"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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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.

CHAPTER II

DEVELOPMENT OF THE STARCH-GEL ELECTROPHORETIC PROCEDURE

Section I:

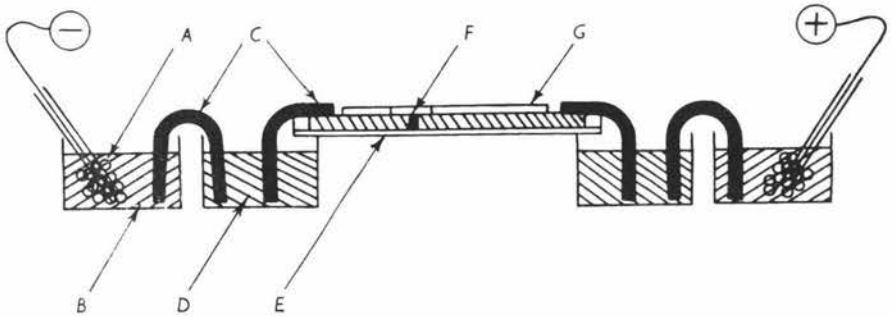
METHODS AND MATERIALS

A. Construction of the Horizontal and Vertical Starch-Gel Electrophoretic Apparatus and Some Modifications in the Process.

The design and set-up of both apparatus followed basically Smithies' description [3,5] with some minor modifications. In the actual process, Wettex cloth was used for contacts between the gel surface and the buffer compartment of the electrode vessels at either end, instead of filter-paper, since Wettex cloth offers less resistance to the current than does the filter-paper [33]. The slicing technique followed was that of Wake and Baldwin [7], but the cutting device was a piece of No. 33 gauge resistance wire (diameter 0.25 mm.), instead of the No.26 wire [7], since this facilitated slicing.

The slots in the gel, containing the sample, were covered by small pliofilm strips, and petroleum jelly at 45°C was poured over the strips. The rest of the gel surface (except the portions covered by the Wettex cloth contacts) was covered by a sheet of pliofilm to reduce evaporation. In the vertical method, however, the whole of the gel surface was covered with petroleum jelly [5] and a plastic lid was applied with the help of elastic bands.

GENERAL LAYOUT OF HORIZONTAL STARCH-GEL ELECTROPHORETIC APPARATUS



A, platinum electrode;

B, borate buffer (pH 8.6) in electrode compartment;

C, Wettex cloth bridges soaked in borate buffer;

D, compartment containing borate buffer (pH 8.6);

E, urea starch-gel contained in perspex tray;

F, position of sample insertion;

G, pliofilm seal to prevent loss of water during electrophoresis.

(see SMITHIES, O. (1955) : Biochem.J. 61 : 629.

WAKE, R.G. and BALDWIN, R.L. (1961) : Biochim.Biophys.Acta, 47 : 225).

It was found necessary to apply either a thin polythene strip or a layer of petroleum jelly on the gel sides in the horizontal method, during electrophoresis, to prevent undue evaporation and shrinkage. The bottom of the trays were slightly roughened by sand paper so that the gel could have a firm contact with the tray bottom, which facilitated the slicing technique.

Usually three runs were made with any one lot of buffer solution[33] in the electrode vessels, each time the polarity of the electrodes were changed to reduce the accumulation of products resulting from hydrolysis of the buffer solutions. The gel formula employed was that of Wake and Baldwin [7] who used a discontinuous buffer system, as described by Poulik [34] for better resolution of the protein zones.

Gels were stained in a saturated solution of Napthalene Black 10B in methanol:water:acetic acid (50:50:10) for 5 minutes. The stained gels were then washed in methanol:water:glacial acetic acid (50:50:10) containing 10% $^{v}/_{v}$ glycerol [7].

The washing solvent was decolourised by passing through a mass of activated charcoal contained in a 1-litre Büchner flask; the colourless solvent coming out of the top after passing through a cotton filter contained in a plastic tube placed just under the rubber cork. The principle of decolourising was proposed by Pert et al. [6]. A syringe, worked by lever action, acted as the pump, discharging 80mls/minute of the colourless solvent.

The procedure adopted was to shake the stained gel for fifteen minutes in the washing solvent in a reciprocating shaker and to discolourise the solvent in the decolourising apparatus before putting back into the shaker.

In the early stages of washing, fifteen minutes was allowed in the shaker to remove the excess stain. In subsequent washes this was increased to 1-1½ hours. Washing was continued till the space between the parallel adjacent protein zones, arising from adjacent slots, became distinctly clear. This method was found to be better than attempting to standardize the time of washing, since the elution of excess dye from the gel appeared to take a variable amount of time. Approximate washing time was 6-8 hours.

Use of the decolourising apparatus proved very economical, since a solvent could be used indefinitely. It was possible to use a Büchner funnel containing activated charcoal on Whatman No. 42 filter-paper, and connecting the flask to a water pump. The procedure was much quicker than using the syringe pump. However, the composition of the washing solvent would have altered through application of a vacuum, and in time a new batch of solvent would have been necessary.

B. Preparation of Acid Casein

The method followed was basically due to Hipp et al. [35] and Wake and Baldwin [7].

Five gallons of freshly separated bovine skim-milk from a bulk milk supply was obtained from the Dairy Research Institute (N.Z.) dairy factory. The pH was 6.7. The skim-milk was warmed to about 30°C and the precipitation was done at pH 4.5 in 6 gallon plastic containers, using approximately 1 N HCl (two subsequent reprecipitations were done at pH 4.6).

The precipitate was separated from the whey (and subsequently from the wash water) by means of a clothes spin drier, using close meshed cotton filter bags. Washing was done in the same machine. The first precipitate was washed

five times with distilled water. It was dissolved in distilled water at pH 8.0, using approximately 1N NaOH for pH adjustment. The second precipitate was washed three times, and the third five times with distilled water.

The pH was not allowed to go over 8.0 or below 4.5, and due care was taken when adding the alkali and acid to prevent local excess.

The wet casein was frozen at -30°C in a deep freeze overnight and freeze-dried until it was noticeably powdery with no hard lumps. Final weight of casein obtained from 5 gallons of bovine skim-milk equalled 454 gms.

Analysis indicated that moisture content equalled 1.9%, and ash content equalled 2.0%.

C. Preparation of Potato Starch

Potatoes supplied by Mr.H.Wenham of Massey University College were grown locally.

The extraction of starch was done by the method of Schoch (36).

The starch was then defatted by a procedure described by Schoch (36), although potato starch does not seem to be associated with fatty acids as do starches of cereal origin (37).

D. Starches of Cereal Origin

(i) Winson's Maize Cornflour was obtained at Palmerston North. Kjeldahl determination indicated that protein content was about 1.5%. No details of the method of extraction or processing are available.

(ii) Beaker's Wheaten Cornflour was obtained at Palmerston North. Kjeldahl determination indicated about 0.5% protein. No details of processing are available.

(iii) Thin Boiling Wheat Starch was obtained from Starch Products Limited, Christchurch. This was an acid hydrolysed product, but again details of processing are lacking.

Section II:

EXPERIMENTAL AND RESULTS

The Use of Indigenous Starches as an Alternative to Imported Hydrolysed Starch in Starch-Gel Electrophoresis.

The attempt to use indigenous starches for the electrophoretic process was based on economical grounds. Smithies [1,3] recommended the use of hydrolysed starch prepared by Connought Medical Laboratories, Canada. This starch had to be specially imported. On the other hand, a free supply of potatoes was made available locally, from which starch was extracted, and the cereal flours obtained locally were relatively much cheaper compared with the imported hydrolysed starch.

A. Potato Starch

The defatted starch, described in the previous section, was hydrolysed by a mixture of acetone: concentrated hydrochloric acid (100:1) at a temperature of 38.5°C. The experiment was first conducted on a pilot scale to find the optimum hydrolysis time for this particular batch of starch, as

suggested by Poulik and Smithies [4]. Samples containing approximately 10g starch were withdrawn at intervals of 30, 45, 60, 75 and 90 minutes; the reaction being stopped immediately with molar sodium acetate solution. The washed and dried hydrolysed starch samples were sent to Dr.Esam Moustafa at the Department of Scientific and Industrial Research for determining the optimum hydrolysis time. Dr.Moustafa's Horizontal Gel Electrophoretic Apparatus enabled a comparison to be made of the starch samples sent to him, with the imported hydrolysed starch under similar conditions of electrophoresis of serum proteins, since the tray could be subdivided into parts to receive different gel types. Based on his report, a hydrolysis time of 40 minutes was chosen as being optimum, and the bulk starch was hydrolysed for this time at 38.5°C.

Both Horizontal and Vertical Starch-Gel methods were then tried with the modified starch. It was discovered, however, that this starch would not give a firm gel at the same concentration as used by Wake and Baldwin [7] using the imported starch. It would form a weak gel at this concentration if urea was absent, or at a slightly higher concentration if the formula had 4.8M urea instead of 7M, but in a 7M urea gel, at least, 50% more starch had to be incorporated to obtain a firm gel. Generally a concentration of approximately 13.42% W/W of starch in the final gel mixture was necessary, compared to approximately 9.37% W/W specified by Wake and Baldwin [7] for the imported starch. The gels from the local starch were much more opaque than the imported starch gels, presumably due to the higher concentration of starch used.

Electrophoresis experiments with the local starch proved a failure. Wake and Baldwin [7] had reported 17-20 components in their study on casein, but hardly three were noticeable in this gel and this was observed only once.

Usually migration was poor, there would be considerable tailing, and the gel structure did not appear to be conducive to the slicing technique employed.

Discussion

There are several possible reasons for the failure to obtain a satisfactory hydrolysed starch comparable to the imported starch.

Smithies [3] stated that potato starches from different manufacturers varied in their properties (cf. Pert et al. [6]). Some were not satisfactory for serum electrophoresis and others required different hydrolysis times. It was possible to remove small differences in electrophoretic behaviour between different starch batches by varying the starch and/or buffer concentrations [3]. It now seems obvious that a better approach would have been to obtain potatoes of different varieties and, perhaps, from different localities to elucidate the best type for electrophoresis, instead of obtaining them from only one source and attempting to equate this starch with the imported starch, trusting more or less to chance.

The temperature of hydrolysis needed to be strictly controlled, since the temperature coefficient for hydrolysis was high [3]. Pert et al.[6] stated that a temperature variance of 0.2°C was approximately equivalent to a 5-minute change in hydrolysis time. While the same thermometer and temperature unit was employed for both pilot scale and bulk starch hydrolysis, one cannot guarantee that a variation of 0.2°C or more did not occur. An overhydrolysed starch gives a weak gel and is difficult to handle [4,6]. The increased concentration of local starch necessary to give a gel of the same mechanical strength, as that of the imported starch, indicated that overhydrolysis had occurred, and the greater opacity of the local starch-gel may be explained by the higher concentration of starch utilized.

The increased starch concentration may also explain the considerable tailing and the poor separation obtained.

Most electrolytes tend to weaken the associative forces between the starch micellae [37], possibly through breaking hydrogen bonds. The presence of ions like calcium and magnesium would, therefore, weaken a gel structure. This problem should not have arisen since distilled water was used throughout the starch making process. However, during part of the starch making process some of the potatoes had to be left soaking overnight in artesian bore water. The potassium starch of potato, known for its cation exchange properties [37], may have exchanged some of its potassium ions for either hydrogen or calcium/magnesium ions. In either case, the gel obtained eventually from such a starch would be weaker than a pure potassium starch of potato [37].

The pilot samples, after the required hydrolysis times, were taken out of a single flask after due shaking. In the hydrolysis of the bulk starch it was attempted to keep similar conditions by shaking at the appropriate time intervals. This factor was not critically understood at the time of the process, and it will be apparent that the conditions may not have been similar to that of the pilot scale experiments. Overhydrolysis could have resulted due to over-shaking, giving a freer access to the acid to attack the starch granules. In the pilot scale experiment, the appropriate amount of starch should have been hydrolysed in different flasks without further shaking. The question of taking out samples from a single flask would not then have arisen.

Furthermore, it must be stated that at the time the local starch was being tried in the electrophoretic process, without much success, even the

imported starch-gel was giving far from promising results, compared to that of Wake and Baldwin [7], because the technique had not been worked out fully. Consequently, the failure to obtain good patterns with the local starch may have been magnified to some extent.

Summing up, the main reasons for the failure to produce hydrolysed potato starch of comparable properties to imported starch appear to lie in the lack of adequate control in some of the critical aspects of the starch hydrolysing process and the unknown properties of the starch itself. If the experiment was to be repeated better success would certainly be achieved. However, the starch should not be obtained from any manufacturer since manufacturing and storage details of either the potatoes or the starch are generally unknown [37].

Potato starch from the British Drug Houses Limited has been used for the preparation of hydrolysed starch [3] and could be used as a source.

B. Starches of Cereal Origin

(i) Beaker's Wheaten Cornflour

This flour, most of which was presumed to be starch, was tried at concentrations between 6.07% and 12.44% W/W in the final gel mixture, using the same basic formula given by Wake and Baldwin [7].

The gelatinisation point appeared to be between 67°C - 70°C. The starch solution obtained was extremely viscous. The gels, which formed on cooling, were opaque in direct contrast to the crystal clear urea gels obtained with imported starch and showed extreme 'stickiness'. When the starch concentration was increased from 6.07% W/W to 12.44% W/W it increased the rigidity, but it also tended to increase the stickiness, and this factor made the gels very difficult to handle.

The Wheaten Cornflour was defatted [36] with methanol and hydrolysed [4] as described for potato starch. This was to see whether the above treatment would make possible its use in electrophoresis by removing the defects mentioned earlier. The hydrolysis times used ranged from 15 minutes to 75 minutes. The gelling properties of the final product were compared to those of the imported starch, using 4.8 M and 7.0 M urea in the gel composition.

As expected, the viscosity of the starch solutions decreased with increasing hydrolysis times. However the gels were only slightly less opaque than when untreated flour was used.

Hydrolysis at 15 and 30 minutes did not appreciably reduce the stickiness in the final gels, but at 60 and 75 minutes there was a noticeable reduction in stickiness. The improvement, however, was not great enough to justify further studies.

It was stated earlier that the protein concentration, based on the Kjeldahl determination, was about 0.5% in the Wheaten Cornflour. It was thought that the stickiness observed in the gels may be due to the wheat proteins. The reduced stickiness in the gels at 60-75 minute hydrolysis times is possibly due to denaturation and aggregation of part of the protein and its removal at washing.

(ii) Winson's Maize Cornflour

An experiment on the untreated flour was conducted on similar lines to that on untreated Beaker's Wheaten Cornflour.

The gelatinisation point appeared to be between 70°C and 73°C. The starch solution was very viscous and the gel obtained on cooling

was opaque and granular in texture. Stickiness was again present, but to a lesser extent than in the Wheaten Cornflour, although the protein content was higher (1.5% against 0.5%). It was felt that these preliminary observations did not justify further treatment, namely, defatting and hydrolysis, as in the case of the Wheaten Cornflour.

(iii) Thin Boiling Wheat Starch

This was an acid hydrolysed product, but details of processing and hydrolysis are lacking.

When the starch suspension was heated, the solution did not attain as high a viscosity at the gelatinisation point, as was observed with the other cereal and potato starches. This was indicative of overhydrolysis of the starch as far as electrophoresis was concerned and this point was confirmed by the fact that the gel, on setting, showed less mechanical rigidity. Electrophoresis was attempted, and although the gel set at a starch concentration, as used in the Wake and Baldwin formula [7], and the casein separated into five or six bands, the picture was a diffused one. The gel itself was more opaque than the one made with imported starch and the gel sections, stained as usual, showed a lot of background blue which was difficult to remove.

At this stage, electrophoretic patterns obtained with the imported potato starch gels were comparable to Wake and Baldwin's patterns [7], since the technique had been properly worked out by now. Failure to obtain a good pattern with the thin boiling starch reflected faults in the starch (see discussion below) and it was considered advisable not to proceed with experimenting with the indigenous starches, but to use imported hydrolysed potato starch for further experiments.

Discussion

The failure to adapt cereal starches for Starch-Gel Electrophoresis may be due to their fundamental differences from potato starch in chemical and physical properties. The way these properties may affect the suitability for electrophoresis is, however, not clear. The situation is further complicated by the fact that even in potato starches wide differences may be found in their suitability for electrophoresis [3], depending on the source of potatoes.

Hofstee and DeWilligan [37] have pointed out some differences between potato and cereal starches and these are briefly described below.

(a) Potato starch contains no fatty acid. Corn, wheat and rice starches contain 0.84, 0.50 and 0.59% fatty acids respectively. A defatting procedure reduced it to 0.12%, 0.03%, 0.03%. Further reduction apparently was not possible. Schoch and Elder [4] point out that the "linear fraction in starch forms insoluble complexes with any polar organic substance of the nature R-OH R-COOH , etc., and these complexes cause a loss of gel strength". The increased opacity in cereal starch-gels may be attributable to the residual fatty acids. This may also explain the fragility of the cereal starch-gels obtained in the experiments.

(b) The phosphorus of potato starch appears to be as a monoester and is not extractable, but a methanol treatment greatly reduces the phosphorus content of cereal starches.

(c) Gelatinisation temperature for potato starch is about 61.5°C., as against 65.0°C, 68.5°C and 75°C for corn, wheat and rice starch. The heat of gelatinisation of potato starch is also much higher than cereal starches.

(d) The average size of the starch-grain of potatoes is 35μ, compared to 16, 13 and 4 for corn, wheat and rice starch.

It is not clear how the above properties affect the suitability of a certain starch for electrophoresis. Except for (a), the others may or may not play a part. It is possible that the amylose to amylopectin ratio may be important since it is the amylose or straight chain component of starch which retrogrades and gives the gelling properties [37,40]. The differences in the amylose:amylopectin ratio between potato and cereal starches are not known.

The manufacturing details for the cereal starches are not known. The possible effect of the presence of cereal protein has already been pointed out earlier. This will be a definite impurity for electrophoresis.

While the substitution of cereal starches for potato starch in Starch-Gel Electrophoresis does not appear very promising, it is difficult to evaluate the results based on one experiment only and more evidence based on a broader field of study, viz: obtaining cereal starches from different sources, reducing the protein and fatty acid content, etc., is necessary.

On the other hand, locally obtained potato starches may offer a more fruitful field for study for their suitability in electrophoresis.

Section III:

The Vertical Starch-Gel Electrophoretic Method and its Use on the Caseinate System of Milk.

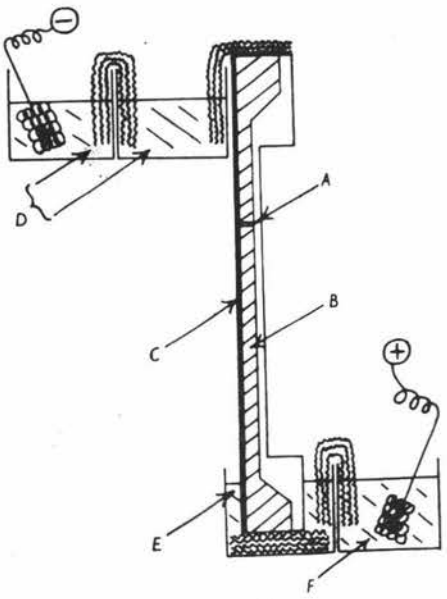
Smithies [5] introduced this method, wherein the protein zones move vertically downwards on passage of current, to overcome the phenomenon of 'electro-decantation' which tended to occur in the horizontal method giving diffused electrophoretic patterns (see Chapter I). Further, it is possible that the vertical method reduces or eliminates 'electro-osmosis' as in the case of Starch-Block Electrophoresis [22]. However, this phenomenon has not been reported widely with the horizontal method, except in one case for serum γ-globulins [1].

To prevent electro-decantation in the horizontal method the protein sample introduced into the slots usually contained some starch granules, or a filter-paper saturated with the sample was inserted in the gel. Both methods tended to reduce resolution of the proteins [1] (see Chapter I). In the vertical method however, the sample can be inserted into the gel without any supporting substance, and the resolving power and the reproducibility of the method is, therefore, improved.

The method, however, proved unsuccessful, despite several attempts. The best separation obtained was as shown on Plate I.

In the early attempts, trouble was experienced with the stability of the gel in the vertical position. The gel would either break at the slots or just above at the bend on the tray. This was, in the main, due to the construction of the gel tray. Even when the bend was 'smoothed' out, the

GENERAL LAYOUT OF VERTICAL STARCH-GEL ELECTROPHORETIC APPARATUS



A, position of sample slots;

B, urea starch-gel;

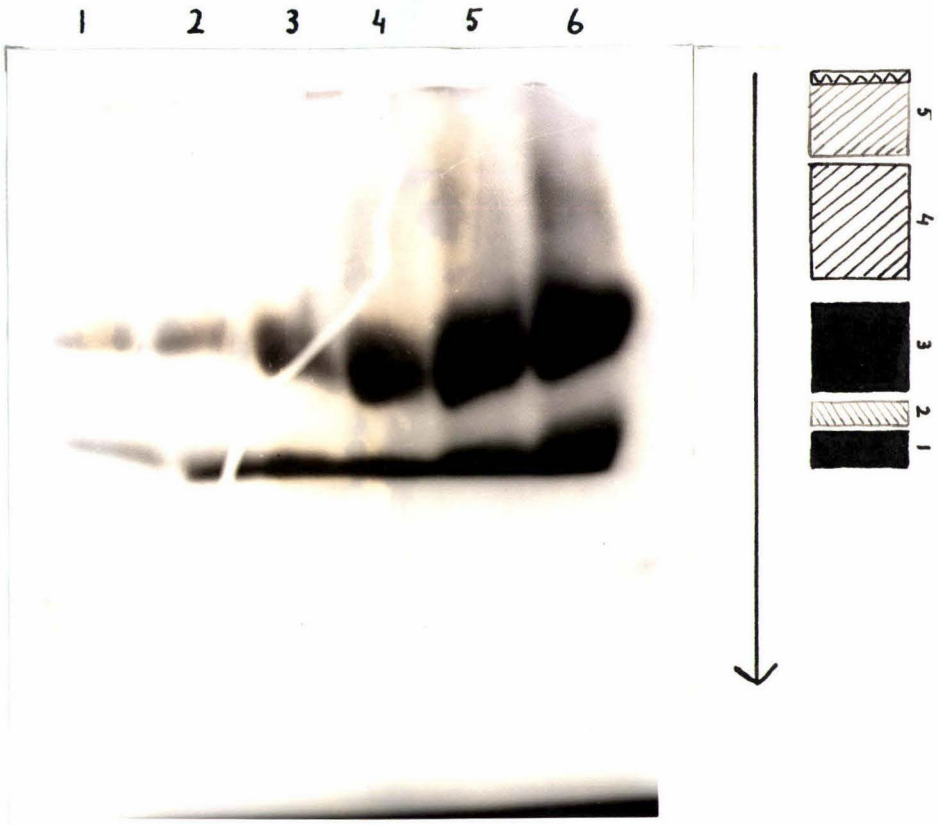
C, petroleum jelly seal to prevent loss of water during electrophoresis;

D, compartments containing borate buffer (pH 8.6). Outer compartment contains the electrode.

E,F, compartments containing borate buffer (pH 8.6). The zig-zag lines show the positions of Wettex cloth bridges.

(see SMITHIES, O. (1955): Biochem. J. 71:585.
WAKE, R.G. and BALDWIN, R.L. (1961) : Biochim.Biophys.Acta, 47:225).

PLATE 1



VERTICAL STARCH-GEL ELECTROPHORESIS OF ACID CASEIN

1	ACID	CASEIN	(0·5%)
2	"	"	"
3	"	"	(1·0%)
4	"	"	"
5	"	"	(2·0%)
6	"	"	"

16h, 150V, 6°C, BORATE BUFFER pH 8·6

SEE PLATE 2'A' FOR COMPARISON WITH HORIZONTAL STARCH-GEL ELECTROPHORESIS

tendency to break at the slits persisted on occasions. The wooden stand carrying the buffer vessels and the tray was improvised with adjustable screws at the bottom, so that the tray would be at right angles during the run. However, the refrigerator in which the whole apparatus was placed was also being used for other purposes, and the occasional opening and closing of the door could very well have upset the position of the tray.

The tray itself could not be called 'perfectly proportional', since there was a distinct diagonal slant if one viewed it from the front. The Wettex cloth contacts were, therefore, not quite in line. This would give an uneven voltage gradient across the gel and lead to poor separation. This may perhaps be borne out by Plate I.

By this time, however, the horizontal electrophoretic technique was beginning to work well using Wake and Baldwin's paper [7] for comparison, and since these workers had reported separating at least 20 components as against 17 by Neelin et al. [44], who used a two dimensional technique using vertical Starch-Gel Electrophoresis, it appeared that rather than spend more time and precious materials on trying to develop the vertical electrophoretic technique, efforts should be concentrated on horizontal electrophoresis.

Section IV.

Use of Starch-Gel Electrophoresis on the Caseinate System of Milk and on Purified Enzymes, and its possible use on the Study of Protein Hydrolysates.

A. Methods and Materials

Acid Casein:- Preparation of acid casein has been briefly described in Section I of this chapter.

Alkaline Milk Phosphomonoesterase:- This enzyme was prepared from buttermilk by the method of Morton (42) and will be discussed in Part II of this thesis.

Commercial Cheese Rennet:- This was supplied by the New Zealand Rennet Co. at Eltham and is used for cheese manufacture in this country.

Preparation of Rennin from Commercial Cheese Rennet:- The method followed for the purification of rennin in commercial rennet was principally due to Ernstrom (43) (see Part II for details).

B. Study of Caseinate System

Originally it was intended to use the method of Starch-Gel Electrophoresis to study the changing electrophoretic pattern of casein due to the action of various enzymes like pepsin, rennin, bromelin and also micro-organisms commonly found in cheese. The aim was to discuss these changes in relation to the ripening process in cheese. Furthermore, a concurrent study

could have been made on enzymic casein hydrolysates. Enzyme hydrolysates of casein are known to have a very bitter taste and attempts to remove or distinguish the bitter tasting fraction in the hydrolysates have not proved very successful, as will be evident from the study on casein hydrolysates by Baker and his co-workers (44-49).

For the above study, an accurate knowledge of the casein complex as it exists in milk was essential, but the picture presented was rather confusing. The complexity of the substrate, together with the difficulties and possible dangers associated with Starch-Gel Electrophoresis, some of which have already been mentioned earlier, made it advisable to change the line of study to the more specific action of rennin on casein. The reasons for this change became more evident by the following discussion on casein studies and on the electrophoresis of enzymes. The electrophoretic pattern of casein was used as an 'indicator' for the study on enzymes.

(i) The Caseinate Complex

A brief review of literature on the studies on casein is necessary, as this will be pertinent to the discussion later.

Prior to 1939, solubility studies on the caseinate of milk indicated that casein was heterogeneous, but it was Mellander (51) who, using Moving-Boundary Electrophoresis, showed that casein consisted of at least 3 components. These were designated α, β and γ casein in a decreasing order of mobility.

Other workers (52,53) have attempted to separate α, β and γ caseins based on their differential solubility in 50% alcohol containing salt, or in aqueous urea at different temperatures and pH. Hipp et al. (53)

found single electrophoretic peaks in Moving-Boundary Electrophoresis for their separate components and presumed that α-, β- and γ-caseins were homogeneous.

Waugh and von Hippel [54], who used calcium chloride for precipitating α- and β-caseins, found a new component which they called k-casein. k-casein was stable in 0.2 M $CaCl_2$ solution and moved very close to the original α-casein in Free-Boundary Electrophoresis. It was postulated that k-casein was in complex with the α_s-casein (α_s = α casein without k-casein) and it acted as a 'protective colloid' to the α_s micelles (see Part II).

McKenzie and Wake [50] confirmed the relationship between the α_s-casein and k-casein on both Free-Boundary and Filter-Paper Electrophoresis. They reported a total of 8 components on Filter-Paper Electrophoresis.

Wake and Baldwin [7], who used Horizontal Starch-Gel Electrophoresis and incorporated urea (7 M) in the gel formula, separated casein into 17-20 components.

(ii) Starch-Gel Electrophoresis of Acid Casein

The method was basically due to Wake and Baldwin [7] with some minor modifications already listed elsewhere. Casein solutions were made in Tris - 7 M urea - buffer.

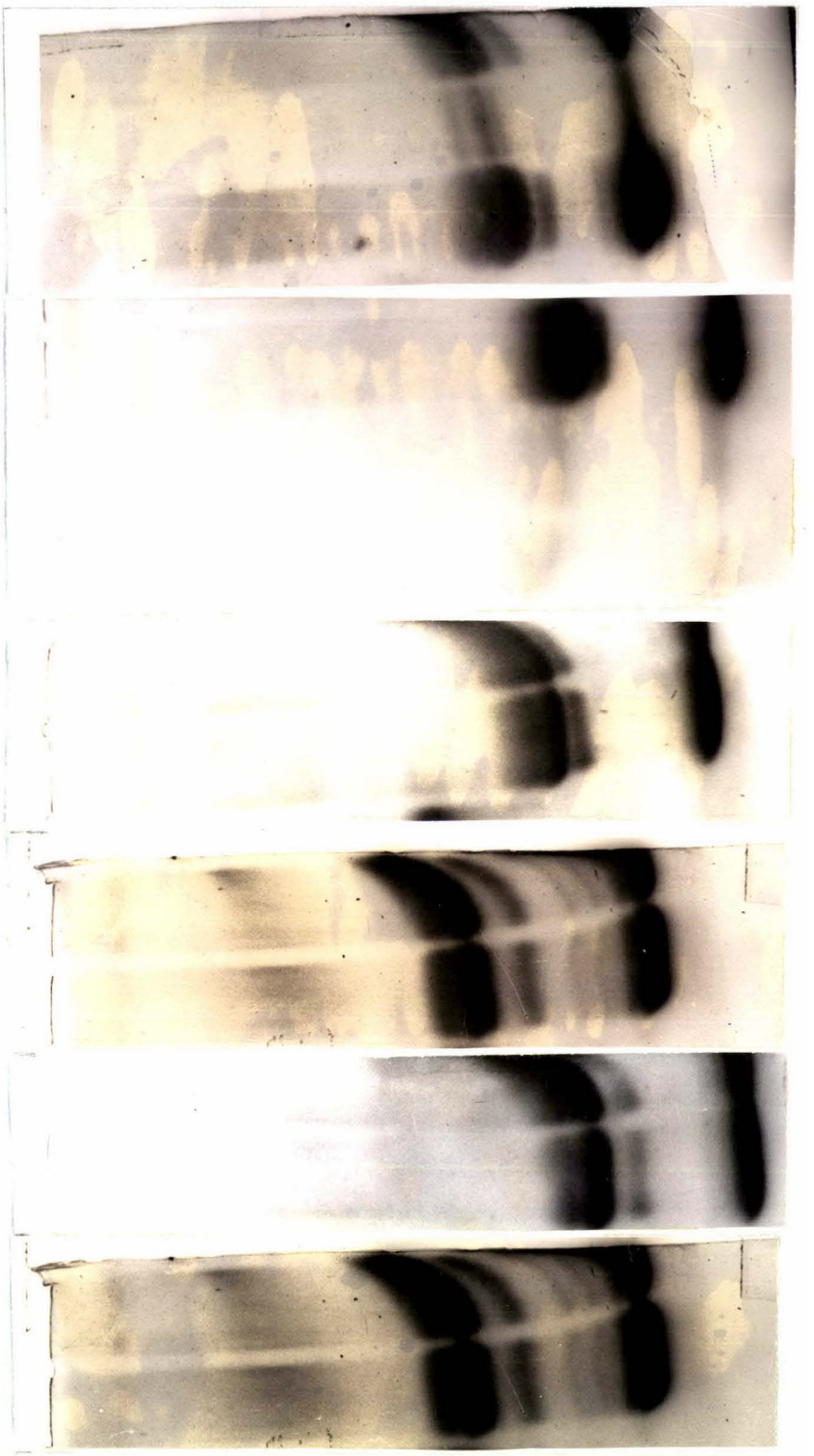
The results are shown on 6 photographic strips (Plate 2A) and can be compared with Wake and Baldwin's [7] diagram of the separated components obtained in their experiments (see Plate 2B). Results appear comparable to theirs, indicating that the method had been developed successfully.

PLATE 2'A'

	%
CASEIN	1·0
"	0·5
"	2·5
"	2·0
"	0·5
"	0·5
"	1·5
"	1·5
"	1·5
"	1·5

STARCH-GEL ELECTROPHORESIS OF ACID CASEIN

{ 16 h, 130 V, 6°C
BORATE BUFFER pH 8·6
TRIS-CIT. " " }



A DIAGRAMMATIC REPRESENTATION OF THE ELECTROPHORETIC PATTERNS OF CASEIN. REFER TO PHOTOGRAPHIC STRIPS ABOVE.

(SEE ALSO PLATE 2'B' FOR COMPARISON)

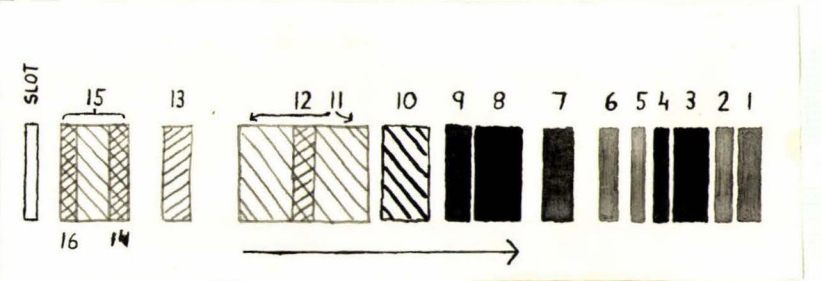


PLATE 2'B'

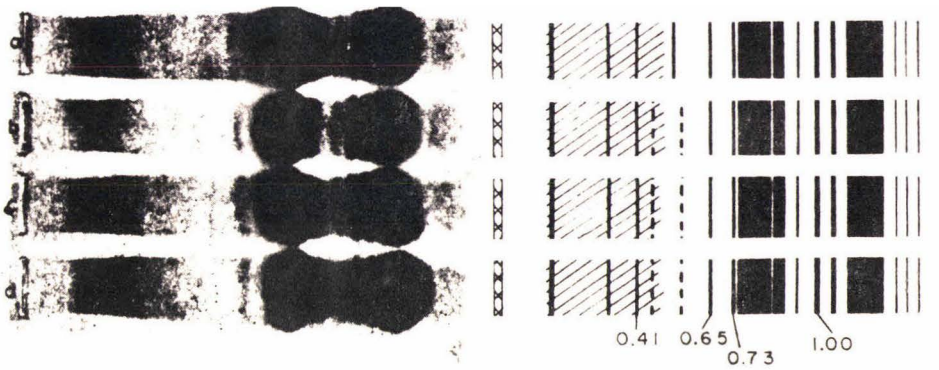


Fig. 3. A test for artifacts of preparation: comparison of whole casein prepared in three different ways. a, acid casein I; b, acid casein II; c, Na_2SO_4 pptd casein; d, first cycle soluble casein.

STARCH-GEL ELECTROPHORESIS OF WHOLE CASEIN

[AFTER WAKE R.G. AND BALDWIN R.L. (1961), BIOCHIM. BIOPHYS. ACTA 47, 225]

COMPARE WITH PLATE 2'A'

In some of the photographic strips the minor components have not shown up well, although visible in the original gel. Each strip represents a different run with fresh acid casein solutions. The acid casein separated into 15-17 components. However, some of the minor components are not always visible and may be missed in some patterns.

Originally the gel strips were examined for percentage transmission changes in the Beckman D.U. Spectrophotometer. This was done by removing the cuvette holder and placing instead a specially made wooden frame to carry the gel strip. The gel strip could be moved a millimeter at a time and the percentage transmission changes recorded directly on graph paper placed on a small platform attached to the main frame. The graph then showed peaks of low percentage transmission whenever a stained zone passed in front of the slits. The wave-length used was 612 mμ.

This process, however, was cumbersome and laborious. The movement of the gel strip which was done manually was not always accurate, and it was possible to move the gel strip too far and thus miss separated components close to one another.

The method may be useful to find changes in the major protein components, either through enzyme or bacterial action, as indicated on the graph paper, but since this line of study was not going to be followed, photography of the gels was undertaken as a better and more permanent record of the original gel.

Details of photographing the gels have already been given in Chapter I.

C.

Starch-Gel Electrophoresis of Commercial Rennet, Rennin and Alkaline Milk Phosphomonoesterase.

The enzyme preparations were all freeze-dried. Details of their preparation and isolation are given in Part II of this thesis. Commercial Rennet (dried) contained 73% salt, while the rennin preparation contained 68% salt.

Generally the method of Wake and Baldwin [7] was followed in electrophoresis using similar conditions as for casein. Enzyme and casein solutions were made in a Tris- 7 M urea buffer.

Plate 3 shows an electrophoretic pattern of 5.4% commercial rennet solution (allowance being made for salt concentration), 1% and 3% phosphomonoesterase solutions and 1.5% casein solution.

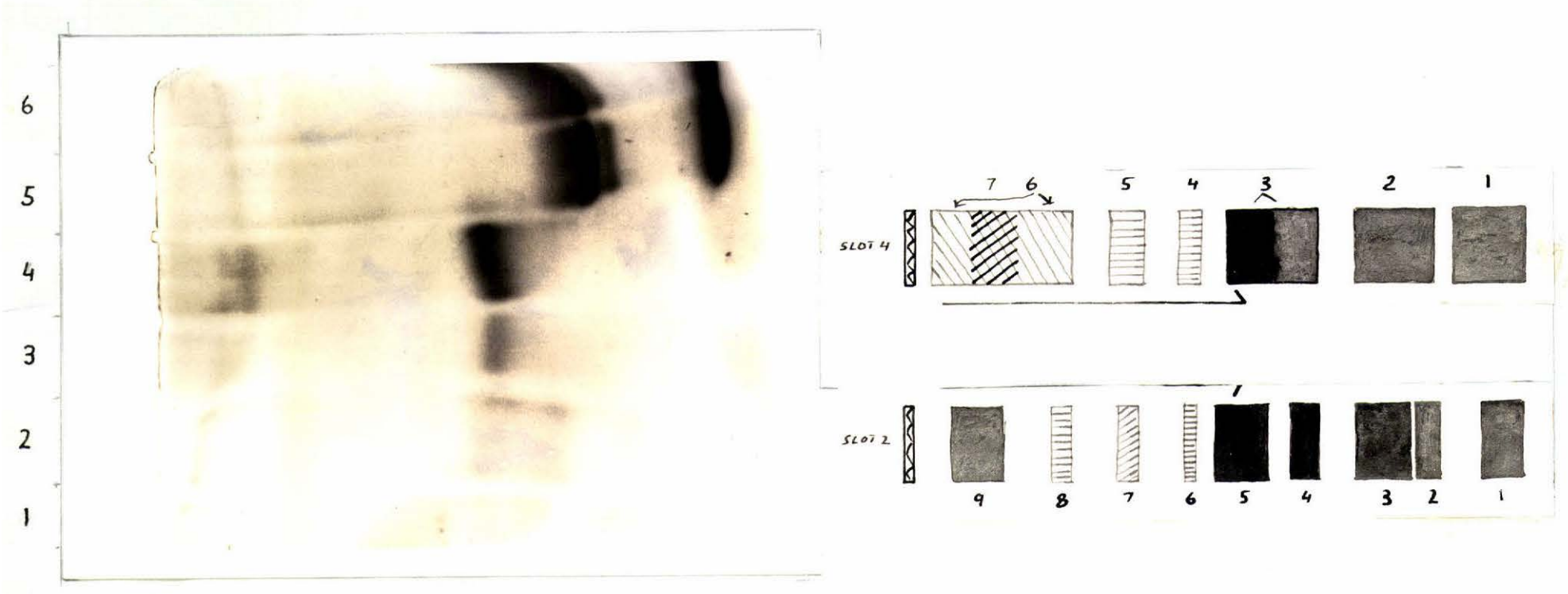
Commercial Rennet indicates 7-9 bands with two major ones about the middle of the plate (slot 2) and a tailing band near the slots.

The phosphatase preparation (slots 3 and 4) shows about 6-8 bands, with 1 major band in the centre of the plate, possibly accounting for over 60-70% of the preparation (purely on visual estimation), and another towards the slots.

Plate 4 shows a pattern of 1% and 3% rennet solution (containing 0.27% and 0.81% rennet respectively) in slots 1 and 2; 1% and 3% rennin solution (containing 0.32% and 0.96% rennin respectively) in slots 3 and 4, and 1.5% casein solution in slots 5 and 6.

The pattern for rennet has been described already. The concentrations used here was to get a comparison with rennin in regard to the depth of staining of the major bands.

PLATE 3

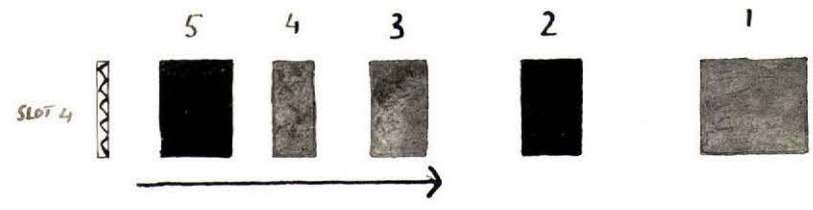


STARCH-GEL ELECTROPHORESIS OF CASEIN, ALKALINE MILK PHOSPHOMONOESTERASE (A.M.P.) AND COMMERCIAL RENNET (R^t.)

1 AND 2.–: R^t. (5·4%)
3 » 4.–: A.M.P. (1·0, 3·0%)
5 » 6.–: ACID CASEIN (1·5%)

16h, 130V, 6°C, BORATE AND TRIS-CIT. BUFFER pH 8·6

PLATE 4



STARCH-GEL ELECTROPHORESIS OF CASEIN, RENNIN AND COMMERCIAL RENNET (R^t)

1 AND 2 –: R^t (0·27, 0·81%) } SEE TEXT

3 » 4 –: RENNIN (0·32, 0·96%) }

5 » 6 –: ACID CASEIN (1·5%)

16h, 130V, 6°C, BORATE AND TRIS-CIT. BUFFER pH 8·6

The rennin preparation shows two major bands (2 and 5), one about the middle of the plate (slots 3 and 4) and the other near the slots. The marked variation in staining of the major zones between the rennet and rennin preparations at similar concentrations (both containing an almost similar salt concentration) indicates that considerable purification had been achieved. Three minor bands may be seen too.

It was thought possible that the two major zones seen in the rennin preparation could be in equilibrium with each other and that separation may have been due to their structural differences; the molecular sieving effect of the starch-gel coming into play.

If this was the case, then eluting each of the two zones from the unstained gel on the tray and running another electrophoresis on each should again give two zones at the same positions as in the first electrophoresis.

The following method was used: The top slice of the gel was stained and washed as usual (Plate 4). When the zones were visible, pieces of gel were removed from the gel tray from positions indicated by the two stained zones (2 and 5) on the top slice, allowance being made for the slight contraction which occurs in the washing of a stained gel. Due care was taken to prevent contamination from adjoining slot areas. The gel pieces were placed into Gooch porcelain crucibles and frozen at -15°C for 4 hours. At the end of the period the crucibles were placed in the centrifuge tubes and wired to the latter to keep them in position. When the gel pieces had thawed, the tubes were placed in the International Type C Centrifuge and centrifuged at 150 g for 15 minutes. Only a few drops of

the liquid were obtained in the tubes. A few drops of Tris citrate buffer was, therefore, added to the tubes to increase the volume. The solutions were then applied in the slots of a newly prepared starch-gel and electrophoresis run as usual (Plate 5).

Plate 5 shows an electrophoretic pattern of 1.5% casein solution (slots 1 and 2); eluant 5 (or zone 5 from the previous run - slot 3); eluant 2 (or zone 2 from the previous run - slot 4) and 0.32% rennin (slots 5 and 6).

It is clear from the Plate that both zones 2 and 5 separated again into 2 zones at the same positions as the original zones 2 and 5, as shown by the 0.32% rennin pattern (slots 5 and 6). It appears, therefore, that rennin may exist in two polymeric forms. It remains to be seen whether both have equal clotting and proteolytic activity.

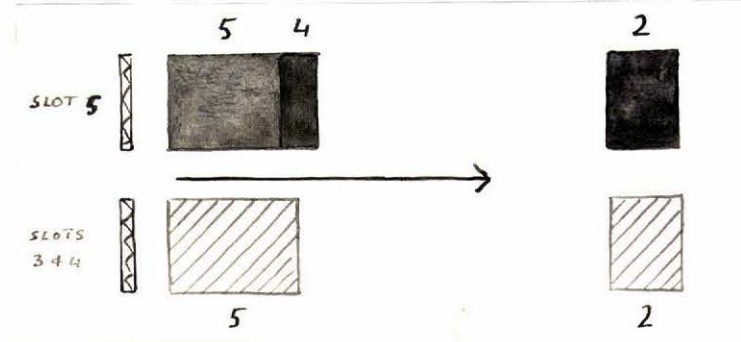
D. Filter-Paper Electrophoresis of Rennin

The electrophoresis was done in a veronal-buffer pH 8.6; ionic strength 0.1.

A 1.0% rennin solution was applied to the paper through a micropipette and electrophoresis was carried out at room temperature for 14 hours at 120V.

The rennin sample shows 2 major bands corresponding to the two major bands indicated in Starch-Gel Electrophoresis. The difference was that while the two bands moved very close together on Filter-Paper Electrophoresis, they were much further apart in Starch-Gel Electrophoresis (see Plate 4), confirming that separation on the latter was based on molecular sieving more than on charge differences. It appears that even on Filter-Paper Electrophoresis

PLATE 5



STARCH-GEL ELECTROPHORESIS OF CASEIN AND RENNIN

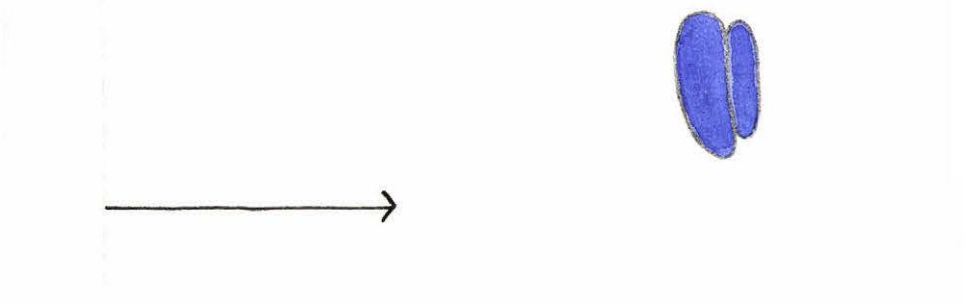
1 AND 2 –: ACID CASEIN (1·5%)

3 –: ELUTE '5' FROM PREVIOUS GEL
4 –: " '2' " " "
} SEE TEXT

5 " 6 –: RENNIN (0·32%)

16 h, 130 V, 6°C, BORATE AND TRIS-CIT. BUFFER pH 8·6

FILTER PAPER ELECTROPHORESIS OF RENNIN



FILTER PAPER ELECTROPHORESIS OF RENNIN AT ROOM TEMPERATURE IN VERONAL-ACETATE BUFFER ($pH\ 8.6, \mu = 0.1$) FOR 14 HOURS AT 120 V.

polymer separation is possible. No other minor bands were visible.

E. Electrophoresis on Rennin Treated Casein.

A 3.5% casein solution at pH 6.6 was treated with 8 mg rennin for 1 hour at an enzyme: substrate ratio = 1:87.5. It was then diluted in a Tris - 7M urea buffer to give a final concentration of 1.5%. In another sample, similarly treated by rennin, 0.1 N HCl was added to pH 4.6 to precipitate the caseinate, it was filtered and urea, to the extent of about 7 M, was added to the filtrate. Electrophoresis was done on the samples.

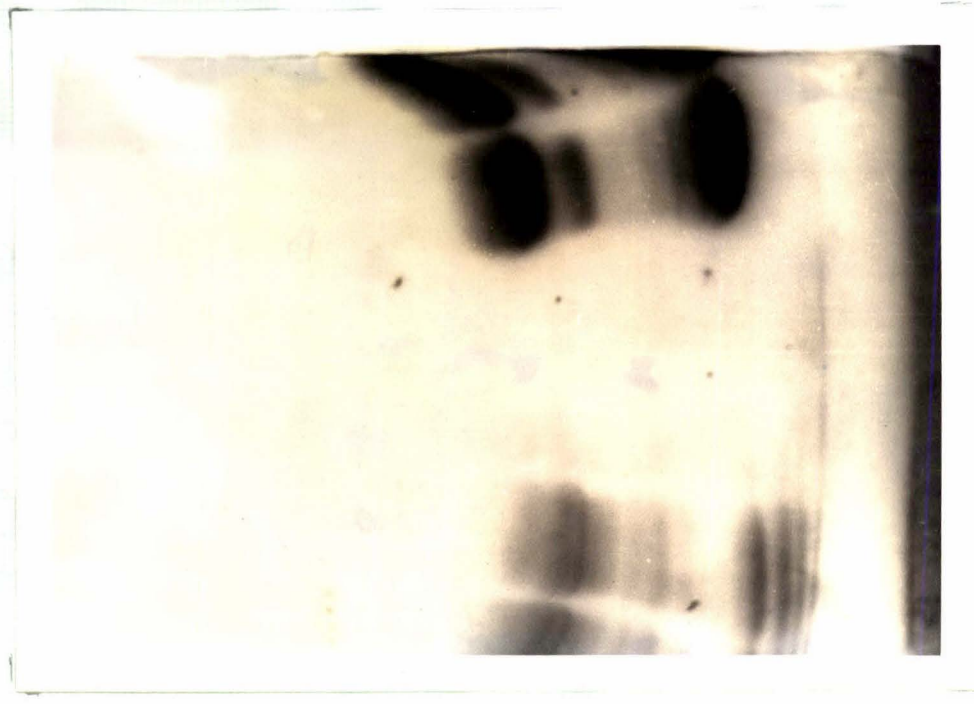
Plate 6 shows an electrophoretic pattern of 1.5% casein (similarly treated), but without rennin as above (slots 5 and 6) and rennin treated casein 1.5% (slots 1 and 2).

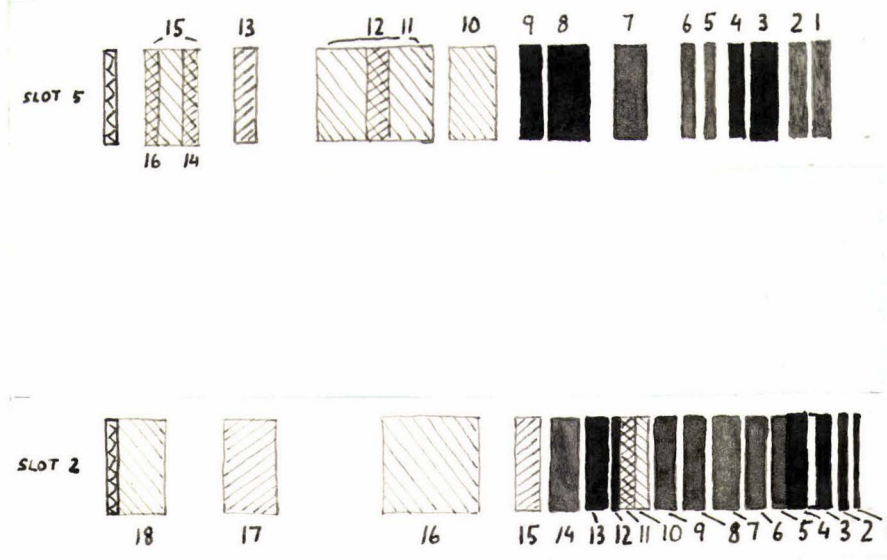
The pattern shows that some proteolysis has occurred and both the major bands of casein, thought to be α- and β-caseins (Neelin et al.) [41], have been attacked; the former to a greater extent.

Wake and Baldwin's [7] observation on the attack of rennin on κ-casein appears to have been confirmed, but it could still be seen after an hour's reaction with rennin contrary to their findings. κ-casein generally occupies a bread zone behind β-casein, midway between it and the slots.

The pattern also indicates that it would be impossible to distinguish as to which of the breakdown protein zones comes from which of the original casein components and that, unless the 'pure' casein components are treated separately with rennin and then analysed electrophoretically, deductions on the electrophoretic pattern of rennin treated casein, or for that matter on any proteolytic enzyme treated casein, is not possible. The picture may be

PLATE 6





STARCH-GEL ELECTROPHORESIS OF CASEIN AND P-CASEIN (16h, 130V, 6°C, BORATE AND TRIS-CIT. BUFFER pH 8·6)

1 AND 2 = P-CASEIN (1·5%)

3 " 4 = FILTRATE AFTER pH 4·6 PRECIPITATION OF P-CASEIN

5 " 6 = ACID CASEIN (1·5%)

further complicated by the fact that some breakdown products, whether from α or β -caseins, may run together on one occasion but separate slightly on another occasion, if conditions of electrophoresis are not absolutely constant, giving a false impression of increased proteolysis.

Discussion

The electrophoretic pattern for acid casein obtained in this study shows a good comparison with Wake and Baldwin's pattern [7]. The pattern need not be absolutely coincident to theirs. In fact, electrophoretic patterns of casein obtained by workers in different countries is not likely to be exactly the same as theirs. Several reasons are possible for this variation.

1. α_s, β and k -caseins can evidently form aggregates with themselves and with one another, the interaction being dependent on pH, temperature and ionic strength of the buffer used in analysis [55]. A lower pH towards the iso-electric point of the caseins increases interaction between α_s and β -caseins [55]. Increasing the pH and lowering the temperature reduces interaction between the casein components.

2. Different casein preparations, although made by the same method, appear to have different properties [55], possibly through "local pH variations during precipitation and dissolution procedures affecting aggregation and proportion of components in various preparations".

During the preparation of casein the temperature of the process appears important since a complexing between β-lactoglobulin and the α_s k-complex is possible (56). The complexing has been reported at temperatures over 65°C., but it is not known whether a complexing can occur at temperatures of 40°C or lower, normally used in casein precipitation (7). Also, some of the minor components may be lost during the preparation of acid casein.

3. Aschaffenburg (57) has shown that β-casein could be split into 3 bands on Paper Electrophoresis which he named A, B and C. These could appear as A and C, A and B, or B alone. Guernsey cows tended to give all 3 types. Jerseys showed only A and B. Breed differences, therefore, could account for differences in the electrophoretic pattern.

4. The same worker (57) also found 2 bands in the γ region which varied considerably in strength between individual cows, or may be absent in some cow's milk which appears to be a genetically controlled property. However, in pooled milk casein they should all be present in an electrophoretic pattern.

Points 2 and 3 above appear to be the only uncontrollable factors affecting the variation in electrophoretic patterns. Point 1 has been tackled in Starch-Gel Electrophoresis of casein (7), partly by using a high pH (8.6) and running the electrophoresis at low temperatures (2°C), but mainly by using concentrated urea in the gel composition (7).

Since the interaction between the casein components is mainly due to H-bonding and van der Waal's forces of attraction, Wake and Baldwin [7] incorporated urea to the extent of 7 M in their gel formula. Urea's hydrogen bond forming power [58] helps separate the different components. It also prevents the characteristic aggregation of denatured proteins through its dispersive action [7,59]. These properties of urea, combined with the molecular sieving effect of the starch-gel accounted for the separation of casein into the large number of components, not hitherto observed in any of the other electrophoretic processes.

The use of urea for the study of casein was possible because the latter bears no free sulphydryl group [60], and no new compound formation is possible through sulphydryl (-SH) and disulphide (-S-S-) reaction [61].

However, the separation of acid casein into 16-20 components in Starch-Gel Electrophoresis does not, as yet, mean that each component is a distinct protein entity. Some of them could be metastable polymers of a monomer - this has been discussed in Section III - (vi) with reference to Franglen and Gosselin's finding on bromocresol green [29] and confirmed in this work on the enzyme rennin (see Plates 4 and 5). Wake and Baldwin [7] did elute some bands from the gel and did not find any more separation into components in a second electrophoresis. However, there are 16-20 bands to account for and re-examination of 2-3 bands is not adequate proof that the rest of them are distinct protein entities. Waugh et al. [62] obtained α_s-casein free from k-casein and fractionated it on D.E.A.E. - cellulose to obtain 2 major components, as indicated on Starch-Gel Electrophoresis, naming them α_{s1} and α_{s2} respectively, in order of mobility. However, they state

that the adsorption-elution characteristics on D.E.A.E.-cellulose and the properties of the 2 components are so similar that they could be genetically related. Only Starch-Gel Electrophoresis revealed the 2 components. It was a pity these 2 components were not eluted from the starch-gel and re-run a second time to find whether each again separated into 2 components. A somewhat similar result to Waugh et al. has been found on the citrate condensing enzyme by Broder and Srere [88].

The incorporation of urea, while useful for disaggregating the casein complex, poses its own problem.

Urea forms inclusion compounds with fatty acids and other compounds containing C atoms linked in a straight chain [63, 64], mainly through hydrogen bonding. However, for inclusion compound formation, there must be at least 4-6 carbon atoms linked in a straight chain and the presence of a benzene ring in the chain inhibits 'inclusion'. The longest carbon straight chain is in lysine with 4 C-atoms, but the ε-NH_2 group is likely to prevent the formation of an inclusion compound, and further there should be enough benzene rings on the casein molecule to inhibit inclusion compound formation. Brown [64], however, states that a complexwith a 3-4 C-atom compound can occur at low temperatures. If a complexing does occur, it is not clear how the electrophoretic pattern will be affected. It is possible that the relative migration of the components, particularly the minor ones, may be affected depending on the extent of adduct formation.

Another more likely problem arises from the fact that aqueous urea solutions undergo isomeric transformation into ammonium cyanate [65] - equilibrium stage is reached very slowly at room and lower temperatures, but at 100°C it is reached in half an hour. An 8 M urea solution at equilibrium

may have 0.02 M ammonium cyanate and the cyanate reacts with any free $-NH_2$ group on the protein and possibly also with the -SH group [66, 67]. Prepared urea solutions held at refrigerator temperatures do not form cyanate, however[41], and freshly made urea solutions would also be free of cyanate [66]. Manson [68] found a definite reaction between the cyanate in aqueous urea solutions and α_s-casein. In Starch-Gel Electrophoretic studies of casein [7, 41], or its components [62, 69], no mention has been made as to whether cyanate formation is possible during the preparation of the Starch-Gel. This could be important since the urea (about 7 M) is added during the heating phase of gel preparation. For the de-aeration step, the gel solution is boiled and, subsequently, after it is poured on to the perspex trays, it remains warm for quite a period giving enough opportunity for a certain amount of cyanate formation.

The copper sulphate - pyridine test for cyanates [70] was carried out on the gel solution prior to its setting. The test was sensitive to a cyanate concentration of 1:20,000. Assuming the urea had attained complete equilibrium during the gel preparation, the amount of the gel solution used in the test would have had 3-5 times more cyanate than required to give a definite positive test. The test was negative. However, this cannot be taken as firm evidence for absence of cyanate, since the urea in the gel mixture would not be expected to attain full equilibrium with ammonium cyanate. Full equilibrium may have been obtained if it had been boiled for half an hour [65]. The cyanate concentration could easily have been 1/5th the amount at full equilibrium and this would not be detected by the test. A greater quantity of gel solution than used in the test causes turbidity and gives a deposit making the test even less sensitive.

Another point that needs clarification is that if cyanate is formed, would it react with the casein at refrigerator temperatures at which electrophoresis is carried out.

A further point worth mentioning is that even if the cyanate does react with the casein components, the addition to the molecular-weight of a component (which runs into thousands), due to the carbomylation reaction with the cyanate [71] is relatively small. Further, during the reaction, although the $-NH_2$ group of the protein is masked, a new $-NH_2$ group is added on. Consequently, the net charge on the protein may be little affected and likewise its electrophoretic mobility. However, if enzyme hydrolysates of casein are being studied, it may be imperative to prevent carbomylation, since one is dealing with breakdown products of lower-molecular-weight whose mobility could change depending on the extent of carbomylation.

Starch-Gel Electrophoresis has introduced a problem of nomenclature for the caseins. Wake and Baldwin [71] suggested that the distance moved by one of the casein components, which is always present in an electrophoretic pattern of casein, may be taken as 1 (i.e. band 1) and the distances moved by the other casein components may then be related to band 1, e.g., say, band 1 has moved 10 cm; another component may have moved 5 cm or 12 cm, and then it would be called 0.5 or 1.2 in relation to band 1. This is, however, not tenable when enzyme hydrolysates are being studied. For band 1, in a casein pattern, may disappear or change its position due to an enzyme attack. For a similar reason, it would be difficult to relate any band indicated on a hydrolysate pattern to any particular casein component, as is indicated by Plate 6. The obvious and ideal thing to do is to isolate and elute each individual casein

component (assuming each is a distinct protein entity) and then react it with the enzymes before re-examining the hydrolysates in electrophoresis. The Starch-Gel Electrophoretic method, as it stands to-day, is not capable of such an elaborate preparative procedure and, therefore, its use is limited to that of an analytical tool, mainly for the study of the proteins.

The classical nomenclature of casein based on Free-Boundary Electrophoresis, namely α, β and γ as elaborated by Mellander [51] still stands. To this may be added k-casein [54], in which case the original α-casein becomes α_s-casein. This nomenclature should be tenable until points raised in this discussion are clarified with regard to Starch-Gel Electrophoresis.

It was because of the uncertainities present in the method that it was thought advisable not to proceed with the study of enzymic hydrolysates of casein using Starch-Gel Electrophoresis, and efforts were concentrated on aspects of rennin's action on casein which is discussed in Part II of this thesis.

PART II

CHAPTER III

REVIEW OF LITERATURE ON THE ACTION OF RENNIN ON CASEIN

The nature of rennin coagulation of milk is not yet fully understood. At best, it is recognized that the mechanism can be resolved into two stages, namely, the enzymic conversion of casein into paracasein and the subsequent coagulation in the presence of calcium ions (72).

However, as early as 1929, Linderstrom-Lang (73) had suggested that one of the casein components acted as a 'protective colloid' and kept the other casein components in solution through its own stability to milk salts. The general evidence pointed to α-casein as being the protective colloid (72).

Berridge (74), however, stated that the protective colloid theory did not explain "the time required for the clotting phase, nor the dependence of the clotting on temperature". He put forward a denaturation hypothesis wherein it was postulated that proteolytic enzymes hydrolysed chemical bonds to give rise to unstable molecules which, at a sufficiently high temperature, denatured. The polypeptide chains of these degraded and native molecules then combined with one another, linking the micelles into a gel network. Evidence given by Berridge appears to support his hypothesis.

However, thoughts moved back to the 'protective colloid' theory with Waugh and von Hippel's (54) discovery of a new casein component which they termed k-casein. The k-casein appeared to be in complex with the α-casein (54,75) (now called α_s-casein); the weight ratio of the α_s-k complex suggested was 4:1 for micelle stabilization. The authors (54) concluded that k-casein was

the most important single factor responsible for micelle stabilization and was the protein on which rennin acted immediately.

Waugh [75] found that κ-casein lost 20% of its molecular weight due to rennin action and this was the only protein altered during normal rennin coagulation. Further, para κ-casein (term used for the rennin altered casein) was insoluble in the absence or presence of calcium ions in contrast to κ-casein which was soluble in the presence of 0.03 M calcium at about neutral pH. The weight ratio of 4:1 of α_s: κ suggested that the molecular ratio was 3:1 (M.W. of κ = 16,300; M.W. of α_s = 23,300)[75]; the linking of the molecules being through hydrogen bonds and non-polar side chains, which masked the phosphate groups on α_s-casein from calcium ions, and so prevented inter-complex links. Waugh [75] suggested a scheme for the α_s - κ casein complex wherein the α_s-casein occupied the three corners of a triangle with the κ-casein in the centre. One end of the κ-casein monomer was suggested as being outside the domain of the α_s - casein monomers and it was this end which rennin hydrolysed [75], giving rise to "insolubility of the para-κ-casein and decreased complex solubility".

It is difficult to contest Waugh's scheme for the α_s - κ casein complex but at the same time there is little evidence for it to be accepted unquestionably. The actual mechanism of clotting is still anybody's guess.

Nitschmann and his co-workers [76] found that about 1.5% non-protein nitrogen (soluble in 12% trichlor-acetic acid) and 4% N.P.N. (soluble in 2% T.C.A.) were rapidly split off from whole casein by rennin at 25°C. The 12% T.C.A. soluble nitrogen proved to be mainly a glyco-macropeptide of molecular-weight 6,000 to 8,000. This glyco-macropeptide was soluble at

pH 4.7 when the rest of the proteins were precipitated from the reaction mixture.

Wake [72] confirmed the release of 'glyco-macropeptide' and traced its origin to κ-casein. He suggested that the primary action of rennin on casein was to release the macropeptide from κ-casein, destroying the latter's micelle stabilizing properties. However, he found no evidence for the rupture of peptide bonds for the release of the macropeptide.

The issue, however, has become slightly confused by the suggestion of Beeby and Nitschmann [77], and Beeby [78] that κ-casein was not a single protein but a complex, and that the action of rennin was first to open the secondary bonds responsible for the stability of the complex [77]. Further, the glyco-macropeptide recognized earlier [72,76] came from one of the κ-casein complex components which was soluble at pH 4.7 and was probably co-valently bound to this component. This pH 4.7 soluble component of the κ-casein complex was also obtained by urea treatment or by repeated iso-electric precipitations of κ-casein. Beeby [78] calculated the molecular-weight of the κ-casein 'complex' as being 50,000 and, basing it on Waugh's value of 16,000 given to κ-casein [75], he suggested that the complex consisted of 3 units of 16,000 each.

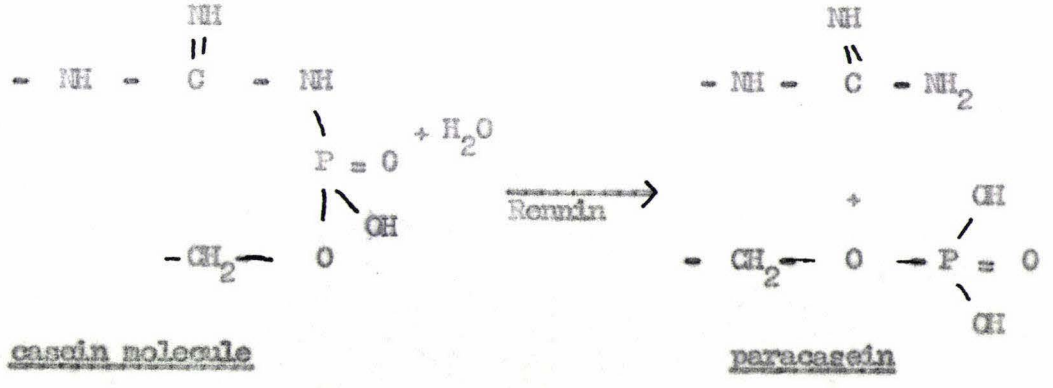
However, it is not clear whether the κ-casein 'complex' exists as such in the casein micelles and Waugh's scheme [75] for the α_s-κ casein complex needs to be reviewed again after clarification of the above point.

While workers, in general, have concentrated on the 'protective colloid' theory over the last few years, Dyachenko [79] in 1959 proposed the theory of phosphoamidase action of rennin for clotting. Based on electrometric

titration he showed that the isoelectric point of casein lay between pH 4.6 to 4.7, while that of paracasein was between pH 5.0 to 5.2. He also showed that the essential difference between the titration curves for the two caseins lay in the "divergence in the strongly alkaline region starting from pH 10.2 when the ε-NH_2 groups of lysine and the guanidine groups of arginine exhibit their buffering effect". Since lysine amino groups have been shown not to increase on rennin action (80), the appearance of strongly alkaline groups in paracasein was attributed to arginine guanido groups (79). Dyachenko (79) postulated that rennin splits the -P-N- link between the phosphoric acid and the guanido group of arginine, exposing the hydroxy groups on the former to react with calcium ions to form a coagulum.

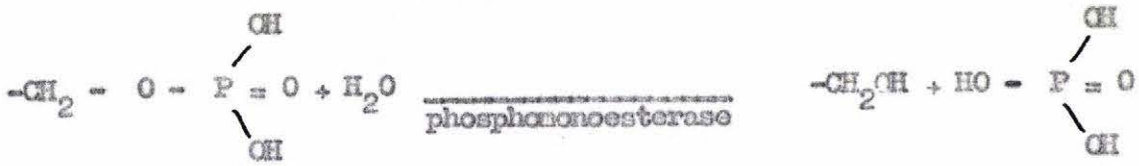
It was decided to make a study of Dyachenko's postulation by using purified rennin and alkaline milk phosphomonoesterase enzymes. The principle of the possible reactions involved are given below:-

A.



casein molecule paracasein

B.



paracasein orthophosphate

Reaction (A) is as per Dyachenko's [79] postulation.

In Reaction (B) it was hoped to release the orthophosphate from the paracasein molecule by the use of alkaline milk phosphomonoesterase.

CHAPTER IV

A STUDY OF THE ACTION OF RENNIN ON THE CASEINATE SYSTEM OF MILK USING PURIFIED RENNIN AND ALKALINE MILK PHOSPHOMONOESTERASE ENZYMES.

Section I:

MATERIAL AND METHODS

A. Preparation of Rennin from Commercial Rennet.

The method of Ernstrom (43) was followed. The last three steps (6, 7 and 8) (43) were, however, eliminated since they were not considered worthwhile for the very small increase in specific activity obtained by following them. For centrifuging, an International Refrigerated Centrifuge was used; the temperature being set at 5°C.

The commercial rennet was supplied by the N.Z.Rennet Co.Ltd., Eltham.

At the end of step 5, the rennin was dissolved in a minimum quantity of distilled water and spread over a stainless steel tray, which was then put in the deep freeze for 24 hours to be frozen. It was then freeze-dried for 48 hours until quite powdery, transferred to a glass bottle and held at -15°C in the deep freeze for the rest of the experimental period.

Starch-Gel Electrophoresis (7) was done on the rennin sample and the result has already been reported in Chapter II, Section IV (C) of Part I of this thesis. Apart from the two major bands (Plate 3) which appear to be in equilibrium with each other (Plate 4), three other minor bands are also reported. This heterogeneity was not surprising since even rennin

crystals showed 4 or 5 electrophoretic peaks [43] in Free-Boundary Electrophoresis. Ernstrom [43] has listed examples which have more than one electrophoretic form of an active enzyme.

Commercial rennet indicates 7 - 9 bands in Starch-Gel Electrophoresis (see Plate 3).

Salt (NaCl) analysis by the method of Volhard [82] indicated that the freeze-dried rennin and commercial rennet samples contained 68% and 73% salt respectively.

B. Preparation of Alkaline Milk Phosphomonoesterase

The method of Morton [42] was followed, but only up to the dialysis stage in step 4.

Fifty percent fat cream was separated from fresh, bulk, unheated milk supplied to the Dairy Research Institute (N.Z.) factory. Distilled water was added to reduce the fat content to 40%. After overnight holding at 7°C, the cream was churned in a small laboratory type churn (42 lbs. cream, 40% B.F. ——→ 13 churnings) at a temperature of 10°C. Two washes with small quantities of chilled water (10°C) were usually given after each churning. The buttermilk and washings were preserved for the isolation of the enzyme.

The rest of the method was as per Morton [42]. After dialysis in step 4, the solution was frozen in stainless steel trays at -15°C for 5 hours and then freeze-dried for 48 hours. The dried product was transferred to glass bottles and held in the deep freeze at -15°C for the rest of the experimental period.

Micro-Kjeldahl nitrogen determination [81] indicated that the buttermilk, plus washings, had a N content of 3.6 mg/ml. and for the enzyme solution, after the dialysis stage in step 4, it was 0.22 mg/ml.

To determine the specific activity, the Aschaffenburg-Mullen test (Tramer and Wight's modification) [84] was undertaken. Liberation of p -nitrophenol was followed by an All-Purpose Lovibond Comparator and Disc. The dilution of the sample to give a liberation of 18 μg p-nitrophenol in ten minutes was determined.

For buttermilk this dilution was 10^{-2}, i.e. the enzyme concentration in a 10^{-2} dilution of the buttermilk gave a liberation of 18μg p-nitrophenol in 10 minutes (i.e. 1,800μg/ml. of the neat solution). The specific activity for buttermilk was:

$$\mu g/mg \text{ N per mg N.} \quad \text{or} \quad \frac{1800}{3.6} = 500 \text{ units}$$

The enzyme solution gave a reading of 18 at a dilution between 10^{-2} and 10^{-3}, i.e. at neat concentration 1800 - 18000μg p-nitrophenol would have been liberated. An arbitrary figure of 8,000 was selected as being the nearest.

$$\text{Specific activity} \quad \therefore \quad \text{was} \quad \frac{8000}{0.22} = 33,363$$

$$\text{Purification achieved} = \frac{33,363}{500} = 66\text{-}67 \text{ fold}$$

Starch-Gel Electrophoresis [7] of the phosphomonoesterase indicated two major and 4-5 minor bands (see Plate 3 in Chapter II, Section IV (C) of Part I of this thesis).

It is not known whether all or only one of them have phosphomonoesterase activity, or whether the two major components are in equilibrium with each other, as was the case with rennin.

C. Preparation of Acid Casein

Details of the preparation have already been given elsewhere (Part I, Chapter II).

Analysis indicated that the ash content was 2.0% and the moisture content was 1%.

Starch-Gel analysis indicated 15-17 components which was comparable to the results of Wake and Baldwin [7] and Neelin et al.[41] (see Plate 2 in Chapter II, Section IV (B) of Part I of this thesis).

Phosphorus analysis of the acid casein indicated 0.74% P; a lower figure than normally given by various workers (about 0.8%). The determination of phosphorus (as P_2O_5) was based upon methods prescribed in the Fertilizers and Feeding Stuffs Regulations, 1960, Seventh and Eighth Schedules, and the method sheet (No.60) was supplied by UNICAM Instruments Ltd., York Street, Cambridge, U.K. [83]. The method for determination of phosphoric acid in feeding stuffs was followed.

D. Determination of Liberated Phosphate from Casein*

(i) Preparation of casein solution

A paste was prepared by mixing a weighed quantity of the freeze-dried acid casein with a small quantity of distilled water and any large lumps were broken down by means of a glass rod. This helped to give a more even and quicker dissolution of the casein subsequently.

* By 'casein' will be meant the whole caseinate complex in this thesis.

More distilled water was then added to disperse the casein. The required quantity of 0.1N NaOH was added dropwise through a burette; the casein dispersion being constantly stirred by means of a magnetic stirrer. The solution at a pH of 6.75 was then made up to the desired volume by distilled water. The final pH was generally between 6.70 to 6.75 at room temperature. During the final stages of 0.1N NaOH addition, nisin [85] (supplied by Aplin and Barret Ltd., Yeovil, England), an antibiotic, was added to the casein solution at a final concentration of about 100 p.p.m. Nisin did not affect the enzymes rennin or phosphomonoesterase, as determined in the milk and the Aschaffenburg-Mullen test respectively. Thus, 20 mls. of pasteurized milk containing 100 p.p.m. nisin and about 20 μg rennin, coagulated in about 5-10 minutes, which was similar to a control sample not containing nisin, and a 0.01 mg per ml. solution of the phosphomonoesterase gave a reading of 18+ in 10 minutes on the Lovibond Comparator whether nisin (100 p.p.m.) was present or not. The tests have no quantitative significance.

(ii) Determination of liberated phosphate

The general technique developed is described below.

Twenty mls. of a 3.5% casein solution (pH 6.7) was taken in 15 x 2 cm pyrex test tubes, which were held by clamps in a water-bath maintained at 38°C. When either rennin or commercial rennet was tested alone on casein they were usually added after solubilizing in distilled water; 5 mls. of the enzyme solution in distilled water being added to the casein solution to give a final volume of 25 mls. in the reaction tubes. After the desired time, the reaction was stopped by pouring the test tube contents into a stoppered measuring cylinder containing 25 mls. vanadium molybdate [83]. The latter

served a threefold purpose, namely, precipitating the casein, inactivating the enzyme and reacting with the liberated orthophosphate to give the soluble, orange coloured vanadium-phosphomolybdate complex. The test tubes were washed three times with distilled water into the measuring cylinder and the final volume in the latter was made up to 75 mls. The whole was then filtered in a Büchner funnel using retentive filter-paper, e.g. Whatman No.42 or 3.

The samples were usually set aside for 2 to 4 hours for full colour development. It was found that using twice as much vanadium molybdate gave a much quicker colour development, although orthophosphate liberation with reference to a suitable standard was similar to that of a sample treated with 25 mls. of vanadium molybdate. However, due to the high acidity in the molybdate reagent it was considered advisable to use the smaller quantity.

It was found necessary to re-filter the solutions before examining in the spectrophotometer for percentage transmission measurements, since part of the casein degradation products were precipitated only later after prolonged contact with the molybdate reagent due to slow aggregation. Repeated filtrations between 4 to 24 hours after colour development of a sample did not appreciably affect the final percentage transmission reading.

The clear orange coloured solutions were examined for percentage transmission in the Beckman Model D.U.Spectrophotometer at 420 mμ using a standard phosphate solution containing 0.1 mg P_2O_5 / 100 mls. solution as a reference (100% transmission) (83). Percentage phosphorus liberated was then calculated from the standard curve (Figure 1 - see later). When necessary, the samples were diluted suitably so that percentage transmission figures could be

related to the standard curve. A fresh reference standard was prepared for each series of readings on the spectrophotometer.

When phosphomonoesterase had to be incorporated in the reaction mixture a slight modification was necessary.

To 20 mls. of a 3.5% casein solution (pH 6.7) the desired quantity of rennin or rennet was added as a powder, using a camel hair brush to minimize the trace of rennin or rennet left behind in the weighing boat. At the appropriate time the phosphomonoesterase was added as a solution in 5 mls. of double strength $Na_2CO_3/NaHCO_3$ buffer [84]. This increased the pH to 9.5 when rennin or rennet would be inactivated, whereas this pH was about the optimum for phosphomonoesterase for most substrates [42]. The reaction was stopped as before in 25 mls. of vanadium molybdate and phosphate liberation measured in the spectrophotometer.

In reactions where casein was first reacted with phosphomonoesterase, and then with either rennin or commercial rennet, the procedure was further modified.

Fifteen mls. of 4.66% casein solution in double strength carbonate buffer at pH 9.5 was taken in the reaction tubes. To this was added 5 mls. of the phosphomonoesterase solution in the same buffer. After the appropriate time interval, 5 mls. of approximately 0.2 N HCl was added to the tubes and mixed well with the contents by inversion of the tube. After each inversion the cork had to be removed to let out the CO_2 gas formed, due to acid/carbonate reaction. The pH dropped to 6.65 to 6.70 and rennin or rennet was then added as a powder. The final volume and the casein concentration was the same as in the other reactions. The rennin or rennet powder could not be added with the acid since it had to be made certain that the pH of the reaction mixture was

below 8.5 before they were introduced to reduce inactivation of the enzymes.

Where casein was reacted with phosphomonoesterase only, the enzyme was dissolved in 5 mls.of distilled water and added to 20 mls.of a 3.5% casein solution at pH 6.7. If the reaction was at pH 9.5, the casein solution was made in the double strength buffer and to 20 mls.(3.5% casein) was added 5 mls.of phosphomonoesterase solution in the same buffer. The reaction in all cases was stopped as before in 25 mls.vanadium molybdate and phosphate liberation measured in the spectrophotometer. Control samples were given similar treatments without the enzymes.

Use of double strength carbonate buffer did not appear to affect the activity of the phosphomonoesterase enzyme on the synthetic substrate disodium p-nitrophenyl phosphate.

Section II:

EXPERIMENTAL AND RESULTS

Experiment 1: Plotting of a Calibration Graph for Phosphorus Determination

The calibration graph was drawn by plotting percentage transmission on the abscissa against known amounts of P_2O_5 (in mg/100 mls. solution) on the ordinate. The method was based on the Seventh and Eighth Schedules of the Fertilizer and Feeding Stuffs Regulations (83). The concentrations of P_2O_5 were scaled down 50 times to suit the experimental conditions.

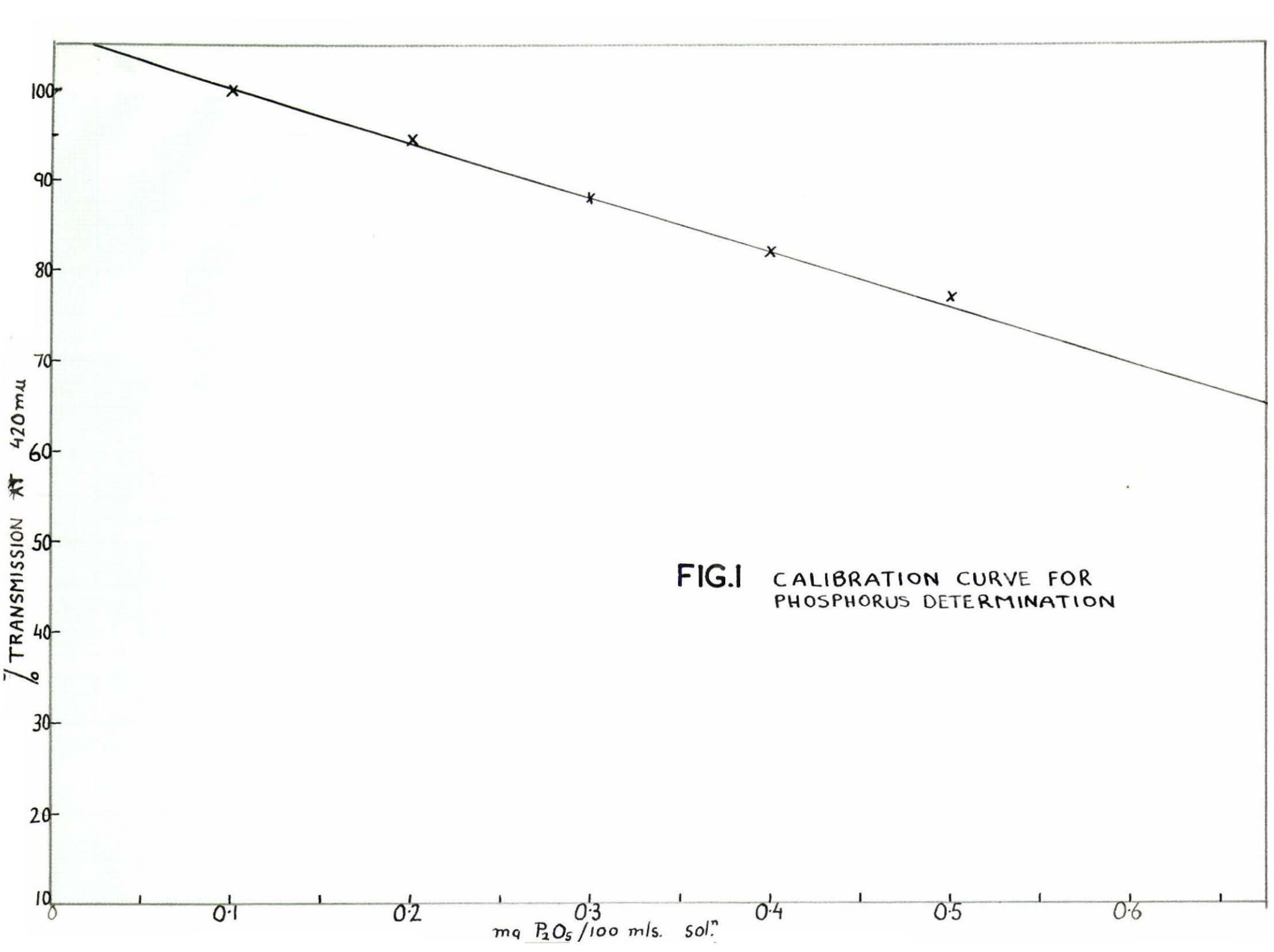


FIG.1 CALIBRATION CURVE FOR PHOSPHORUS DETERMINATION

TABLE I

Percentage transmission of solutions containing known amounts of P_2O_5.

Sample	mg P_2O_5/100 mls. solution	% Transmission at 420 mμ
1	0.1	100
2	0.2	94.5
3	0.3	88
4	0.4	82
5	0.5	77

Result:- A straight line was obtained on the graph (Figure 1). In subsequent experiments, phosphorus liberation was calculated with reference to this calibration curve.

Experiment 2:

Liberation of Phosphate from Casein by Rennin/Rennet and Phosphomonoesterase used individually or together

Details of the technique have already been given elsewhere. Twenty-five mls. of the reaction mixture contained 700 mg casein and about 5.2 mg phosphorus (or 12 mg P_2O_5 equivalent) associated with the casein; 13.5 mg rennet or 8.0 mg rennin (taking NaCl content into account), and 6 mg alkaline milk phosphomonoesterase whenever used. Enzyme:substrate ratios were 1:51.9, 1:87.5 and 1:116.7 respectively (see Table II).

The rennin or the rennet acted on the casein for 1 hour, while the phosphomonoesterase acted for 30 hours. Total reaction time was 31 hours. The extended reaction time for the phosphomonoesterase was based on Perlman's finding (86) that phosphomonoesterases acted only slowly on unfractionated casein.

TABLE II

Liberation of Phosphate from Casein by Rennin/Rennet and Phosphomonoesterase used individually or together.

Sample	Reaction		% Transmission at 420 mμ		P_2O_5 ≡ mg/100 mls.		P_2O_5 ≡ mg/75 mls.		% P (of total) liberated (0.7 g casein has 12 mg P_2O_5 ≡)	
1	C 6.7/1h + 5 mls.D.S.B./30h.		105	106	-	-	-	-	-	-
2	C 9.5/30h + 5 mls.0.2N HCl/1h		105	105	-	-	-	-	-	-
3	R^T + P9.5/30h		104	103	0.03	0.05	0.023	0.038	0.19	0.32
4	R^N + P9.5/30h		105	104	-	0.03	-	0.023	-	0.19
5	C 6.7/1h + P9.5/30h		105	104	-	0.03	-	0.023	-	0.19
6	C 6.7+R^T/1h+ 5 mls.D.S.B./30h.		103	102	0.05	0.07	0.038	0.053	0.32	0.44
7	C 6.7+R^N/1h+ 5 mls.D.S.B./30h.		73	75	0.55	0.52	0.41	0.39	3.4	3.3
8	C 6.7+R^T/1h+ P9.5/30h		103	104	0.05	0.03	0.038	0.023	0.32	0.19
9	C 6.7+R^N/1h+ P9.5/30h		76	74	0.50	0.53	0.38	0.40	3.2	3.3
10	C 9.5+P/30h+ R^T6.7/1h	*	105	103	-	0.05	-	0.038	-	0.32
11	C 9.5+P/30h+ R^N6.7/1h	*	77	76	0.48	0.50	0.36	0.38	3.0	3.2

NOTE:

C = 3.5% casein solution; 6.7 and 9.5 refer to reaction pH; P = phosphomonoesterase; R^N = rennin; R^T = rennet; D.S.B. = double strength carbonate buffer pH 9.5; 1h and 30h refer to time in hours.

* Numbers in red in the main table represent results from a similar but completely independent experiment.

* Rennet and rennin were added in powder form, after 5 mls 0.2 N HCl was added to reaction tubes (see Section I).

Rennin and rennet concentrations indicated have been compensated for salt concentrations.

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Results:

Table II clearly indicates that alkaline milk phosphomonoesterase has little or no effect on the release of 'phosphorus ' from casein. But the interesting result was that rennin itself had liberated 3.0% to 3.4% of the total phosphorus, which was surprising, because of the view held that rennin had no phosphomonoesterase activity (79). Commercial rennet, although used in a much higher quantity (13.5 mg) than rennin, had only a minor effect on 'phosphorus ' liberation, explained wholly by the very low concentration of rennin present in it. This unexpected result with rennin complicated matters as far as Dyachenko's postulation (79), that rennin splits only the N-P linkage, was concerned. It appeared probable that the phosphate group acted as a link between adjacent casein chains and that the bonds hydrolysed by rennin to release the orthophosphate (for which the vanadium molybdate reagent is specific) were other than -O-P- bonds. This point was investigated on synthetic substrates.

Experiment 3:

Phosphatase activity of Rennin, Commercial Rennet and Alkaline Milk Phosphomonoesterase on Synthetic Substrates.

(i) Test on di-sodium p-nitrophenyl phosphate

The modified Ashaffenburg-Mullen phosphatase test (84) was carried out on the three enzymes; the concentrations used being the same as in Experiment 2.

The alkaline test (pH 9.6) was done in the usual carbonate buffer and the acid test (pH 6.2) was done in $Na_2 H PO_4/KH_2PO_4$ buffer (82). Suitable controls were used as reference.

Observations at 2 and 18 hours indicated definite alkaline and acid phosphatase activity in the commercial rennet powder, but rennin did not show activity at either pH. The activity of milk phosphomonoesterase at the alkaline pH was easily the strongest and even at pH 6.2 its acid activity was stronger than the commercial rennet powder.

(ii) Test on glucose-1-phosphoric acid (di-potassium salt)

One-hundred mg of the salt was dissolved in about 50 mls. of distilled water.

Fifteen mls. of the solution was taken in each of three test-tubes. Rennin and commercial rennet powders were added to two (using a similar concentration as in Experiment 2) after dissolving in 5 mls. of distilled water, while in the third the milk phosphomonoesterase was added in 5 mls. carbonate buffer (pH 9.6). Reactions were carried out at 38°C for 1 hour, after which the tube contents were poured into a stoppered measuring cylinder containing 25 mls. vanadium molybdate. The volume in the cylinder was made up to 75 mls.

The rennin preparation showed no phosphatase activity. Commercial rennet showed some, whereas the phosphomonoesterase preparation appeared to release all the phosphoric acid from the salt during the hour. The high acidity of the vanadium molybdate eventually liberated all the phosphoric acid from the glucose salt in the rennin and rennet treated samples when held overnight at room temperature. The acidity, however, did not cause trouble with casein as indicated by the control readings in Experiment 2.

Results:

The qualitative experiment on synthetic substrates indicates that the mode of action of rennin on casein (Experiment 2), compared to that of milk phosphomonoesterase on the synthetic substrates in this experiment was different, although the orthophosphate was liberated in both cases. This difference in action was possibly due to a different type of bond present on the casein as compared to an -O-P- bond in the synthetic substrates.

Rennin, therefore, appears to have no phosphomonoesterase activity on synthetic substrates in which the phosphate is known to be linked by ester type covalent bonds, but can liberate the 'phosphate' from casein (Experiment 2). The latter aspect was studied further, as described in the following experiments. Further studies on the commercial rennet were discontinued.

Experiment 4:

The Phosphatase Action of different Rennin Concentrations on Casein at a Fixed Reaction Time (1 hour).

3.5% casein solution at pH 6.7 was prepared, as described earlier. To 20 mls. was added 5 mls. of rennin solution in distilled water. Reaction was stopped in vanadium molybdate as usual.

Rennin concentrations of 0.32, 0.64, 0.96, 1.92, 3.84 and 8.0 mg were used giving enzyme:substrate ratios of 1:2188, 1:1094, 1:728, 1:364, 1:182 and 1:87.5 respectively. (See Table III)

TABLE III

The Phosphatase Action of different Rennin Concentrations on Casein at a Fixed Reaction Time (1 hour)

Sample	Reaction	% Transmission at 420 mμ		P_2O_5 ≡ mg/100 mls.		P_2O_5 ≡ mg/75 mls		% P(of total)liberated (0.7 g casein has 12 mg P_2O_5 ≡)	
1	C 6.7 + 5 mls.dist.water/1h.	105	105	-	-	-	-	-	-
2	C 6.7 + R^N 0.32mg/1h	103	106	0.05	-	0.038	-	0.32	-
3	C 6.7 + R^N 0.64mg/1h	100	103	0.1	0.05	0.075	0.038	0.63	0.32
4	C 6.7 + R^N 0.96mg/1h	100	101	0.1	0.08	0.075	0.06	0.63	0.5
5	C 6.7 + R^N 1.92mg/1h	95	97	0.18	0.15	0.135	0.113	1.1	0.94
6	C 6.7 + R^N 3.84mg/1h	85	87	0.35	0.31	0.264	0.23	2.2	1.9
7	C 6.7 + R^N 8.0mg/1h	72	75	0.57	0.52	0.427	0.39	3.6	3.3

NOTE:

C = 3.5% casein solution; R^N = rennin; 6.7 = reaction pH; 1 h = 1 hour.

Rennin concentrations indicated have been compensated for salt concentration.

Values in red in the main table represent results from a similar independent experiment.

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Results:

Phosphorus liberation at the end of 1 hour was variable at the lower enzyme concentrations 0.32 to 0.96 mg (enzyme:substrate ratios between 1:2188 to 1:728). Due to the small quantity being liberated, small variations in technique would magnify variations in the calculated phosphorus liberation values. In an independent experiment it was found that 0.32 mg rennin (E:S = 1:2188) liberated 3.1% of the total phosphorus in casein in 52 hours. Because of the time factor involved, it was decided that further studies on the phosphatase action of rennin on casein be limited to the higher enzyme concentration, i.e. 1.92, 3.84 and 8.0 mg (E:S of 1:364, 1:182 and 1:87.5 respectively).

Phosphorus liberation of 3.3% to 3.6% for the 8 mg rennin concentration compares favourably with results obtained in Experiment 2 (3.0% to 3.4%).

Experiment 5:

Phosphatase action of Rennin using three Enzyme:Substrate Ratios at varying Reaction Times.

The experiment was conducted on similar lines as before, using 20 mls. of 3.5% casein solution at pH 6.7 and adding rennin dissolved in 5 mls. distilled water. 1.92, 3.84 and 8.0 mg rennin concentrations were used (E:S of 1:364, 1:182 and 1:87.5 respectively).

TABLE IV

Phosphatase Action of Rennin using three Enzyme:Substrate Ratios at varying Reaction Times

Sample	Reaction	* Dilution factor.	% Transmission at 420 mμ	P_2O_5 ≡ mg/100mls	Corrected for dilution.	P_2O_5≡ mg/75mls	% P (of total) liberated. (0.7 g casein has 12mg P_2O_5 ≡
1	C6.7 + 5 mls.dist.water/1h	-	109	-	-	-	-
2	C6.7 + R^N 1.92mg/1h	-	102	0.07	-	0.053	0.44
3	C6.7 + R^N 3.84mg/1h	-	94	0.20	-	0.15	1.3
4	C6.7 + R^N 8.0 mg/1h	-	77	0.48	-	0.36	3.0
5	C6.7 + 5 mls.dist.water/2h	-	108	-	-	-	-
6	C6.7 + R^N 1.92mg/2h	-	91	0.25	-	0.19	1.6
7	C6.7 + R^N 3.84mg/2h	-	79	0.45	-	0.34	2.8
8	C6.7 + R^N 8.0 mg/2h	-	62	0.73	-	0.55	4.6
9	C6.7 + 5 mls.dist.water/4h	-	110	-	-	-	-
10	C6.7 + R^N 1.92mg/4h	-	73	0.55	-	0.41	3.4
11	C6.7 + R^N 3.84mg/4h	2	80	0.43	0.86	0.65	5.4
12	C6.7 + R^N 8.0mg/4h	2	69	0.61	1.22	0.92	7.7
13	C6.7 + 5 mls.dist.water/24h	-	110	-	-	-	-
14	C6.7 + R^N 1.92mg/24h	3	84	0.37	1.11	0.83	6.9
15	C6.7 + R^N 3.84mg/24h	3	79	0.45	1.35	1.01	8.4
16	C6.7 + R^N 8.0 mg/24h	3	76	0.50	1.50	1.13	9.5

NOTE:

C = 3.5% casein solution; R^N = rennin; 6.7 = reaction pH; 1h, 2h, 4h, 24h = time in hours.

* Dilution factor = samples diluted 2 or 3 times original volume before reading % transmission.

Rennin concentrations indicated have been compensated for salt concentration.

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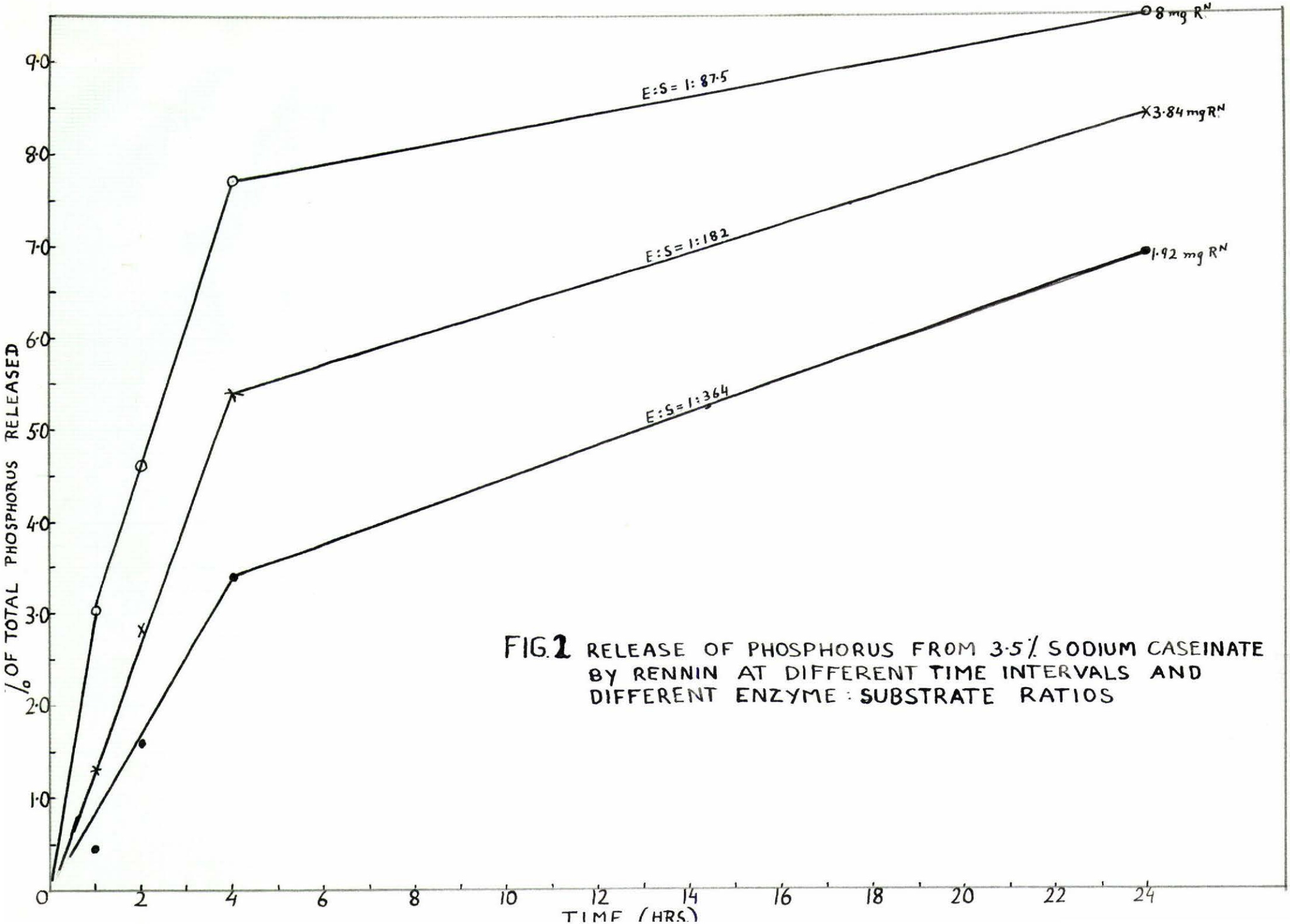

FIG. 1 RELEASE OF PHOSPHORUS FROM 3·5% SODIUM CASEINATE BY RENNIN AT DIFFERENT TIME INTERVALS AND DIFFERENT ENZYME : SUBSTRATE RATIOS

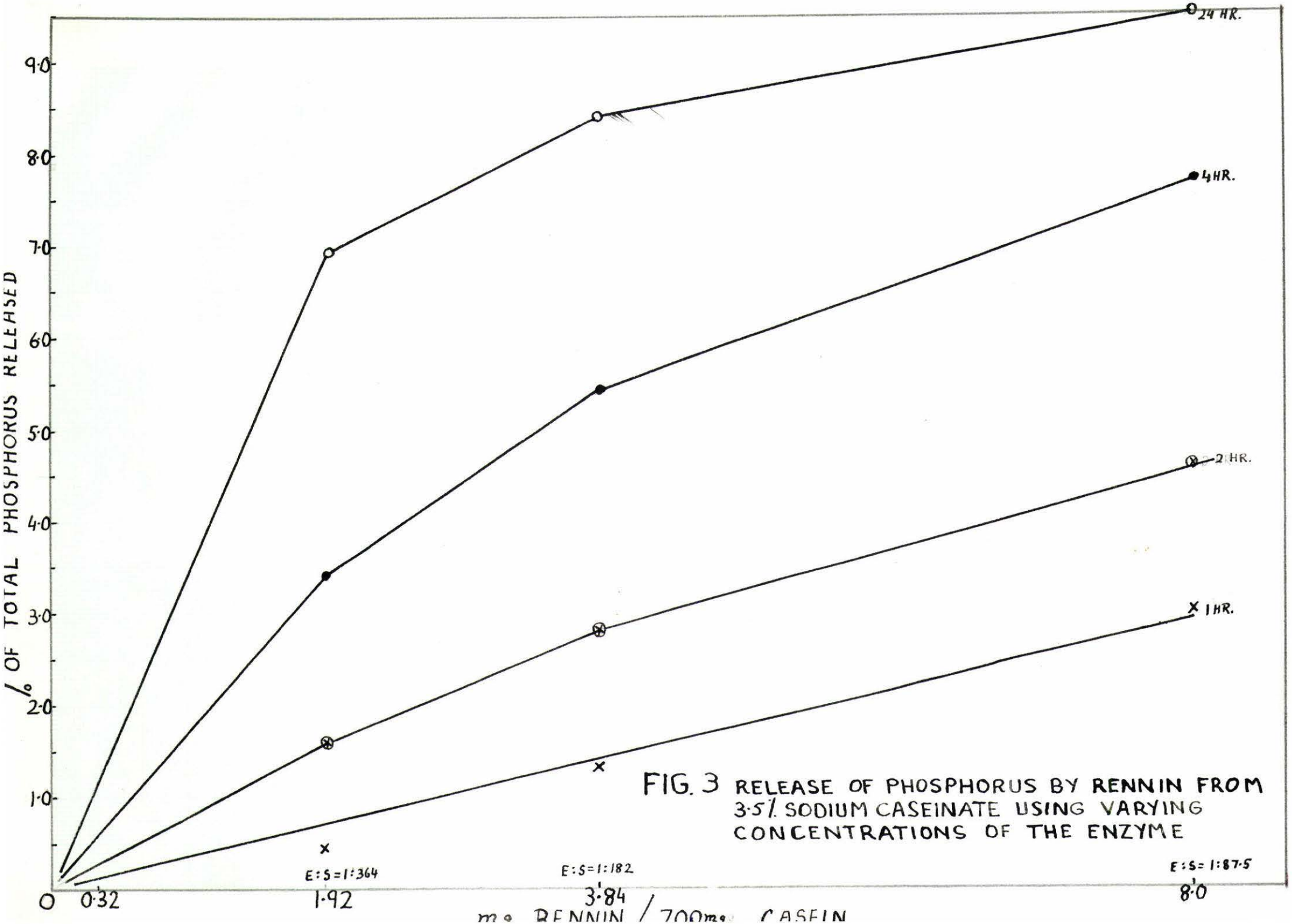

FIG. 3 RELEASE OF PHOSPHORUS BY RENNIN FROM 3·5% SODIUM CASEINATE USING VARYING CONCENTRATIONS OF THE ENZYME

Results:

Percentage (of total) phosphorus liberation figures given in Table IV have been plotted against time in Figure 2 and against rennin concentrations at the different reaction times in Figure 3.

Figure 2 shows that in the early stages of the reaction with 1.92, 3.84 and 8.0 mg rennin, the reaction follows zero order kinetics (for approximately 4 hours), after which first order kinetics are followed. At the end of 24 hours, all the 3 curves are still rising, although the steepness of the rise diminishes with increasing rennin concentration, explained by first order kinetics, namely, that at any given time there is less of the substrate available to the higher rennin concentration as compared to the lower concentration.

It was mentioned under Experiment 4, that in an independent experiment 0.32 mg of rennin (E:S = 1:2188) had liberated about 3.1% of the total phosphorus from a 3.5% casein solution in 52 hours. In Figure 3, the 24-hour curve indicates that 0.32 mg rennin would have liberated about 1.2% to 1.4% of the total phosphorus in 24 hours, which compares favourably with the 3.1% liberated in 52 hours.

Figure 3 confirms that up to almost 4 hours the liberation of 'P' followed first order kinetics and at the 24-hour level only those tubes containing 0.32 to 1.92 mg rennin would follow zero order reactions.

Experiment 6:

Phosphatase action of Rennin on a 1% Casein Solution using Enzyme:Substrate Ratio of 1:25.

The previous experiment indicated that given time, more than 9.5% of the total phosphorus could be liberated from casein (see Figure 2). In this experiment, the enzyme:substrate ratio was set at 1:25 to find out whether this increased ratio would indicate how much more phosphorus could be liberated.

Conditions for making the 1.0% casein solution and addition of enzymes were similar to those described previously. To 20 mls. 1% casein solution (pH 6.7) was added 8 mg rennin in 5 mls. of distilled water.

TABLE V

Phosphatase action of Rennin on a 1% Casein Solution using Enzyme:Substrate Ratio of 1:25 at varying reaction times.

Sample	Reaction	Dilution factor	% Transmission at 420 mμ	P_2O_5 ≡ mg/100 mls	Corrected for dilution	P_2O_5 ≡ mg/75 mls	% P (of total) liberated (0.2g casein has 3.43mg P_2O_5 ≡)	% P Corrected for Control Values
1	C6.7 + 5 mls.dist.water/½h.	-	103	0.05	-	0.038	1.1	-
2	C6.7 + 5 mls.dist.water/8h.	-	100	0.10	-	0.075	2.2	-
3	C6.7 + 5 mls.dist.water/24h.	-	103	0.05	-	0.038	1.1	-
4	C6.7 + R^N 8mg/½h.	2	89	0.28	0.56	0.42	12.3	11.2
5	C6.7 + R^N 8mg/1h.	2	87	0.31	0.62	0.47	13.7	12.6
6	C6.7 + R^N 8mg/2h.	2	87	0.31	0.62	0.47	13.7	12.6
7	C6.7 + R^N 8mg/4h.	2	87	0.31	0.62	0.47	13.7	12.6
8	C6.7 + R^N 8mg/8h.	2	86	0.33	0.66	0.50	14.6	12.4
9	C6.7 + R^N 8mg/24h.	2	87	0.31	0.62	0.47	13.7	12.6

NOTE: C = 1.0% casein solution; R^N = rennin; 6.7 = reaction pH;

½h, 1h, 2h, 4h, 8h, 24h = time in hours.

Rennin concentrations indicated have been compensated for salt concentration.

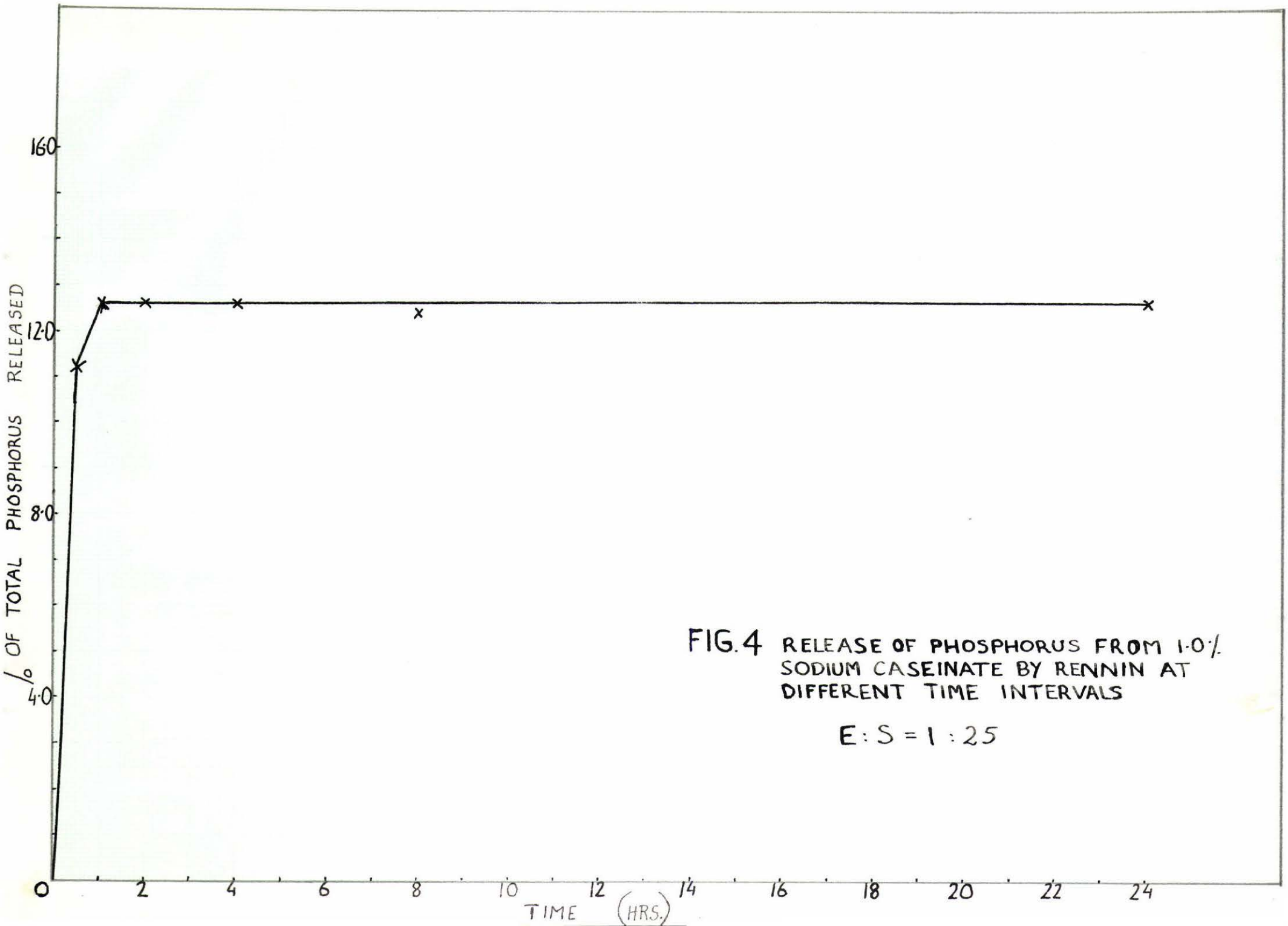

FIG. 4 RELEASE OF PHOSPHORUS FROM 1·0% SODIUM CASEINATE BY RENNIN AT DIFFERENT TIME INTERVALS

E : S = 1 : 25

Results:

Percentage (of total) phosphorus liberation figures given in Table V have been plotted against time in Figure 4.

The flattening of the curve at the end of the first hour is rather marked and suggested that rennin could liberate a maximum of 12.5% to 13.0% of the total phosphorus in casein.

Also, at the high enzyme:substrate ratio of 1:25, all or most of the phosphorus appears to be liberated in the first hour.

A note of caution is, however, necessary. The slope of the calibration curve (Figure 1) indicates that small differences in percentage transmission would magnify the differences in percentage phosphorus liberation figures, and this is noticeable between samples 7 and 8 respectively (Table V) where a difference of 1% Transmission has given a difference of 0.9% in percentage 'P' liberated (almost an 8% change). The complete flattening may, thus, be slightly misleading and it is possible for the curve to be moving in a slightly upward direction.

Control samples, No. 2 and 8, (8 hr. reaction) have both given rather high percentage 'P' liberation figures, although when the control value is taken off the reaction value, the latter comes in line with the other reaction values. It is difficult to explain the higher values obtained in these tubes, since the same casein solution was used throughout.

Experiment 7:

The Effect of Casein Concentration on the Liberation of Phosphorus by Rennin.

Since there appeared to be a tendency for a greater release of phosphorus on increasing the enzyme:substrate ratio, the final study of the phosphatase action of rennin on casein was directed to finding the effect of different casein concentrations on the liberation of phosphorus by rennin. Rennin concentration chosen was 8 mg. since this liberated the maximum amount of phosphorus in 24 hours. The technique followed for preparation of casein solutions and other conditions of the experiment remained the same as before. Casein concentrations used were 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 3.5%; 20 mls. of each containing 1.71, 3.43, 5.14, 6.86, 8.57, 10.29 and 12 mg P_2O_5 equivalent respectively.

TABLE VI

The Effect of Casein Concentration on the Liberation of Phosphorus by Rennin

Sample	Reaction	Dilution factor	% Transmission at 420 mμ	P_2O_5 ≡ mg/100mls.	Corrected for dilution	P_2O_5 ≡ mg/75 mls. (or 60 mls.)	% P(of total) liberated. (0.7g casein has 12 mg P_2O_5 ≡)	% P corrected for control values
1	0.5% C6.7 + 5mls.dist.water	-	104	0.03	-	0.018/60	1.10	-
2	0.5% C6.7 + R^N, 8mg/24 h	-	80	0.43	-	0.26/60	15.2	14.1
3	1.0% C6.7 + 5mls.dist.water	-	105	0.02	-	0.015	0.40	-
4	1.0% C6.7 + R^N, 8mg/24 h	2	88	0.30	0.60	0.45	13.1	12.7
5	1.5% C6.7 + 5mls.dist.water	-	109	-	-	-	-	-
6	1.5% C6.7 + R^N, 8mg/24 h	2	84	0.37	0.74	0.55	10.9	10.9
7	2.0% C6.7 + 5mls.dist.water	-	102	0.052	-	0.039	0.57	-
8	2.0% C6.7 + R^N, 8mg/24 h	2	74	0.53	1.06	0.8	11.7	11.1
9	2.5% C6.7 + 5mls.dist.water	-	110	-	-	-	-	-
10	2.5% C6.7 + R^N, 8mg/24 h	2	71	0.58	1.16	0.87	10.1	10.1
11	3.0% C6.7 + 5mls.dist.water	-	103	0.05	-	0.038	0.37	-
12	3.0% C6.7 + R^N, 8mg/24 h	2	67	0.65	1.30	0.98	9.5	9.1
13	3.5% C6.7 + 5mls.dist.water	-	108	-	-	-	-	-
14	3.5% C6.7 + R^N, 8mg/24 h	3	76	0.5	1.5	1.13	9.4	9.4

NOTE: C6.7 = casein solutions at the indicated concentrations at pH 6.7

R^N = rennin

24h = time in hours

Rennin concentration indicated has been compensated for salt concentrations

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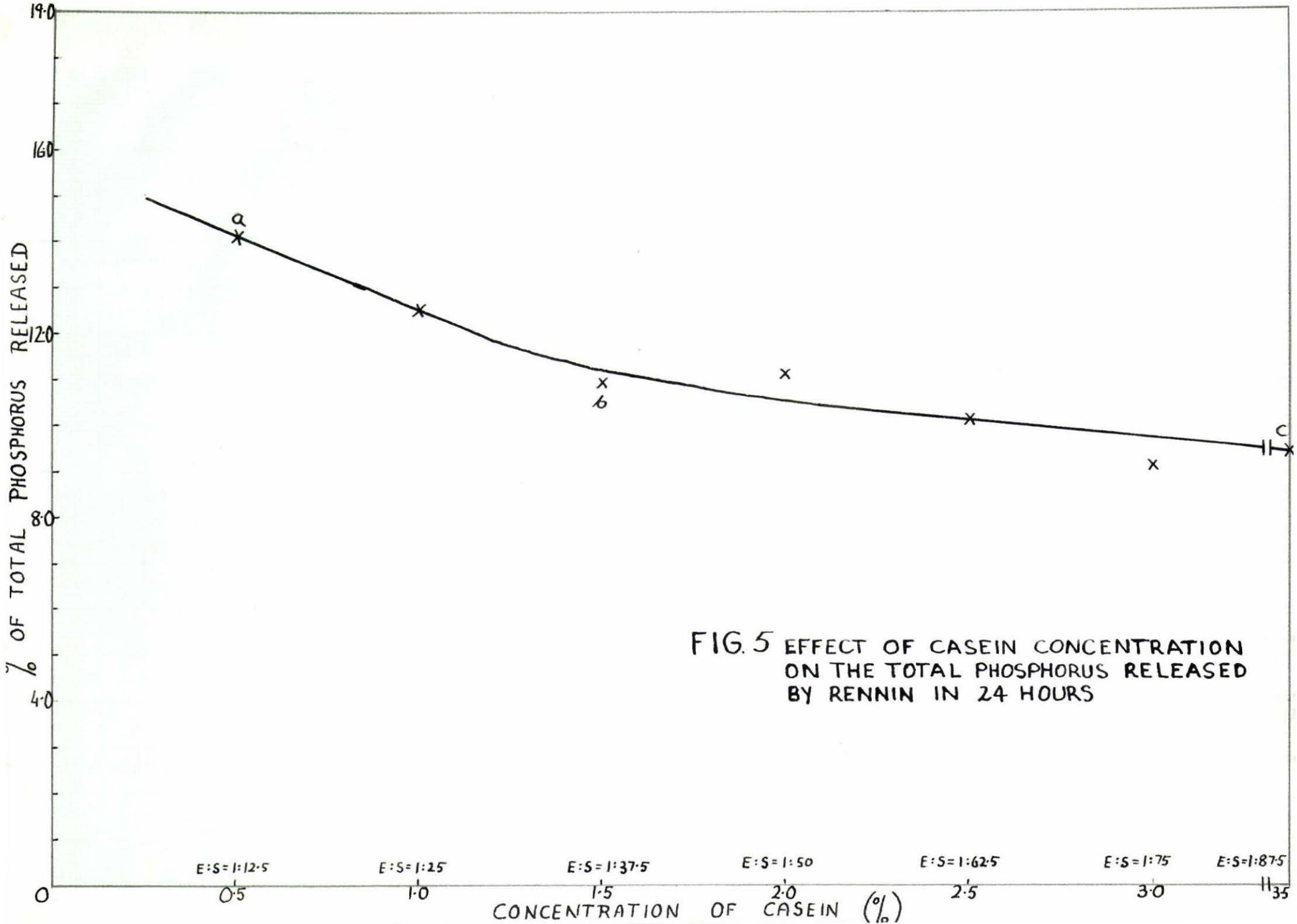

FIG. 5 EFFECT OF CASEIN CONCENTRATION ON THE TOTAL PHOSPHORUS RELEASED BY RENNIN IN 24 HOURS

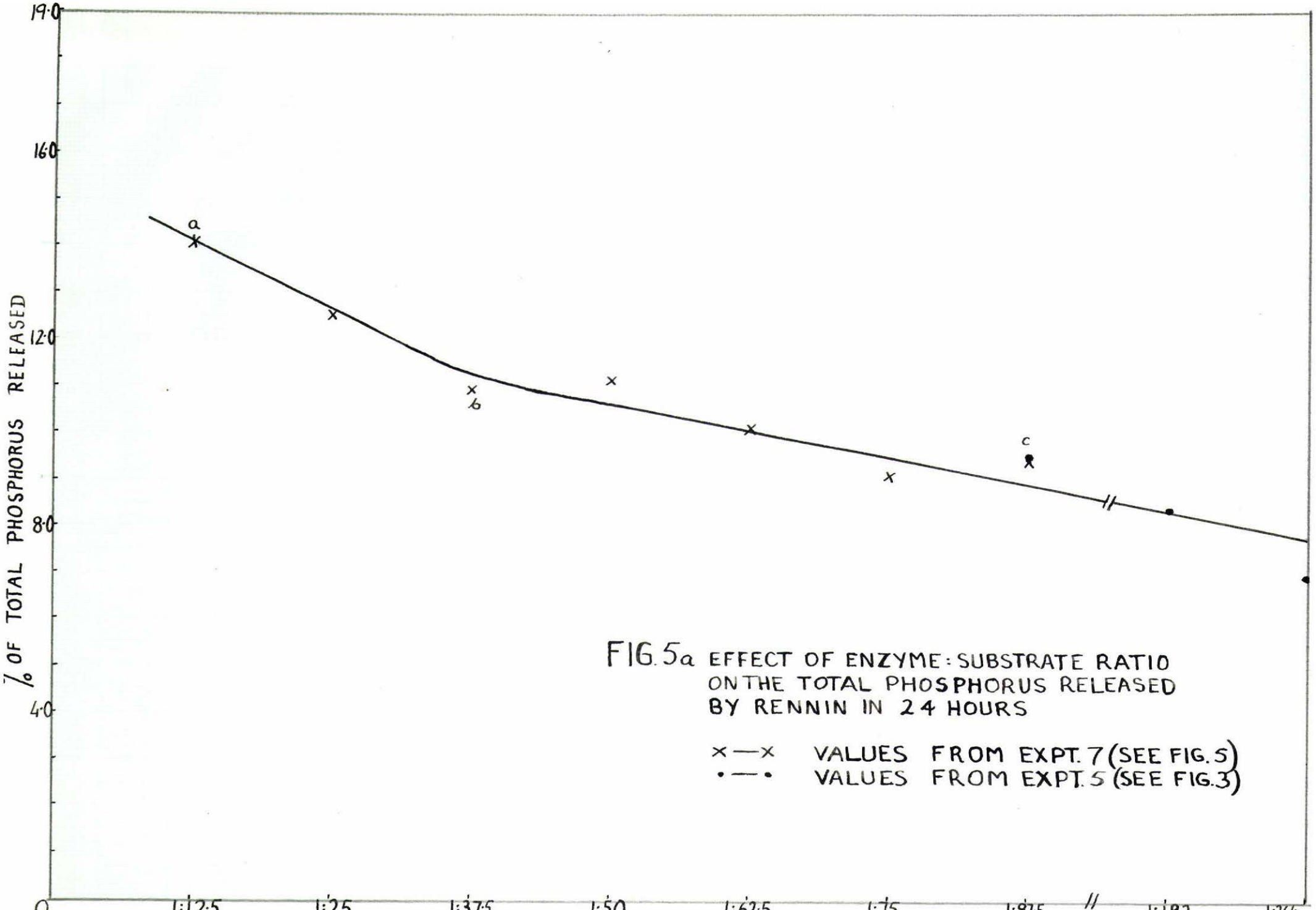

FIG. 5a EFFECT OF ENZYME : SUBSTRATE RATIO ON THE TOTAL PHOSPHORUS RELEASED BY RENNIN IN 24 HOURS

×—× VALUES FROM EXPT. 7 (SEE FIG. 5)
•—• VALUES FROM EXPT. 5 (SEE FIG. 3)

Results:

Values for percentage (of total) phosphorus liberated given in Table VI have been plotted against casein concentration (%) in Figure 5.

Between 1.5% to 3.5% casein concentration (E:S = 1:37.5 to 1:87.5) the phosphorus liberated varies little between 9% to 11%, but between 1.5% and 0.5% casein concentration (i.e. E:S = 1:37.5 to 1:12.5) the phosphorus released has gone up from 11% to 14%. The slope of the curve suggests that not more than 15% to 17% of the total phosphorus in casein would be released by rennin action. Although the graph has been drawn as a curve, it is quite possible that points 'a', 'b' and 'c' (see Figure 5) are on straight lines with a 'break' occurring at point 'b'. If the values uncorrected for the corresponding blank are used (and this may be justifiable in view of the zero blanks obtained in some instances), the points on the graph for casein concentrations of 1.5% and higher would be more nearly on a straight line, with the exception of the 2.0% casein level which appears to be adventitious. Using the uncorrected data and straight line plotting, the maximum release of phosphorus appears to be about 17% of the total.

It is interesting to note that the 24-hour curve in Figure 3 (Experiment 5) represents an extension of this experiment into the more dilute enzyme:substrate ratios. Figure 5 'a' represents a combined curve for values obtained in experiments 5 and 7 respectively. The general slope of the curve for the lower enzyme:substrate ratios has been drawn as being slightly more steep than indicated in Figure 5, but the overall picture presented remains basically the same.

Experiment 8:

The Effect of a Buffer on the Phosphatase Action of Rennin on Casein.

In previous experiments, no buffer was incorporated in the reaction mixture; the buffering action of the casein itself being taken as adequate for the purpose. The pH of the solutions after 24 hours' reaction with rennin generally dropped from 6.7 to 6.2-6.3, with the formation of a precipitate of casein.

In this experiment, rennin was dissolved in a veronal-acetate buffer (87) at pH 6.7 before addition to the reaction tubes, and the results were compared to a non-buffered system.

TABLE VII

The Effect of a Buffer on the Phosphatase Action of Rennin on Casein

Sample	Reaction	Dilution factor	% Trans-mission at 420mμ	P_2O_5 ≡ mg/100 mls.	Corrected for dilution	P_2O_5 ≡ mg/75 mls.	% P(of total) liberated. (0.7g.casein has 12 mg P_2O_5 ≡)	% P corrected for control values
1	3.5% C6.7 + 5mls.dist. water.	-	106	-	-	-	-	-
2	3.5% C6.7 + R^N 8mg/24h + 5mls.dist. water.	3	75	0.52	1.56	1.17	9.8	9.8
3	3.5% C6.7 + 5mls.buffer (6.7)	-	101	0.08	-	0.06	0.5	-
4	3.5% C6.7 + R^N 8mg/24h + 5mls.buffer (6.7)	3	73	0.55	1.65	1.24	10.3	9.8

NOTE: C6.7 = casein solutions at indicated concentration at pH 6.7

R^N = rennin

24h = time in hours

Rennin concentration indicated has been compensated for salt concentration.

Results:

It appears from Table VII that incorporation of a buffer to stabilize the pH at 6.7 caused little change in percentage phosphorus release. Even if this result is based on only one experiment, at least it indicates that no great deviation from the values of percentage phosphorus released by rennin, cited in previous experiments, may be expected.

A certain amount of casein precipitate formed in the buffered system, similar to the one in the non-buffered reaction tube, after about 8 hours, but most of the precipitate was seen only after 20-24 hours reaction. Prior to precipitate formation, the casein solutions containing rennin became distinctly opaque after about 4 hours reaction, as compared to the controls. The development of the opaqueness and the eventual formation of the precipitate may partly be explained by denaturation and aggregation of the casein, as reactive groups are exposed. If calcium ions were present, it is possible that the aggregation (through calcium acting as a intermolecular bridge) would have been faster, and the opaqueness and precipitate development much quicker than observed in the reaction tubes, particularly since α_s and β caseins are sensitive to the presence of calcium ions (54, 75).

Section III:

The Phosphatase Action of Alkaline Milk Phosphomonoesterase on Casein.

In Section II, Experiment 2, it was shown that the phosphomonoesterase had little or no effect on the release of phosphorus from casein. Further, it was found that rennin had no phosphatase activity on the synthetic substrates, whereas the phosphomonoesterase was very active (Section II, Experiment 3).

Zittle and Bingham [89], however, have reported an 80% dephosphorylation of casein with alkaline milk phosphatase prepared by the method of Zittle and Della Monica [90], in direct contrast to the finding in this study on the phosphomonoesterase preparation prepared by the method of Morton [42]. Zittle and Bingham also reported that the greatest activity of their phosphatase preparation lay between pH 6-7 in contrast to over pH 9.0, normally expected for this enzyme. This surprising report made it imperative to study further the action of the monoesterase on casein.

Experiment 9:

The Phosphatase Action of Alkaline Milk Phosphomonoesterase on Casein using three pH values.

A 3.5% casein solution at pH 6.6 was prepared as previously described. It was divided into three 140 ml. portions. Each was treated as under:-

Portion A:	Added 2.3 mls. distilled water.	Final pH = 6.6
Portion B:	Added 0.7 mls. distilled water, plus 1.6 mls. N NaOH	Final pH = 8.0
Portion C:	Added 2.3 mls. N NaOH	Final pH = 9.5

The antibiotic, nisin, was added during the initial preparation (100 p.p.m.). The reason for using sodium hydroxide to adjust the pH instead of the carbonate-bi-carbonate buffer used previously was because of the possible inhibitory effect of carbonate ions on the phosphomonoesterase (42), although this was the buffer used in the Aschaffenburg-Mullen phosphatase test for milk (84).

To 20 mls. of the casein solution, as treated in A, B and C above, was added 6 mg phosphomonoesterase dissolved in 5 mls. of $MgCl_2$ solution (stock solution had 1 g $MgCl_2$/100 mls. solution). The concentration of Mg^{++} in the reaction mixture was approximately 10^{-1} M for maximum activation of the enzyme (42). The addition of $MgCl_2$ reduced the pH of reaction mixtures A, B and C to pH 6.05, 7.35 and 8.75 respectively. Reaction tubes A, B and C contained about 0.69 g casein and 6 mg phosphatase to give an enzyme:substrate ratio of 1:115.

TABLE VIII

The Action of Alkaline Milk Phosphomonoesterase on Casein using three pH values

Sample	Reaction	% Transmission at 420 mμ			% (of total) phosphorus released from casein
		A	B	C	
1A, 1B, 1C	+ 5 mls $MgCl_2$/6h	110	110	108	negligible
2A, 2B, 2C	+ 5 mls $MgCl_2$/24h	109	110	108	
3A, 3B, 3C	C + P, 6mg/1h	108	106	107	
4A, 4B, 4C	C + P, 6mg/2h	110	107	107	
5A, 5B, 5C	C + P, 6mg/6h	109	108	107	
6A, 6B, 6C	C + P, 6mg/12h	109	107	105	
7A, 7B, 7C	C + P, 6mg/24h	107	108	106	

NOTE: 1-7 = tube number

A, B and C = reactions at pH 6.05, 7.35 and 8.75 respectively

1h, 2h, etc. = time in hours

P = alkaline milk phosphomonoesterase

- - - - - -

Results:

Table VIII shows that there is no obvious release of phosphorus from casein at the three reaction pH, even at the end of 24 hours reaction with the phosphomonoesterase, inspite of adding Mg^{++} and not using the carbonate buffer. The pH (initially 6.05, 7.35 and 8.75 respectively in tubes A, B and C) dropped to 6.0, 7.15 and 8.15 respectively). The drop in pH without a corresponding release of phosphate indicated that it was a denaturation phenomena with the unmasking of acidic groups. Some precipitate had formed after 24 hours reaction, which again could be explained by denaturation followed by aggregation, particularly since Mg^{++} ions were present.

It was decided to repeat the experiment using higher enzyme concentrations and reacting for longer times.

Experiment 10:

The Action of Alkaline Milk Phosphomonoesterase on Casein using a Higher E:S ratio (1:34.5) and Longer Reaction Times.

The technique used was similar to that used in Experiment 9, except that the final reaction pH was 6.5, 8.3 and 9.6 in reaction tubes A, B and C respectively. Twenty mg of the phosphomonoesterase was added to give an enzyme;substrate ratio of 1:34.5. Results are given in Table IX.

TABLE IX

The Action of Alkaline Milk Phosphomonoesterase on Casein using a Higher E:S ratio 1:34.5 and Longer Reaction Time.

Sample	Reaction	% Transmission at 420 mμ	P_2O_5 ≡ mg/100 mls.	P_2O_5 ≡ mg/75 mls.	% P (of total) liberated (0.69 g casein has 11.8 mg P_2O_5 ≡)	% Phosphorus released - corrected for control values
1A	C6.5 + 5 mls $MgCl_2$/54h	100	0.10	0.075	0.63	-
2A	C6.5 + P, 20mg/54h	98	0.13	0.098	0.82	0.19
1B	C8.3 + 5 mls.$MgCl_2$/54h	97	0.15	0.113	0.94	-
2B	C8.3 + P, 20mg/54h	94	0.2	0.15	1.25	0.31
1C	C9.6 + 5 mls.$MgCl_2$/54h.	93	0.22	0.165	1.38	-
2C	C9.6 + P, 20mg/54h	95	0.18	0.135	1.13	-

NOTE: C = casein solutions

6.5, 8.3, 9.6 = pH values

P = phosphomonoesterase

54h= time in hours

- - - - - -

Results:

It is evident from Table IX that little phosphorus was released from casein by the phosphomonoesterase. The high readings for the controls at the higher pH values may be explained by the lability of the phosphate group on phospho-proteins in alkaline conditions (86).

In an independent experiment a casein solution incubated with the phosphomonoesterase for 48 hours was checked for phosphatase activity, immediately after adding the enzyme and after 48 hours reaction at 38°C. The substrate used was di-sodium p-nitrophenyl phosphate. There appeared to be no loss of phosphatase activity at the end of 48 hours, indicating that the enzyme had not been destroyed in the reaction.

In a further experiment, conditions approximating those of Zittle and Bingham's (89) were employed; namely, a similar enzyme:substrate ratio, veronal-acetate buffer (87) at the same concentration as in their experiment and a pH of 7.0. Concentration of magnesium ions was also similar. Only the method of phosphorus determination was different. However, no evidence of phosphorus liberation was found with this phosphomonoesterase preparation, as compared to the finding of 80% dephosphorylation by Zittle and Bingham (89).

Discussion

THE ACTION OF RENNIN AND ALKALINE MILK PHOSPHOMONOESTERASE ON CASEIN

Experiments to date have established the release of about 15% to 17% phosphorus from casein through the action of rennin, but no release of phosphorus was evident through phosphomonoesterase action. Purified rennin did not have phosphatase action on synthetic substrates, such as di-sodium p-nitrophenyl phosphate and glucose-1-phosphoric acid (di-potassium salt), whereas the phosphomonoesterase was extremely active on them. Commercial rennet had some activity - this was not unexpected, since it is possible for contaminating enzymes from the calf mucosa to get into the rennet during its commercial extraction.

The reasons for the inability of the phosphomonoesterase to release any phosphorus, as compared to 80% dephosphorylation of casein attributed to this enzyme by Zittle and Bingham [89], are not clear. These workers used an enzyme prepared differently [90] from that of Morton [42]. Commercial rennet was used for precipitating the casein from skim-milk and it was quite possible for some rennin to have been co-precipitated along with the phosphomonoesterase. However, at the most, this would account for only 20% to 21% of the phosphorus reported by them [89], if rennin liberated a maximum of 17% from casein. It is, however, unlikely that rennin contamination would be high enough to account for even 20% of their reported figure.

The other possibility was that the two enzyme preparations were, in fact, two different enzymes with different specificities and are present, as such, in milk. Only a study of the two enzymes together would confirm such a supposition.

Casein treated with alkaline phosphomonoesterase for some hours and then precipitated with vanadium molybdate was observed to give a deep yellow precipitate. The vanadium molybdate reagent contains nitric acid, and this acid is known to react with aromatic amino acids to give a yellow colour or yellow flakes with proteins containing aromatic amino acids [92]. A rennin treated casein or casein by itself did not give a yellow precipitate unless heated at 50°C for half an hour or so, in contrast to the yellow precipitate obtained immediately on precipitation of phosphomonoesterase treated casein. This was possibly because the nitric acid in the vanadium molybdate reagent had quicker access to the aromatic amino acids in the phosphomonoesterase treated casein, as compared with the rennin treated or untreated casein; perhaps through some hydrolytic action associated with the phosphomonoesterase preparation. It is not known whether the release of the aromatic amino acids through such an action, either free or associated with a soluble peptide, was the cause of the blue colour which developed in Sumner's method [91], as used by Zittle and Bingham [89] for phosphorus determination. The aromatic amino acids are known to give varied colour reactions [92] with somewhat similar reagents as used in this method [89, 91]. However, no satisfactory explanation can be given for the completely contrasting result obtained in this study, as compared to Zittle and Bingham's finding.

Kalan and Telka [93] have, however, indicated an 85% dephosphorylation of whole casein, using calf intestinal mucosa acid

phosphomonoesterase, which was in general agreement with Zittle and Bingham's finding [89] based on the use of alkaline milk phosphomonoesterase which, however, had shown greatest activity on casein at neutral pH.

Sampathkumar et al. [94] have reported 5% dephosphorylation of whole casein using an acid pyro-phosphatase from soya bean, and Sundararajan and Sarma [95] dephosphorylated whole casein to the extent of 77% using a phosphoprotein phosphatase (acid) from rat tissues. This enzyme was active on phosvitin (phosphorus as monoester), but not on β-glycerophosphate, so the exact nature of the enzyme is not clear. These results were comparable to Zittle and Bingham's [89] which indicated that the phosphomonoesterase preparation used in this study and prepared by the method of Morton [42] was a different enzyme to Zittle and Bingham's preparation, although it acted rapidly on synthetic substrates, such as di-sodium para-nitrophenyl phosphate, and behaved as a monoesterase. Overall results from the action of phosphatases on casein appear confusing. However, the results obtained by all the above workers did not account for about 15% to 20% of the total phosphorus in casein and it was on this that results with rennin in this study assume significance.

In this study, rennin was found to liberate 15% to 17% of the total phosphorus in casein, which appears to resist the action of phosphatases in general.

Perlman [86] indicated that 40% of the total phosphorus in α-casein was linked as a phosphoamide diester with a -N-P-O- bond and Dyachenko [79] postulated a phosphoamidase action of rennin on casein for clotting to occur.

The phosphorus was suggested as being linked to the guanido group of arginine. Further study was, therefore, directed to finding whether rennin action on casein exposed more guanido groups on the molecule than those already free, and to attempt to correlate phosphorus liberation with exposure of guanido groups of arginine due to rennin action.

Section IV:

Exposure of Guanido Groups of Arginine on Casein through Rennin Action.

To study the exposure of arginine guanido groups on casein, due to rennin action, the Sakaguchi test [92, 96] for arginine was selected. The test was specific for arginine and, moreover, was also specific for the guanido group [96]. It involved the addition of sodium hydroxide, 0.1% α-naphthol in 70% alcohol solution, and 0.5% sodium hypochlorite to a solution containing arginine when a deep red colour developed.

Experiment 11:

Development of the Sakaguchi test for Arginine

A 3.5% casein solution (pH 6.7) was prepared, as described in Chapter II Section I. To a small portion, varying quantities of the reagents specified above were added. A red colour developed indicating that part of the arginine at least on the casein had its guanido group unattached. Preliminary experiments were then carried out to find the optimum quantities of the reagents α-naphthol and sodium hypochlorite which were necessary to be added to 2 mls. of a 2.8% casein solution (20 mls. 3.5% casein solution + 5 mls. distilled water = 2.8% casein solution - see later experiments), and

0.5 mls. 1 N NaOH to obtain maximum colour development. The reaction was done at 38°C. After several observations, the following procedure was adopted:-

1. 2 mls. of a 2.8% casein solution (pH 6.7) were taken in small colorimeter test tubes; 75 mm x 15 mm held in a water bath maintained at 38°C.

2. 0.5 mls. of 1 N NaOH was added.

3. The alkali was followed by 0.5 mls. of 0.1% α naphthol in 70% alcohol solution, and

4. 1 ml. of 5% sodium hypochlorite solution.

Stock solutions of α-naphthol and sodium hypochlorite were held in dark bottles inside cupboards and taken out only when needed.

After colour development at 38°C, the tubes were taken out of the water bath and allowed to cool on their own to room temperature. Percentage transmission was read in the Beckman Model D.U.Spectrophotometer about $\frac{1}{2}$ hour to 1 hour after colour was first developed. This was to allow maximum colour development.

Selection of optimum wavelength

The wavelength at which maximum absorbtion of light occurred for the above system was found to be 420 mμ, relative to distilled water set at 100% transmission.

There was a tendency for bubbles to stick to the sides of the cells containing the sample and affect percentage transmission readings. It was found important to invert the cells twice or thrice to dislodge the bubbles, particularly from the reflecting sides before taking readings in the Beckman.

Opacity study of the sample.

The biggest problem encountered was the opaqueness of the coloured casein solutions, as examined in the Beckman. A preliminary examination of an untreated and rennin treated casein solution indicated a definite lowering of percentage transmission in the rennin treated sample. This lowering of percentage transmission could have been due to two reasons:

(a) Increased opacity of the casein solutions due to rennin action. It was stated elsewhere that rennin action did increase the opacity and caused the eventual formation of a precipitate at the end of 24 hours in the reaction tubes.

(b) Increased colour development in the Sakaguchi reaction, due to exposure of more guanido groups of arginine on the casein molecule.

The following experiment was done to examine this point.

A 2.8% casein solution was reacted with rennin (enzyme: substrate ratio = 1:87.5) for 4 and 24 hours. To 2 ml. portions were added 0.5 mls. 1 N NaOH and 2 mls. distilled water. The samples were then checked for percentage transmission at 420 mμ.

Results are shown below:

Tube	Time of rennin treatment	% Transmission
1	Control	100
2	4 hours	98
3	24 hours	98

The experiment indicated that the opaqueness was a constant factor, whether a casein solution was treated with rennin or not. This was evidently due to the solubilizing action of sodium hydroxide used in the Sakaguchi reaction, even on the casein precipitate which formed at the end of a 24-hour reaction with rennin. The marked decrease in percentage transmission of the colour developed rennin treated sample, observed earlier, must have been due to increased colour development, rather than increased opacity. The result was sufficiently encouraging to pursue the matter further.

It was not possible to remove the casein by precipitation and measure the colour in the clear supernatant, since arginine was not released into solution by rennin action, but remained attached to the P-casein. This was clearly indicated when to the red coloured casein solution 20% trichloroacetic acid was added to precipitate the protein. The supernatant was colourless, whereas the precipitate was a yellowish colour. The supernatant of a rennin treated sample tested by the Sakaguchi reaction indicated no large scale release of free arginine.

Stability of the colour developed

Colour was developed in a 2.8% casein solution by the method described earlier, and after various time intervals, percentage transmission

was measured and compared with a freshly prepared sample. Results are given below:

Sample	Time interval after colour development	% Transmission at 420 mμ with sample1 = 100% T.
1	$5\frac{3}{4}$ hrs.	100
2	$3\frac{1}{4}$ hrs.	80
3	$2\frac{1}{2}$ hrs.	66
4	$\frac{1}{2}$ hr.	63

It was evident from samples 3 and 4 that the colour was stable for about 2 hours. The samples were kept away from direct light and the colorimeter tubes were well stoppered by means of rubber corks prior to examination in the Beckman Spectrophotometer. This technique was followed in subsequent experiments.

Experiment 12:

Calibration Curve for Arginine

120.92 mg. of arginine monohydrochloride (laboratory reagent grade) was dissolved in distilled water; the pH of the solution was brought up to 7.0 by $^{N}/_{20}$ NaOH, and the volume made up to 100 mls. The arginine content in this solution was taken as 100 mg. % $^{W}/_{v}$.

Two mls. of a 2.8% casein solution were taken in each of 6 colorimeter tubes held in a water bath at 38°C. The solution of arginine was then added in varying volumes to obtain the desired arginine concentrations in the various colorimeter tubes (see Table X). Distilled water was added to

TABLE X

Plotting of a Calibration Curve for Arginine

Tube No.	Arginine solution added (mls.) and Arginine ≡ (mg.)	Distilled water added (mls.)	% Transmission at 420 mμ	
			A	B
1	-	1	100	100
2	0.2 mls. (≡ 0.2 mg.)	0.8	86.5	88
3	0.4 mls. (≡ 0.4 mg.)	0.6	77	77
4	0.6 mls. (≡ 0.6 mg.)	0.4	66	65
5	0.8 mls. (≡ 0.8 mg.)	0.2	52.5	58
6	1.0 mls. (≡ 1.0 mg.)	-	42	49

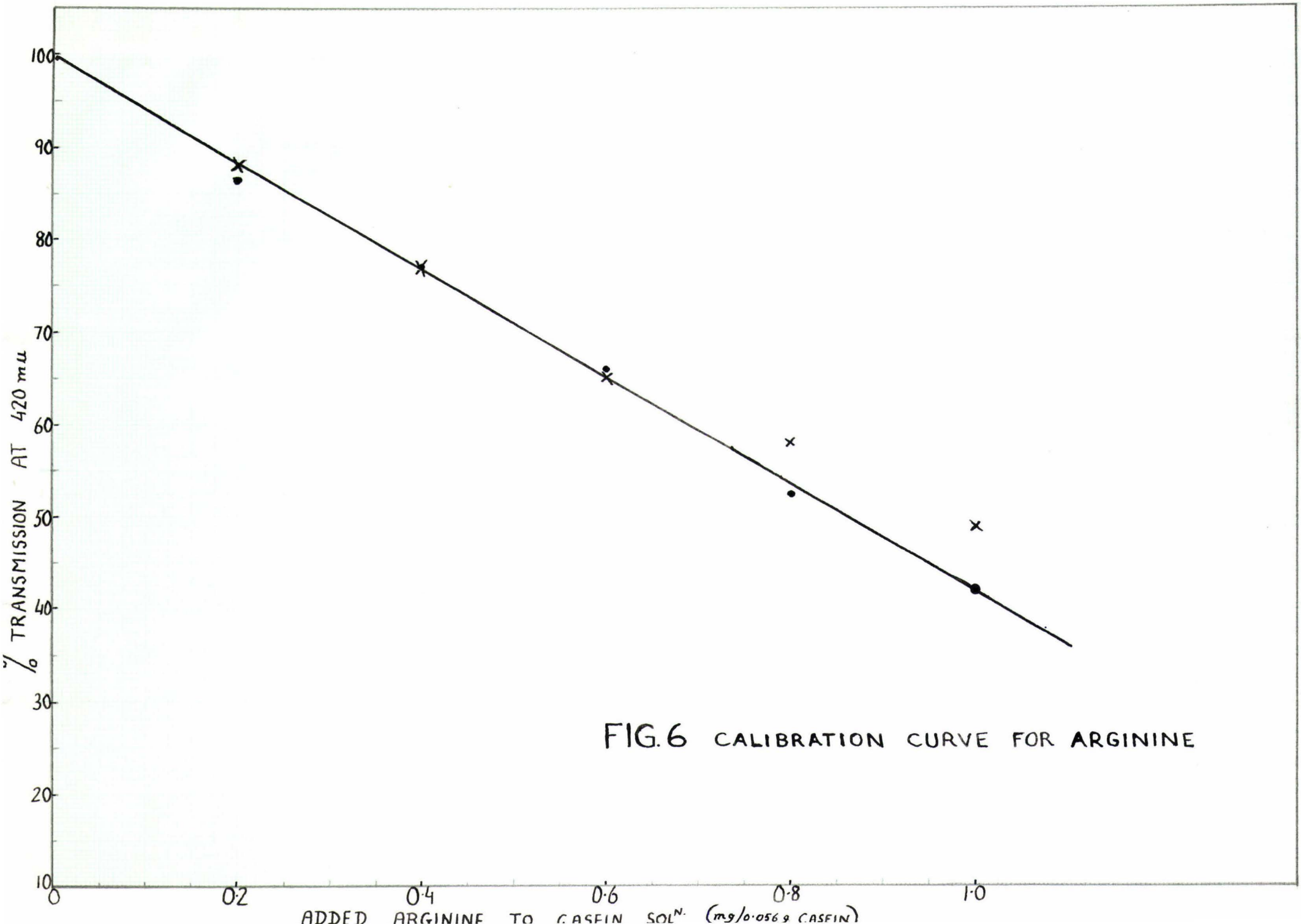

FIG. 6 CALIBRATION CURVE FOR ARGININE

equalize volumes in all the tubes, followed by the reagents used in the Sakaguchi test, in concentrations and volumes described earlier. Total volume in the colorimeter tubes was 5 mls. Percentage transmission was read half an hour after colour development at 420 mμ in the Beckman Model D.U. Spectrophotometer at room temperature. Casein solution was used in this experiment to get the same opacity as in subsequent experiments when the action of rennin was tested. Results from duplicate experiments are given in Table X.

Results:

Percentage transmission was plotted against mg arginine added (Figure 6). Although there was some variation in the percentage transmission figures, the line joining the plots was taken as arising from the origin, i.e. 100%, and a straight line was drawn as being a reasonable representation.

Experiment 13:

Release of Phosphorus and exposure of Guanido Groups of Arginine during Rennin Action on Casein.

A 3.5% casein solution (20 mls.) at pH 6.7 was reacted with rennin (enzyme:substrate = 1:87.5) which was added as a solution in 5 mls. of distilled water. Tubes were set up in duplicate - one for phosphorus liberation and the other for arginine exposure studies.

Reaction involving phosphorus studies were stopped, as previously described in vanadium molybdate, and the colour developed was measured in the Beckman Model D.U. Spectrophotometer at 420 mμ.

TABLE XI

A: Phosphorus released

Release of Phosphorus and Exposure of Guanido Groups of Arginine during Rennin Action on Casein

Tube No.	Reaction	Dilution factor	% Transmission at 420 mμ	P_2O_5 ≡ mg/100mls.	Corrected for dilution	P_2O_5 ≡ mg/75mls.	mg.Phosphorus liberated/100g casein	moles 'P' released/100 g casein
1-4	C6.7 + 5 mls.dist.water	-	106 +	-	-	-	-	-
5	C6.7 + R^N 8mg/1h	2	93	0.22	0.44	0.32	20	65×10^{-5}
6	C6.7 + R^N 8mg/2h	2	80	0.43	0.86	0.65	41	132×10^{-5}
7	C6.7 + R^N 8mg/4h	2	70	0.60	1.2	0.90	56	180×10^{-5}
8	C6.7 + R^N 8mg/24h	2	60	0.76	1.52	1.14	71	230×10^{-5}

NOTE:

C6.7 = 3.5% casein solution at pH 6.7

R^N = rennin

1h,2,4h,24h = time in hours

Rennin concentrations indicated have been compensated for salt content.

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B: Arginine exposed

TABLE XI (continued...)

Release of Phosphorus and Exposure of Guanido Groups of Arginine during Rennin Action on Casein

Tube No.	Reaction	% Transmission at 420 mμ	mg.arginine exposed/0.056g casein	mg.arginine exposed/100g casein	Moles arginine exposed/100g casein	Ratio	moles arginine expos / moles 'P' released
1A - 4A	C6.7 + 5mls.dist.water	100	-	-	-		
5A	C6.7 + R^N 8mg/1h	76	0.44	730	420×10^{-5}	$\frac{420}{65}$	= 6.5
6A	C6.7 + R^N 8mg/2h	72	0.48	860	500×10^{-5}	$\frac{500}{132}$	= 3.8
7A	C6.7 + R^N 8mg/4h	68	0.55	980	560×10^{-5}	$\frac{560}{180}$	= 3.1
8A	C6.7 + R^N 8mg/24h	61	0.675	1210	700×10^{-5}	$\frac{700}{230}$	= 3.0
9A	C6.7 + R^N 8mg/48h	62	0.65	1160	670×10^{-5}		-
10A	C6.7 + 6.25 mls.1N NaOH + R^N 8mg/24h	102	-	-	-		-

NOTE:

C6.7 = 3.5% casein solution at pH 6.7

R^N = rennin

1h, 2h, 4h, 24h = time in hours.

Rennin concentrations indicated have been compensated for salt content.

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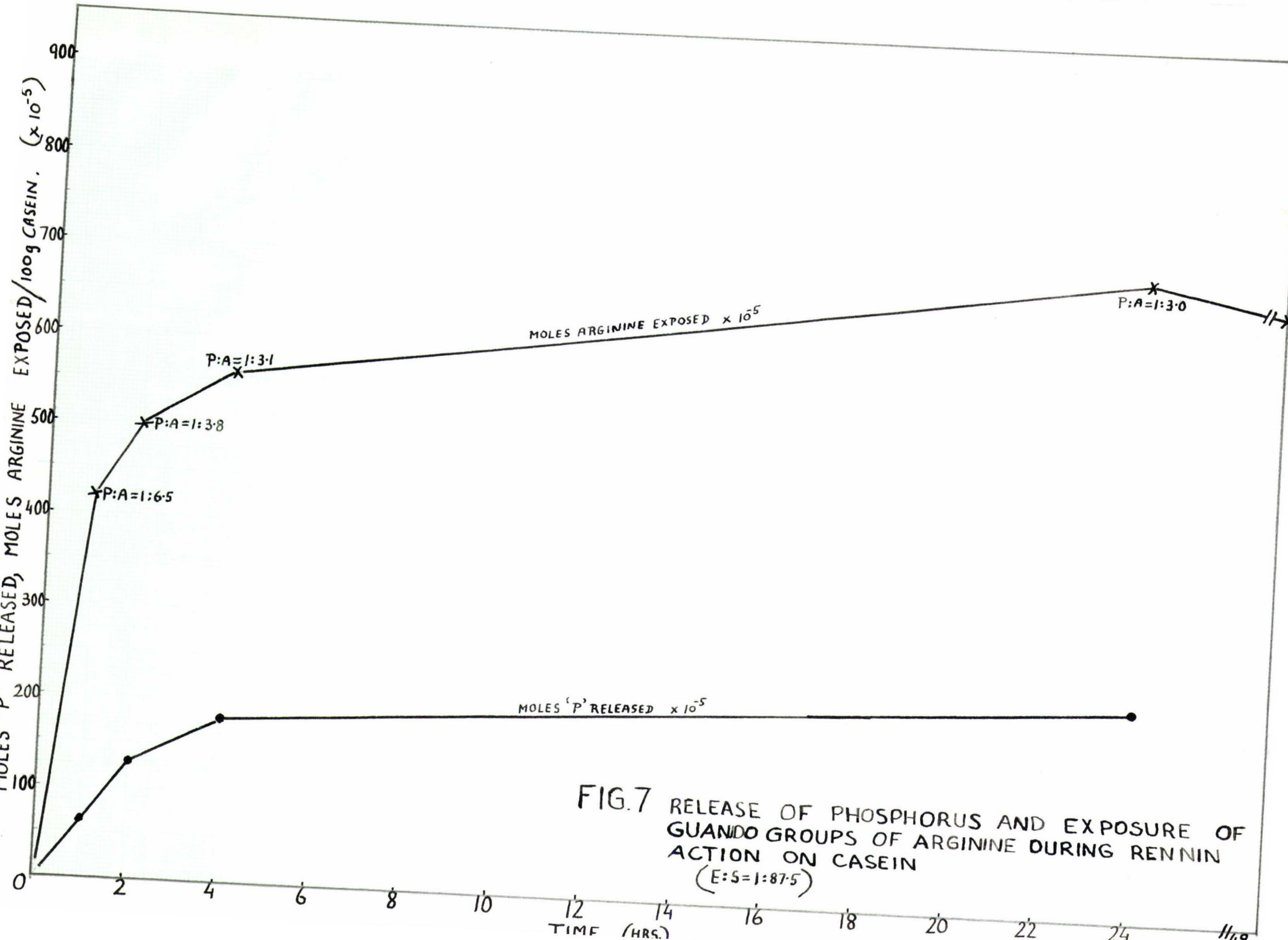


FIG. 7 RELEASE OF PHOSPHORUS AND EXPOSURE OF GUANIDO GROUPS OF ARGININE DURING RENNIN ACTION ON CASEIN
(E:S=1:87·5)

In reactions involving arginine studies, 2 mls. of the reaction mixture were taken out at the appropriate times (reaction time same as for phosphorus liberation studies) and pipetted into colorimeter tubes containing 0.5 mls. 1 N NaOH, which stopped rennin action immediately. 1 ml. of distilled water was added (cf. standard arginine curve, Table X) and this was followed by the reagents used in the Sakaguchi test. Colour was developed at 38°C and measured in the Beckman, as described earlier. Percentage transmission figures were related to the arginine standard curve and moles arginine exposed/100 g casein was calculated. Moles phosphorus liberated/100 g casein have also been calculated from the standard phosphate curve. Results are shown in Table XI.

Results:

Moles phosphorus released and moles arginine exposed/100 g casein calculated in Table XI were plotted against time (Figure 7).

Specimen calculations are given below to indicate how the figures were arrived at.

(i) Moles 'P' released at 1 hr. (No. 5 in Table XI).

P_2O_5 ≡ released from 0.7 g casein = 0.32 mg.

∴ Phosphorus released = $0.32 \times \frac{62}{142}$ (31 and 142) were taken as the molecular-weights of phosphorus and P_2O_5 respectively).

∴ 'P' released mg/100 g casein = $\frac{100}{0.7} \times 0.32 \times \frac{62}{142} = 20$ mg.

∴ Moles 'P' released/100 g casein = $\frac{20}{31,000} = 0.00065 = 65 \times 10^{-5}$ moles/100 g casein.

(ii) Moles arginine exposed at 1 hr. (No. 5A in Table XI).

mg. arginine exposed per 0.056 g casein (from standard curve) = 0.41

$\therefore$ mg. arginine exposed/100 g casein = $\frac{0.41 \times 100}{0.056}$ = 730 mg.

$\therefore$ moles arginine exposed/100 g casein = $\frac{730}{174.000}$ = 0.0042 =

420×10^{-5}/100 g casein

(molecular-weight of arginine taken as 174)

It would be evident from Table XI and Figure 7 that exposure of guanido groups of arginine, due to rennin action on casein, was much faster than the release of phosphorus. However, the ratio of moles phosphorus released to moles arginine exposed of 1:6.5 at the end of the first hour increased to 1:3.0 at the end of 24 hours, as phosphorus release continued and arginine exposure steadied.

The percentage transmission figures for arginine were the averages of six experiments, since some variation was observed in the Sakaguchi test results. The percentage transmission figures varied from 74-81, 68-74, 67-71 and 59-63 for the 1,2,4 and 24-hour reactions. Experiments on phosphorus liberation were not repeated, since figures given in Table XI were quite comparable to results on phosphorus liberation obtained in Section II.

Very little or no further arginine appeared to be exposed due to rennin attack after 24 hours (Figure 7). It was stated in Section II that 15% to 17% of the total phosphorus in casein may be released by rennin action. Fifteen percent is equivalent to 111 mg. P/100 g casein or 360×10^{-5} moles P/100 g casein. The ratio of moles arginine exposed to moles phosphorus released then becomes almost 2.0, allowing for experimental error.

Experiment 14:

Exposure of Arginine Guanido Groups on Casein in the first hour of Rennin Action.

In experiment 13, it was found that at the end of 1 hour, over 400×10^{-5} moles arginine/100 g casein had been exposed at the guanido group, due to rennin action. In this experiment, a study was made of the exposure

TABLE XII

Arginine Guanido Groups exposed on Casein in the first hour of Rennin Action

Tube No.	Reaction	% Transmission at 420 mμ	mg. arginine exposed/0.056 g casein	mg. arginine exposed/100 g casein	moles arginin exposed/100 g casein
1	C6.7 + 5 mls. dist. water	100	-	-	-
2	C6.7 + R^N 8mg/5 minutes	95	0.08	146	84×10^{-5}
3	C6.7 + R^N 8mg/10 minutes	91	0.15	268	154×10^{-5}
4	C6.7 + R^N 8mg/20 minutes	87	0.22	394	226×10^{-5}
5	C6.7 + R^N 8mg/30 minutes	82	0.31	555	320×10^{-5}
6	C6.7 + R^N 8mg/45 minutes	78	0.375	670	386×10^{-5}
7	C6.7 + R^N 8mg/60 minutes	74	0.45	805	460×10^{-5}

NOTE:

C6.7 = 3.5% casein solution at pH 6.7

R^N = rennin

Rennin concentrations indicated have been compensated for salt content.

- - - - - -

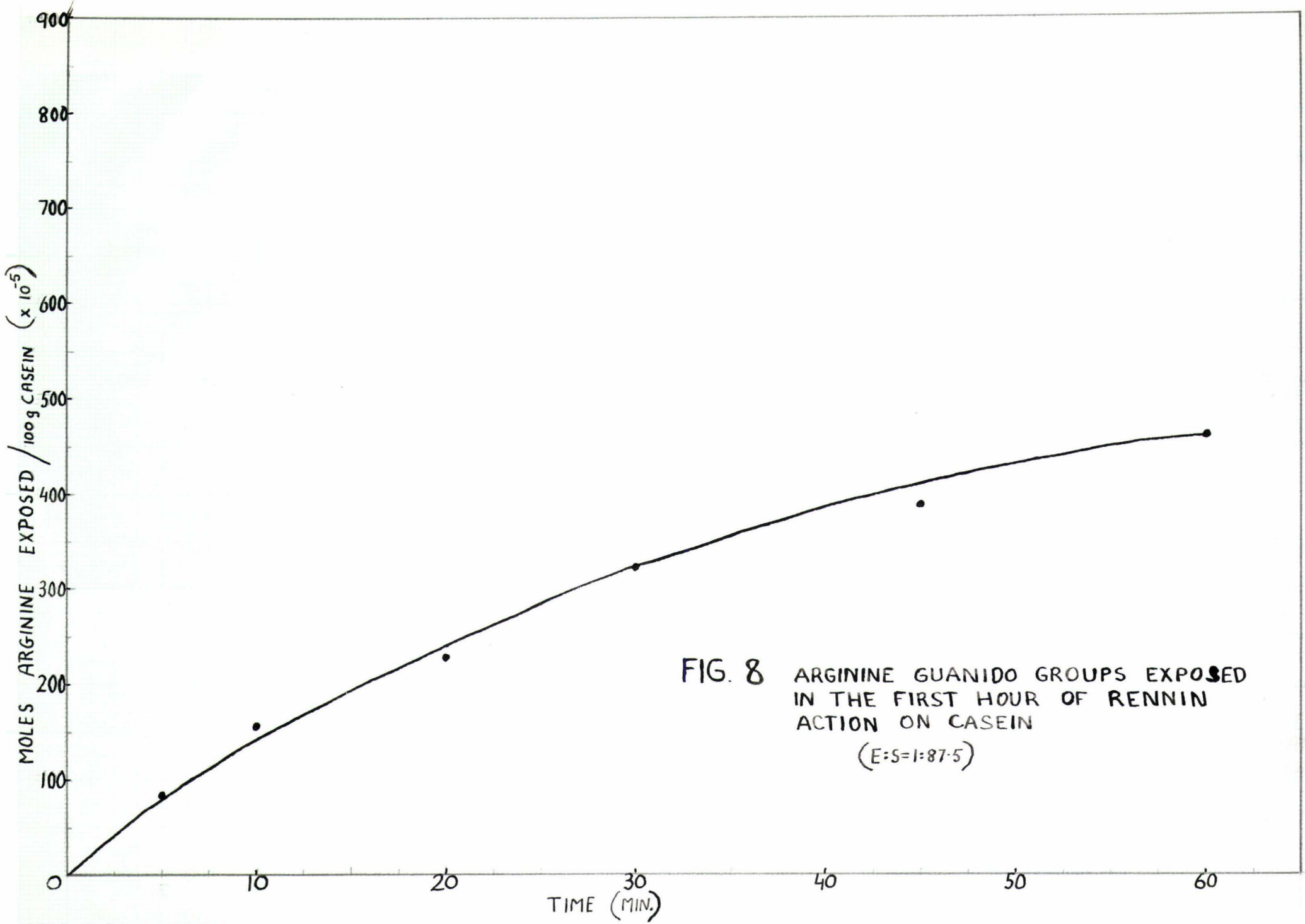


FIG. 8 ARGININE GUANIDO GROUPS EXPOSED IN THE FIRST HOUR OF RENNIN ACTION ON CASEIN

(E:S=1:87·5)

of guanido groups during the first hour of rennin action.

The technique was similar to the one described earlier. Two ml. portions of the reaction mixture (20 mls. 3.5% casein + 8 mg. rennin in 5 mls. distilled water) were taken out at appropriate intervals and pipetted into colorimeter tubes containing 0.5 mls. 1 N NaOH and 1 ml. distilled water. The colorimeter tubes were then transferred to the 38°C water bath and the red colour developed with the Sakaguchi reagents. Percentage transmission was read for the samples as described earlier. Enzyme:substrate ratio was 1:87.5. Results are shown in Table XII.

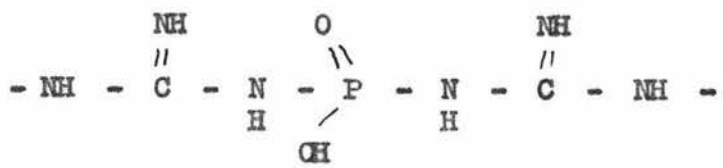
Results:

Values for moles arginine exposed/100 g casein during the first hour of rennin action on casein (Table XII) was plotted against time (Figure 8). Even during the first hour, the reaction followed first order kinetics. This was particularly noticeable after 15-20 minutes reaction.

Section V:

Discussion on the Action of Rennin on Casein

This study on the action of rennin on casein indicated that a possible 15% to 17% of the total phosphorus in casein was released by rennin action. Further, for every mole of phosphorus released, two moles of arginine appeared to be exposed on the casein molecule. This exposure appeared to go at a very much faster rate initially than the release of phosphorus. Thus, the ratio of phosphorus released to arginine exposed (at the guanido groups) was of the order of 1:6 to1:7 after 1 hr. (Table XI, Figure 7). The ultimate 1:2 ratio of phosphorus released to arginine exposed seemed to suggest an -N-P-N- link on the casein molecule, i.e. two arginine residues linked by their guanido groups through phosphorus as follows:



There are two possible reasons which may explain the low ratio of 1:6 to 1:7 obtained initially.

Firstly, it is possible for rennin to break a -N-P- bond on one side faster than the one on the other side, purely on steric considerations and type of amino acid residues attached to either arginine residues on the casein molecule.

Further, it may be possible for the Sakaguchi reagents to react with that guanido group on the ρ-casein molecule to which the phosphoric acid

is still attached, in addition to reacting with the guanido group exposed through the initial action of rennin. If this is so - Poller's work [103] on a number of guanidine derivatives indicates that this is not impossible - the figures quoted for moles arginine exposed in this study have been partly magnified for the initial stages of rennin action on casein and may partly explain the very low phosphorus released to arginine exposed ratio of 1:6 to 1:7 at the end of one hour.

Alternatively, or possibly concurrently, since the Sakaguchi reaction has been carried out in alkaline conditions, the alkaline hydrolysis of the remaining -N-P-link between arginine and the bound phosphate could occur, releasing the guanido group for detection. In the test, the *p*-casein was in a solution of approximately 0.2 N NaOH at 38°C for a short period prior to the addition of the other reagents for colour development, and it is known that the phosphate group in phosphoproteins is labile [86] to 0.25 N NaOH at 38°C and that the -N-P- bond is less stable than a -O-P- bond [104].

The rise in the ratio of phosphorus released to arginine exposed from 1:6 - 1:7 to 1:3.0 at the end of 24 hours is then explained by rennin's action of cleaving the second -P-N- bond, replacing chemical cleavage of the same bond in the Sakaguchi test, i.e. the values given for moles arginine exposed during the initial stages of rennin action are partly due to the enzyme itself and partly through effects produced in the Sakaguchi reaction, while the values given for the final stages of rennin action are attributable mainly to the action of the enzyme.

It should be pointed out that the arginine standard curve was drawn using arginine monohydrochloride at varying concentrations, whereas the

colour developed in ρ-casein solutions and compared to the standard was due to arginine bound to the ρ-casein molecule. The colour intensity given by a free arginine molecule need not necessarily be the same as that given by an arginine molecule bound to a protein. This point needs clarification.

In summation, therefore, it appears that an -N-P-N- bond is present in casein; the phosphorus linking two arginine residues through their guanido groups. The low ratio of moles phosphorus released to moles arginine exposed during the initial stages of rennin action may partly be explained by the characteristics of the enzyme itself, and partly by either or both of the pseudo-effects produced in the Sakaguchi reaction discussed above.

The work of Dyachenko (79) indicated that the basic groups exposed by rennin action on casein stem mainly from arginine, and results from this study support this view.

It may also be pointed out that no new α-amino end group has been demonstrated on the ρ-casein or ρ-k- casein molecules (72), due to rennin action. This would be expected if the phosphorus was attached to guanido groups at either end, as suggested in this study. Cleavage of either -P-N-links would only expose guanido groups and not produce a new α-amino end group.

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