Observations on the Microbiological Determination of Riboflavin in Blood

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The present investigation was undertaken to devise a clinically useful method for the microbiological determination of the riboflavin content of the blood in various rheumatic diseases, and to elucidate certain factors which might influence the results.

The microbiological method of Strong, Feeney, Moore & Parsons (1941) involves the use of whole haemolyzed blood, which, on being autoclaved, produces a large amount of red-brown, fibrinous solid matter. This solid introduces uncontrollable variations into the final result. Further, the medium must be freshly prepared and it is difficult to deal with samples in an out-patient clinic if they cannot be collected into a working group over a period of 24 hr. Strong *et al.* (1941) found that their results were concordant when using tubes containing less than 0.2 ml. blood, but were inconsistent with larger volumes of blood. We find it more satisfactory to make an acid extract of blood, and so to remove a large proportion of the solid matter before the material is added to the assay tubes. The acid extract is stable and can be kept overnight, which greatly facilitates routine practice.

EXPERIMENTAL

Reagents required. Pure culture of *Lactobacillus casei* ϵ ; 0.1N-HCl; 0.1N-NaOH; 0.02N-acetic acid; sterile saline (0.85% (w/v)); Indicators (British Drug Houses Ltd.): (a) bromo-thymol blue; (b) bromo-thymol blue triple strength (capillator); (c) bromo-cresol green (capillator); culture medium as described by Snell & Strong (1939); stock riboflavin solution (100 μ g./ml.) in 0.02N-acetic acid prepared every 2 or 3 weeks, and stored under toluene in dark brown glass-stoppered bottles; Difco Yeast Extract (Digestive Ferments Co., Detroit); basic lead acetate ('Analar', British Drug Houses Ltd.); ammonia solution 3.1% (w/v); cellulose wadding (B.P. quality, Boots Pure Drug Co. Ltd.).

Organism used in assay. We experienced some irregularity at first with the culture obtained from the National Collection of Type Cultures (N.C.T.C., Lister Institute, Elstree) of *Lactobacillus casei* ϵ (Serial no. 7649 from the American Type Culture Collection). The culture from Elstree, differentiated on Crowe's (1927) chocolate medium, gave three definite morphological variants with differing responses to riboflavin (Fig. 1). Another culture from Mr S. A. Price (Vitamins Ltd.), also from the

N.C.T.C. originally, gave the same three variants (Fig. 1). A culture from Dr J. G. Davis (Shinfield), which was found to be pure, has been used in all our work.

Fig. 1. Growth response of different morphological variants in cultures of *L. casei* ϵ . Curves IA, IIA, IIIA: strains isolated from Lister Institute, Elstree culture, showing differences in acid production with same concentration of riboflavin. Curves IB, IIB, IIIB: similar to series IA, IIA, IIIA, but repeated at a later date with another specimen of Lister Institute, Elstree culture.

The previous nutritional history of the organism also affected the accuracy of the results. At first we carried the organism by daily subcultures into the assay medium containing 1.0 μ g. of riboflavin/10 ml. of medium (cf. Snell & Strong, 1939), returning to the stab culture once a week. We were puzzled by the initial 'kick' in the standard curve, as often a bigger response was given by 0.05 μ g. than by 0.075 μ g. of riboflavin, and differentiation between these small quantities was difficult. These results were far more regular when the inoculating emulsion was prepared from a single growth in liquid

medium, inoculated 24 hr. previously from the stab culture. In a stab culture several days old the organism probably tends to become deficient in riboflavin so that on subculturing in the assay tubes its response will be more nearly linear. On the other hand, in a liquid medium rich in riboflavin, some storage of the vitamin may occur either within the bacterial body or by adsorption on the cell surface. The effect produced is a much more vigorous growth by repeated subcultures in liquid medium, and the acid production is much more erratic. Fig. 2 shows the typical effect obtained with 'deficient' and 'replete' cultures.

Fig. 2. Effect of previous nutritional conditions on the response of *L. casei* to riboflavin. Curve A. 'Deficient' culture, i.e. 24 hr. broth culture direct from a stab culture 4 or 5 days old. Curve B. 'Replete' culture, i.e. 24 hr. broth culture after subculturing 4 days in succession on a broth rich in riboflavin (1 μ g./10 ml.).

Preparation of medium. The alkalinized photolyzed peptone (Snell & Strong, 1939) keeps well under toluene and can be made in bulk and used as required. The yeast supplement must be freshly prepared (from Difco yeast extract) for every batch and used within 24 hr. It appears to contain a labile factor which has a marked effect on the acid production, this being revealed as a central depression in the theoretically linear logarithmic curve. Fig. 3 shows a typical standard curve using a yeast extract several weeks old, compared with a similar curve obtained with fresh yeast extract 24 hr. old.

The yeast supplement is prepared as follows: 2.5 g. Difco yeast extract in 12.5 ml. distilled water are mixed with

3.75 g. basic lead acetate in 12.5 ml. distilled water and the pH adjusted to 10 by a few drops of ammonia (3.1% w/v). It is easy to judge the pH at which maximum precipitation of the solids occurs. The mixture is then filtered through a thin layer of cellulose wadding and acidified with enough glacial acetic acid to ensure complete precipitation

Fig. 3. Effect of ageing of riboflavin-free yeast supplement used in preparation of basal medium on the subsequent response of *L. casei* to riboflavin. Curve X. Deficient culture grown on medium containing freshly prepared yeast supplement. Curve Y. Deficient culture grown on medium containing old yeast supplement stored in the refrigerator for several weeks.

of the lead sulphide by H_2S and to prevent frothing during the passage of the gas. The lead sulphide is filtered off through cellulose wadding and the filtrate tested by bubbling H_2S until no further precipitation of lead occurs. The final solution is filtered through filter paper (Whatman no. 40) and boiled rapidly for 1–2 min. to remove H_2S . When cool, the solution is neutralized to pH 6.8 with $N-NaOH$, made up to 25 ml. and immediately placed in the refrigerator until required. Neutralization of the extract gave more consistent blank values in the assay tubes and the standard curve was more nearly linear. 5 ml. of this solution are required for every 250 ml. of concentrated medium.

Salt solutions are the same as those recommended by Snell & Strong (1939) but are made up in freshly boiled glass-distilled water and stored under toluene at room temperature.

Autoclaving. Precautions should be taken to prevent wetting of plugs during autoclaving. Unless the time and temperature are rigidly controlled, the medium darkens, which causes difficulty in reading the end-point in the final titration.

Preparation of acid extract of blood. Various concentrations of hydrochloric acid were tried in order to determine the optimum concentration for the extraction of riboflavin from blood.

The riboflavin content of the same sample determined direct by dilution with water only was used as a standard and the riboflavin determined on 0.25 ml. of blood, all other conditions being similar.

Table 1. *Effect of concentration of HCl on extraction of riboflavin*

(Results are the average of three replicates for each concentration of HCl.)

Sample	0.1 N-HCl in 10 ml. of extract (ml.)	Riboflavin content of whole blood ($\mu\text{g./100 ml.}$)	Percentage* of riboflavin extracted
X	4.0	35.2	100.0
	3.5	34.4	97.7
	3.0	28.8	81.8
	2.5	24.8	70.4
	2.0	22.8	64.7
	1.5	20.8	59.0
	1.0	16.4	46.5
	0.5	14.8	42.0
	4.0	24.0	100.0
Y	N-HCl in 10 ml. of extract (ml.)		
	4.0	22.0	91.6
	3.0	20.0	83.3
	2.0	16.0	66.6
	1.0	17.0	70.8
	0.75	22.8	95.0
	0.5	20.8	86.6
	0.25	26.0	108.3
	0.1	17.6	73.3
	Nil	20.0	83.3

* The results for percentage extraction were calculated on the 4.0 ml. of 0.1 N-HCl figures.

The results indicate that no advantage is obtained by increasing the concentration beyond 4 ml. of 0.1 N-HCl in 10 ml. extract (Table 1). The acid extract is adjusted to pH 6.8 by addition of NaOH. The addition of the neutralized extract to the medium might cause inhibition of growth on account of the increased concentration of sodium ions. That possibility was ruled out when an excess of either sodium chloride or sodium nitrate was found to have little effect upon growth.

The acid extract of blood thus prepared was checked against the original method of Strong *et al.* (1941) and found to give consistent results within the limits of experimental error (Table 2).

These results, obtained at the beginning of the work, indicate good agreement on the same levels for the whole blood and acid extract. The variation observed at different levels is dealt with later on and is apparently due to another factor added with

Table 2. *Riboflavin content as determined on whole blood compared with that determined on the acid extract from the same sample*

Date and Subject	Fresh whole blood		Acid extract of blood	
	Blood (ml.)	Ribo- flavin content ($\mu\text{g./}$ 100 ml.)	Blood (ml.)	Ribo- flavin content of blood ($\mu\text{g./}$ 100 ml.)
4. vi. 43	0.1	30.0	0.15	33.3
Subject R	0.2	32.5	0.20	32.5
	Mean	31.2	0.30	28.5
			Mean	31.4
4. vi. 43	0.15	41.0	0.15	40.0
Subject L	0.20	35.0	0.20	36.0
	Mean	38.0	Mean	38.0
22. vi. 43	0.10	40.0	0.10	40.0
Subject J (saturated with riboflavin)	0.15	36.6	0.15	36.0
	Mean	38.3	Mean	38.0

the blood. Taken from patients with pathological conditions these samples of blood might be expected to show other variables.

Standardization of volume of inoculum. As a result of parallel unpublished work by one of us (H. C.) on the population of cultures of *Staphylococcus aureus* it was apparent that the curves obtained in these microbiological assays are not 'growth curves' in the sense that measurement of the growth of animals is used in biological assay work. The acid production after 72 hr. incubation on any level of riboflavin is a measure of the maximum population supportable in the medium on that level of riboflavin. At lesser intervals of time the population may not have reached the stable maximum, in which case neither the acid production nor the opacity will bear a direct linear relationship to the concentration of riboflavin originally present in the tube. It has also been observed that the maximum stable population of *Staph. aureus* in a simple peptone medium can vary in direct relationship to the concentration of added food factors, e.g. glucose (cf. Hinshelwood, 1944). Hence to obtain true relative readings of riboflavin content in the Snell & Strong basal medium the following conditions must be assured: (a) time must be allowed for the population to reach a stable maximum; (b) the size of the original inoculum must be standardized; (c) the culture should be in a certain relatively deficient state. Hence we standardize the size of the drop. The weekly variations in growth response may be standardized by using a saline suspension of known opacity measured on the Spekker photoelectric absorptiometer, or by visual comparison with a standard suspension sealed in a centrifuge tube.

It is necessary to shake the tubes at least once during the 72 hr. incubation period in order to break

up bacterial clumps and thus ensure a more even rate of propagation. In measuring the rate of growth by opacity in less than 72 hr., as recommended in some papers, these factors are very important.

Poole & Hinshelwood (1940) and Hinshelwood (1944) were able to observe in cultures of *B. lactis aerogenes* four stages in the development of the stable maximum population: (a) lag-phase reaction, which is concerned with slow building up of some substance necessary for cell division; (b) actual processes involved in cell division; (c) metabolic reactions which go on even when active division has ceased; (d) lethal processes in which the actual death of the cell is accelerated. The growth-rate and stable maximum population which a given medium will support vary in a remarkably parallel manner, and growth of population follows closely the logarithmic law $dn/dt = kn$. At the end of the logarithmic phase growth falls off abruptly. It is therefore important in microbiological assays to select a time for titration at which the bacterial population is at a maximum and when the third phase of fermentation is complete. This occurs before the final or death phase of the culture is reached. In experiments on gas formation, Poole & Hinshelwood (1940) found no measurable amount of gas was collected until the bacteria had attained their stationary phase of growth, when the gas volume (presumably the fermentation stage) increased linearly with time, then abruptly ceased to increase.

The stationary phase is not due to a simple balancing of death-rate and division-rate but depends on actual cessation of division. This may be due to a decrease in the amount of a metabolite necessary for the fermentation reactions, e.g. riboflavin. The authors quoted above were working on the inhibitory action of disinfectants, e.g. phenol, mercuric chloride, etc. which had a toxic action. It is possible that the inhibitory substances which we found in blood may act at any one of the four phases of growth by immobilizing one of the essential metabolites in the development of *L. casei* ϵ .

RIBOFLAVIN IN BLOOD OF RHEUMATIC PATIENTS

Procedure

Preparation of extract. 1 ml. venous blood is mixed, immediately after withdrawal, with 4 ml. 0.1N-HCl in a conical centrifuge tube which is then closed with a waxed cork and inverted several times to mix its contents. The mixture turns dark brown in colour. A cotton-wool plug is substituted for the cork, care being taken to prevent loss of liquid on sides of cork and tube. The tube, which is placed in a tall 400 ml. beaker without lip and protected against moisture, is autoclaved (at a pressure of 15 lb./sq.in.) for 15 min. After autoclaving the tube is immediately placed in a dark cupboard to cool. The extract is stable for at least 24 hr. whereas the unheated acid solution deteriorates

overnight. When the assay is begun the contents of the tube are neutralized with 0.4 ml. of N-NaOH, mixed and back-titrated to pH 4.5 with a few drops of 0.02N-acetic acid (bromo-cresol green as indicator) and centrifuged. It is convenient to use tubes of equal weight for a series of tests. The supernatant liquor is transferred to a standard 10 ml. measuring flask. 2 ml. of glass-distilled water are added to the sediment and the mixture stirred by means of a fine sealed capillary tube and again centrifuged. The washing process is repeated with 2 ml. water, and the combined washings added to the 10 ml. flask. This extract is adjusted to pH 6.7 with a drop or two of 0.1N-NaOH before making up to 10 ml. The final solution may be centrifuged again if necessary.

The isoelectric point of most of the blood proteins is near pH 4.5. Precipitation at this pH ensures that a relatively small proportion of coagulable matter is carried through into the assay tubes.

Riboflavin solutions for standard curve. The stock solution of riboflavin is diluted with glass-distilled water to contain 0.1 μ g./ml. for all subsequent measurements, so that the smallest volume measured is 0.5 ml. in the standard curve.

Preparation of assay tubes. A standard curve is prepared with each batch of tests. Graded quantities of standard riboflavin solution are added to the 5 ml. of medium, and the volume is made up to 10 ml. by running freshly distilled water in from a burette with the mouth of the test-tube held against the tip of the burette and the test-tube rotated rapidly in the left hand. To avoid contamination of the contents of one tube with that of another we always inoculate and measure volumes to the tubes in order, starting with the lowest concentration.

The neutralized acid extract of blood is then added to the assay tubes in the same manner, and portions of 2 and 2.5 ml. in duplicate (equivalent respectively to 0.2 and 0.25 ml. of blood) are satisfactory. With less than 0.2 ml. of blood, the quantity of acid produced was below the lowest measurable quantity of riboflavin on the standard curve. The total volume is made up to 10 ml. as for the standard curve. The assay tubes and the standard tubes are autoclaved for 15 min. at a pressure of 15 lb./sq.in. at the same time and are then ready for inoculation. All measurements are done in a darkened room (diffused daylight) and all tubes, etc. containing riboflavin are protected from light at all stages.

Inoculation. The organism is carried in stab cultures in yeast-water-agar containing 1% of glucose; this is quite satisfactory if a 24 hr. culture in liquid medium is taken direct from stab. After centrifuging for 10 min. the bacterial cells are emulsified with 10 ml. sterile saline and may be checked for opacity on the Spekker photoelectric absorptiometer. To avoid standardization of capillary pipettes a sterile 1 ml. syringe fitted with a no. 10 (Allen & Hanbury Ltd.) needle about 2.5 in. long, from which the oblique tip has been cut, is satisfactory when a number of tubes are to be inoculated. Inoculation is accomplished by holding the tube in a vertical position with the needle at right angles to the axis of the tube and then releasing one drop rapidly. If alternate drops are discarded there is little danger of contamination from the outside air. The contents of the tubes are well and carefully mixed by agitation and rotation.

Incubation. When inoculated the tubes are incubated at 37° for 72 hr. At least once during the incubation period

and preferably after each 24 hr. the tubes are removed and mixed carefully and thoroughly to break up bacterial clumps.

Titration. If there is an appreciable amount of coagulum in the tubes containing blood it may be rapidly removed by filtering through a 1 in. square of cellulose wadding split to a half or a third of its thickness. These squares are washed with distilled water whilst supported in 1.5 in. funnels and the solution is filtered rapidly into 50 ml. hard glass Erlenmeyer flasks. The tube and filter pad are washed twice with 5 ml. of freshly distilled water and the excess moisture expressed from the filter pad by rolling and pressing with a fine glass rod. The contents of the assay tubes thus diluted to 20 ml. with washings are titrated against 0.1 N-NaOH with bromo-thymol blue as internal indicator. In the first instance 10 drops of indicator are added and then as titration proceeds 1 drop of indicator is added for every 1 ml. of alkali used. This keeps the intensity of the colour constant with increasing dilution and gives greater accuracy at the higher levels of riboflavin.

Examination of results. The acid production, plotted against the log of the concentration of riboflavin in the standard tubes, gives a straight line and a truer picture of the bacterial population than the ordinary standard curves of Snell & Strong (1939).

Accuracy of the method. Over a period of 11 months 43 standard curves have been determined. The correlation coefficient r , determined from the mean values of the 43 estimations for each standard concentration of riboflavin, shows a value = +0.85, which suggests a significant correlation. During this time the refinements in technique here described were evolved, so that the correlation coefficient taken on the same basis for the last fifteen curves may be expected to be a more accurate assessment of this technique, and shows a figure $r = +0.98$.

The assay tubes for normal blood usually give a reading equivalent to an amount of 0.075 μg . We derive the figures given in Table 3 from the series of 43 estimations of this amount.

Table 3. *Accuracy of the method*

	Mean value for tube containing 0.075 μg . riboflavin	Standard devia- tion of mean	Standard error of mean	Standard error as % of total
Total from 43 curves	3.88	0.80	0.122	3.14
Last 15 curves	4.42	0.73	0.188	4.25

The small number of readings obtained with a single specimen of blood and the effect of the unknown quantities and qualities of inhibitory substances does not permit a satisfactory statistical analysis of the accuracy of the method. We would estimate roughly from practical experience that the results lie within the range = $\pm 2 \mu\text{g}$./100 ml. of blood ($\pm 0.005 \mu\text{g}$. in the volume estimated). With an average reading of 25 μg ./100 ml. this is equivalent to $\pm 8\%$, the error becoming greater with smaller totals and less with greater total contents. The figure thus obtained is about twice the standard error (Table 3), as might be expected.

Results

The above method was devised to obtain information about the riboflavin concentration in the

blood in chronic rheumatic disease. The results here reported were measured in duplicate on 0.2 and 0.25 ml. of whole blood; eight normal subjects gave an average of 21.2 μg ./100 ml. One normal individual was tested on four occasions and gave the following results:

1943	μg ./100 ml.
May	31.0
June	35.0
September	13.1
September (1 week later)	13.5

This shows the likely variations to be expected over a period of war-time diet.

From 56 cases of chronic rheumatic disease the results, given in Table 4, were obtained.

Table 4. *Riboflavin content of rheumatic blood*

Riboflavin content (μg ./100 ml.)	10-30	31-50	Above 50
No. of cases	40	13	3
Mean riboflavin content (μg ./100 ml.)	21.3	43.7	76.3

Relation between riboflavin content and differential sedimentation test (D.S.T.) of rheumatic blood

D.S.T.* (%)	1-10	11-20	21-30	31-40	41-90
No. of cases	12	1	2	1	3
Mean riboflavin con- tent (μg ./100 ml.)	23.0	(33.5)	22.5	25	22

* The differential sedimentation test was carried out by the method of Coke (1937).

There is no striking difference between the riboflavin contents of normal and of rheumatic bloods and it is difficult to see any significant correlation between the riboflavin content of the blood and any other factor associated with the rheumatic condition, either of the clinical diagnostic type or of the nature of a laboratory assessment such as a differential sedimentation test (Coke, 1937) of the blood. Of particular interest was a patient with acute iridocyclitis who had been saturated with riboflavin. He was excreting 9.5 mg. of riboflavin/100 ml. of urine; his blood riboflavin was only 42 μg ./100 ml. From the clinical aspect one can record only the impression that the blood riboflavin may possibly be related to the patient's resistance or reactive capacity and thus may show a similarity to the results reported by Cottingham & Mills (1943).

SOME FACTORS AFFECTING THE MICRO-BIOLOGICAL ASSAY OF RIBOFLAVIN

Inhibitory factors in blood. To determine the accuracy of the method recovery experiments were carried out. A pooled sample of blood from five

patients was freed from riboflavin by the following method:

5 ml. of the pooled blood were diluted with 20 ml. of 0.1N-HCl and mixed with 5 ml. of a solution containing 1.08 g. of NaOH. (0.08 g. of NaOH is required to neutralize the 20 ml. of 0.1N-HCl and the resulting solution therefore contained 4% NaOH.) The 30 ml. of alkalized blood were

placed in a beaker of 2½ in. diameter and irradiated for 10 min. at a distance of 20 in. from an ultra-violet lamp (Hanovia Ltd.) by direct rays. The solution was transferred to a 50 ml. flask, neutralized with 0.96 ml. of glacial acetic acid, the pH adjusted to 6.7-6.8 and the volume made up to 50 ml.

A sample of this alkalized, photolyzed blood was assayed direct at levels ranging from 0.1 ml. by increments of 0.1 to 0.5 ml. Since no growth was observed at any level it may be assumed that the treatment destroyed the riboflavin and that no other stimulatory substances are present in blood. The resulting liquid was dark brown in colour and graded quantities were added to tubes containing 0.1-1.0 µg. of riboflavin/10 ml. of medium. The work was done on three different batches of pooled blood and the results calculated as percentage loss of riboflavin (Table 5).

It appears that there is some strongly inhibitory substance added with the alkalized photolyzed blood which can be detected with amounts of blood of 0.2 ml. or more. This 'inhibitory' effect is frequently noted in the assay of individual blood specimens (cf. Table 2). Such inhibitory substances are clearly important in the technical assay and in the interpretation of clinical riboflavin-deficiency conditions. Experiments showed that ultra-violet irradiation of the inorganic constituents added with the blood did not produce an inhibitory effect; the indication was that the inhibitory material was derived from the blood.

Effect of calcium salts. Calcium, in concentrations of 0.01-0.04 mg. and of 8-80 mg./10 ml. of medium, had no inhibitory effect.

SUMMARY

1. Some observations on the factors governing the technique of microbiological assays in general have been recorded.

2. A modified method for determining the blood riboflavin in 1 ml. of blood is described.

3. The presence of a factor or factors in blood which interferes with the microbiological assay of riboflavin has been shown.

4. The riboflavin contents of eight normal and of fifty-six rheumatic blood samples have been determined; no striking variations were found.

5. An attempt has been made to correlate the blood riboflavin with the sedimentation rate of rheumatic blood but no relationship has been established.

We wish to thank the Council and Medical Staff of the Charterhouse Rheumatism Clinic for the facilities and helpful atmosphere under which this work was carried out. Also we are grateful to Roche Products Ltd. for a very generous gift of riboflavin.

Table 5. *Effect of alkalized, photolyzed blood on recovery of added riboflavin*

Date	Ribo- flavin added to medium (µg./ 10 ml.)	Blood added to medium (ml./ 10 ml.)	ml. of 0.1N- acid formed*	Ribo- flavin found (µg./ 10 ml.)	Loss (%)
8. x. 43	0.1	0.05	6.95	0.12	(gain 0.02)†
		0.10	6.6	0.10	Nil
		0.15	3.4	0.03	67
		0.20	Nil	Nil	100
		0.25	Nil	Nil	100
8. x. 43	0.2	0.30	Nil	Nil	100
		0.05	8.7	0.22	(gain 0.02)†
		0.10	8.05	0.18	7
		0.15	2.05	0.16	17
		0.20	Nil	Nil	100
29. ix. 43	0.3	0.25	Nil	Nil	100
		0.1	7.9	0.19	35
		0.15	5.9	0.12	61
		0.20	0.7	Nil	100
		0.25	Nil	Nil	100
8. x. 43	0.5	0.30	Nil	Nil	100
		0.40	Nil	Nil	100
		0.1	8.0	0.18	62.9
		0.15	6.7	0.12	76.0
		0.20	1.4	0.01	98
19. xi. 43	0.6	0.25	Nil	Nil	100
		0.30	Nil	Nil	100
		0.40	Nil	Nil	100
		0.1	7.1	0.28	53.0
		0.2	Nil	Nil	100
19. xi. 43	0.7	0.3	Nil	Nil	100
		0.4	Nil	Nil	100
		0.1	7.2	0.29	58
		0.2	Nil	Nil	100
		0.3	Nil	Nil	100
11. ii. 44	0.8	0.4	Nil	Nil	100
		0.10	7.8	0.47	40
		0.15	7.1	0.37	53
		0.20	1.6	0.02	97
		0.30	Nil	Nil	100
11. ii. 44	0.9	0.40	Nil	Nil	100
		0.10	8.05	0.5	44
		0.15	7.4	0.4	55
		0.20	0.9	Nil	100
		0.30	Nil	Nil	100
11. ii. 44	1.0	0.40	Nil	Nil	100
		0.10	8.1	0.5	50
		0.15	7.2	0.48	52
		0.20	Nil	Nil	100
		0.30	Nil	Nil	100
		0.40	Nil	Nil	100

* Mean of duplicate assays.

† A value within the expected experimental error.

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Rennet Hysteresis and the Calcium Phosphate of Milk

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Heated milk exhibits a phenomenon known as rennet hysteresis, i.e. its time of coagulation shows a progressive increase with increase in the interval between heating and addition of rennet. Mattick & Hallett (1929) observed the effect in milk heated to temperatures ranging from 40 to 100°, and showed that a maximal and constant renneting time was attained in about 5 hr. Powell & Palmer (1935) later showed that the phenomenon was in all probability a property of the calcium caseinate-calcium phosphate complex of milk, demonstrable in artificial complexes of this type but not in calcium caseinate alone. They further observed that rennet hysteresis occurred both in solutions of the calcium caseinate-calcium phosphate complex to which gelatin had been added, and in gelatin-protected solutions of calcium phosphate to which calcium caseinate had been added. The nature of the protecting protein thus appeared to be a matter of indifference for the phenomenon, and the two forms of phosphate protection, by caseinate and by gelatin, seemed to differ little if at all. Earlier experiments by the writer (Pyne, 1934) had shown, on the other hand, that these two systems reacted differently with formaldehyde, whence it appeared that the nature of the protection was essentially different in the two cases; protection of calcium phosphate by caseinate in milk being, it was suggested, chemical rather than physical. In view of the apparently divergent behaviour of the system the experiments of Powell & Palmer were repeated by the author, who was unable to confirm them, the type of hysteresis being found to depend on the nature of the protecting protein. An explanation of the apparent origin of these differences is given later.

In view of these findings it was decided to examine further the whole phenomenon. The great sensitivity

of the rennet coagulation to changes in the concentration of calcium ions suggested that the shift in equilibrium of the caseinate-phosphate complex postulated by Powell & Palmer might take the form of a temporary change in the concentration of these ions in milk serum as a result of heating. Heat treatment, especially if severe, is known to produce permanent changes of this kind and it seemed possible that it might also give rise to short-time changes hitherto overlooked owing to the relatively long interval generally elapsing between heating and analysis. The comparative slowness with which phosphate equilibrium is attained in milk, as exemplified by the changes in soluble calcium content which occur when milk is shaken for prolonged periods (Lampitt, Bushill & Filmer, 1937), lends support to such a suggestion.

EXPERIMENTAL

Freshly separated skim milk was used and only one heat treatment was in general employed, 80–85° for 30 min. Calcium caseinate-phosphate complexes were prepared as by Powell & Palmer (1935), colloidal calcium phosphate protected by gelatin by the method of De Toni (1921). Renneting was carried out in a thermostat at temperatures ranging from 30 to 40°, and in general without addition of calcium salts, though such addition was necessary for the artificial caseinate-phosphate-gelatin complexes. Rennet, either as the commercial liquid extract or from tablets, was used in such amounts as to give convenient renneting times (2–10 min.). Coagulation times were determined for larger samples as by Powell & Palmer (1935), and for small samples by direct observation of curd formation.

For conductivity measurements the Kohlrausch bridge and telephone with valve amplification was employed, for freezing-point determinations, the Hortvet cryoscope.

Analytical methods for calcium and phosphate, where not otherwise stated, were those used by Pyne (1940).