresis

A common procedure for using various granular supporting media, like starch grains, is to simply make a homogeneous paste with a buffer and pack it in semi-cylindrical glass troughs or rectangular plastic boxes [22,23]. Electrophoresis is generally horizontal, although a vertical arrangement is recommended for reducing electroosmosis [22]. After electrophoresis, the block is segmented at the parts where zones are present (using a filter-paper pressed against the block and stained, as an indicator of the zones) [22]. The protein zones may be eluted by centrifugation or merely letting the starch settle down to give a supernatant liquid containing the separated protein. Other methods are mentioned for the recovery [23].

Advantages of using granular starch in block electrophoresis, as against silica glass powder or asbestos fibre, is that the starch granules give a very homogeneous packing with little tendency to 'melt' when handling [22,23]. Sharp bands can be applied at the origin.

Bloemendal [22] states that adsorption of most proteins on starch is very small, but other workers [1,23] have contrasting opinions. The elution of proteins from starch segments is easier than from gelatin or agar-gel, an important factor for preparative purposes.

Some disadvantages are that electroosmosis, though lower than in most supporting media [24], may still cause poor separation in this technique [22]. While the method works well for larger sized proteins, those below 30,000 in molecular-weight do not migrate freely - possibly through entrance of these

smaller molecules into the swollen starch granules [23]. Furthermore, "small amounts of soluble carbohydrate, phosphorus compounds, ninhydrin positive materials and ultra-violet absorbing substances cause difficulty in analysing the isolated fractions".[23] Difficulties encountered with specific proteins have been enumerated by Kunkel and Trautman [23].

(v) Agar-Gel Electrophoresis

The technique was originally described by Gordon and his co-workers [25]. About 2% to 3% agar may be used [7,23] in a buffer to form the gel. The gel may be prepared on microscopic slides [23] or on plates 18 cm x 6 cm [27].

Cut sections of the gels are stainable, as in the case of Filter-Paper Electrophoresis. There appears to be little adsorption of the proteins in the path of migration [23,27], but adsorption occurs in the slit where the sample is applied. The technique appears relatively simple. However, there are several disadvantages.

Agar is itself an electrolyte [26] and is liable to form complexes with proteins. Its use is, therefore, limited to those proteins which will not interact with it.

Agar is strongly acidic, due to the presence of an ethereal sulphate for each galactose unit [23], and gives rise to considerable electroosmotic backflow. Preparative work is difficult since traces of carbohydrate and other substances persist following isolation of zones from agar-gel [7, 23].

(vi) Starch-Gel Electrophoresis

A brief description of the actual technique has already been given. In this section the advantages and disadvantages of the method will be discussed and comparisons made with other electrophoretic methods.

Starch-Gel Electrophoresis, which has hydrolysed starch as the supporting medium, has the advantage of zone detection by staining methods characteristic of Filter-Paper Electrophoresis, and does not suffer from the serious adsorption defects of Starch-Grain of Filter-Paper Electrophoresis. It retains the advantage of Agar-Gel and Starch-Grain Electrophoresis in the application of the sample as a narrow initial zone [1] and the separated zones show little distortion [1,3,27]. Like Agar-Gel Electrophoresis, it is adaptable to take small quantities of sample, but appears to give better resolution than any other method.

Thin sections for staining are possible in Starch-Gel Electrophoresis [3], as against Agar-Gel Electrophoresis and Starch-Grain Electrophoresis.

There seems to be very little electroosmosis in Starch-Gel Electrophoresis [9,26] compared to the other methods, possibly due to the greater mechanical hindrance in the gels. Smithies [1,3] reported, however, the osmotic flow of serum Y globulins, particularly in Horizontal Starch-Gel Electrophoresis.

The greatest advantage of the Starch-Gel Electrophoretic method, however, lies in its ability to separate proteins on their molecular size and shape, in addition to the differential charge on them [1,3,9]. In

this it surpasses even Agar-Gel Electrophoresis. A 1% agar, for example, is said to have a free space almost equal to that of free solution [28]. In the starch-gel, the pore size may approach the molecular size of some proteins which are offered greater resistance to movement. Smithies [1] gives experimental evidence to show that the "starch-gel mobilities of proteins are in the inverse order of their molecular sizes, despite the fact that on Filter-Paper Electrophoresis they had approximately the same mobility at a similar pH". In Moving-Boundary Electrophoresis, Filter-Paper Electrophoresis, Starch-Grain Electrophoresis and Agar-Gel Electrophoresis, "the greater frictional retardation of the larger sized proteins is compensated for by a greater net charge". This compensation does not work in Starch-Gel Electrophoresis, due to its own characteristic hindrance to the proteins.

This hindrance could, however, be a disadvantage too. Some proteins are trapped in the slot itself and do not enter the main gel block[9,23]. This could be due to adsorption in the slot as in Agar-Gel Electrophoresis or due to excessive hindrance of the Starch-Gel, as may be the case with certain macro-globulins[23].

The property of Starch-Gel Electrophoresis of superimposing resolution by molecular sizes on resolution by free solution mobility introduces another kind of danger which does not appear to have been solved yet. Franglen and Gosselin [29], working with the dye bromocresol green, made the rather startling discovery that Starch-Gel Electrophoresis separated not only distinctly different substances, but also metastable polymers of a monomer, i.e. separation into different components was no demonstration of heterogeneity by itself. The numerous reports [30] on the heterogeneity of human and bovine serum proteins,

and of milk proteins [7], enzymes and hormones, as based on Starch-Gel Electrophoresis must, therefore, be accepted with reserve until this point is clarified.

Other difficulties pertinent to the other methods remain with Starch-Gel Electrophoresis, although perhaps to a lesser extent. Thus, in the photometric evaluation of the stained gels, less trouble, due to opacity, is encountered with agar and starch-gels than with stained filter-paper, but the trouble is by no means very much less, as will be appreciated from the discussion on modifications of the washing procedure in Starch-Gel Electrophoresis earlier on.

Furthermore, there are differences in the dye binding capacity of different proteins and with stained filter-paper, agar and starch-gel patterns, Beer's law is not followed with regard to concentration and colour intensity [7,9].

Starch-gels also suffer from there being a certain amount of background protein present in the starch originally. Gordon's work [31] indicates that the background protein in a starch-gel could well be about 5% of the concentration of that in the heaviest band in a gel pattern. It is easily possible, therefore, that in a study of any protein by Starch-Gel Electrophoresis, a minor component could be missed, even using spectrophotometric methods of scanning, since the optical density of an area of gel, containing no bands, would not be very much lower than one containing a weak band[9].

The method has not significantly solved the problems of preparative work which was evident in the other methods. Smithies [1] briefly discusses four methods by which the separated proteins may be recovered from the gel, namely:

(i) Pieces of gel could be macerated with a buffer solution. (Difficulties: Incomplete recovery, particularly with large molecular-weight proteins, dilution of proteins).

(ii) Gel frozen, thawed and liquid centrifuged, or sucked, or squeezed out of the sponge like gel. (Difficulties: Pieces of gel may get into the sample, and irreversible adsorption of the proteins to the frozen precipitated starch is possible).

(iii) Recovery of proteins by a second electrophoresis [31,32]. (Difficulties: Proteins diluted and method cumbersome if many fractions have to be isolated but recovery can be almost complete [1,31]).

(iv) A collection slot made 5 cm from the sample slot in the gel. A vertical flow of buffer solution (1ml/hr.) across the collection slot enables proteins to be collected as they reach the latter. (Difficulties: Some dilution, but less than other methods. Not always leak-free (cf. Elton and Ewart [39]).

Of the above, No.(ii) has been used most [7,9], since it is simple and rapid, and dilution with a buffer is not necessary. Obviously, however, the problem of large scale preparative work remains unsolved.