SOME ASPECTS OF RIBOFLAVIN METABOLISM IN THE RUMINANT

A Thesis submitted to the University of Glasgow for the Degree of Doctor of Philosophy in the Faculty of Science

by

LEIGH INGRAM HART

The Hannah Dairy Research Institute,

Ayr.

April 1964

CONTENTS

SECTION

SECTION

LIST OF TABLES

LIST OF FIGURES

SECTION I

INTRODUCTION

1. Historical

Bourquin & Sherman (1931) showed that cow's milk was effective in promoting the growth of rats deficient in vitamin B_2. The constituent of cow's milk possessing vitamin B_2 activity was isolated in a pure form from the whey of cow's milk by Kuhn, György & Wagner-Jauregg (1933), who named it lactoflavin. Proof that lactoflavin was in fact 6,7-dimethyl-9-(1'-D-ribityl)-iso-alloxazine was obtained by Karrer, Schöpp & Benz (1935), who renamed the compound riboflavin.

György, Kuhn & Wagner-Jauregg (1934) observed that most but not all of the riboflavin content of cow's milk was diffusible and was therefore present in the free form. Euler & Adler (1934) found that 20 - 25% of the total riboflavin of cow's milk was non-diffusible, and was therefore bound to some substance of high molecular weight which, they suggested, might be an enzyme. Houston, Kon & Thompson (1940 a, b), using a fluorimetric method for the estimation of riboflavin, observed that about 10% of the total riboflavin of cow's milk was in a bound form, and that this bound riboflavin was released on heating the milk. This observation was confirmed by Hoeflake (1953), who found that about 10% of the total riboflavin of cow's milk was not utilized by the riboflavin-requiring organism *Lactobacillus casei* ATCC 7469, but was converted to a utilizable form by heating the milk.

2. Xanthine oxidase and flavin nucleotides in cow's milk

The first indication of the nature of this bound riboflavin of cow's milk came from the work of Ball (1938, 1939) who isolated the enzyme xanthine oxidase (xanthine: O_2 oxidoreductase, EC 1.2.3.2) from this source, and showed that it was a flavoprotein. He considered that the flavin prosthetic group was similar to, but not identical with, flavin-adenine dinucleotide (FAD). At the same time, Corran & Green (1938 a, b), while attempting to purify milk xanthine oxidase, isolated from cow's milk an enzymically inactive flavoprotein, the flavin moiety of which resembled FAD. Shortly afterwards Corran, Dewan, Gordon & Green (1939) isolated an active preparation of xanthine oxidase from cow's milk and showed that the flavin constituent of this was indeed FAD. Xanthine oxidase activity had been observed much earlier in milk by Morgan, Stewart & Hopkins (1922) but the work of Ball and of Corran et al. (1939) gave the first indication that the enzyme was a flavoprotein. Later, it was shown that the enzyme also contained molybdenum (Totter, Burnett, Monroe, Whitney & Gomar, 1953; Green & Beinert, 1953), and iron (Richert & Westerfeld, 1954).

Modi & Owen (1956) extracted cow's milk with trichloroacetic acid and found, using the paper chromatographic method of Grammer (1948), that the only flavins present in the extract were riboflavin and FAD. They observed also that no FAD was present in ultra-filtrates of cow's milk. Manson & Modi (1957), who also

used Crammer's (1948) method, detected the presence of riboflavin and of flavin mononucleotide (FMN), but not of FAD, in such ultrafiltrates. They observed that free FAD appeared when fresh cow's milk was heated to 95°, and concluded from these observations that all the FAD of cow's milk was present in a bound form. Modi, Owen & Darroch (1959) separated the flavins in trichloroacetic acid extracts of cow's milk by Crammer's (1948) method, and estimated that 16 - 27% (mean 21%) of the total riboflavin of cow's milk was bound as FAD.

Recent surveys have shown that in Britain almost 40% of the total amount of riboflavin in the national diet is derived from milk (Milk Composition in the United Kingdom, 1960). Since the results of Modi et al. (1959) quoted above indicate that about one-fifth of the total riboflavin of cow's milk is in the form of FAD bound to some constituent of the milk, it was obviously important to determine the nature of this constituent. The question arises: Is the bound riboflavin of cow's milk accounted for by the FAD of xanthine oxidase, or are other flavin compounds present in addition to xanthine oxidase? So far this problem does not appear to have been investigated.

3. Evidence for the occurrence of other flavoproteins in cow's milk

Bergel and Bray and their collaborators isolated and studied the properties of many highly purified preparations of xanthine oxidase from cow's milk (Avis, Bergel & Bray, 1956; Bergel & Bray, 1958; Bray, Malmström

& Vånngård, 1959; Bray, Pettersson & Ehrenberg, 1961). Their best preparations were homogeneous by the usual criteria applicable to proteins (Avis, Bergel, Bray, James & Shooter, 1956), but the composition, specific activities and electron-spin resonance properties of these preparations varied considerably from one preparation to another. To account for this variation, Bray *et al.* (1961) postulated that their most highly purified preparations consisted of mixtures of varying amounts of the active enzyme with two other closely related but enzymically inactive metalloflavoproteins (see Table 18, p. 90 of this thesis). Bray *et al.* (1961) did not attempt to determine the origins of these components. Their presence in purified preparations might be explained by one or both of the following possibilities:- (1) they are present in cow's milk together with the active enzyme, and accompany it throughout the purification procedure; (2) they arise by degradation of the active enzyme during the purification procedure. At present, there appears to be no published evidence favouring either of these possibilities.

It is clear from the work of Bergel and Bray and their collaborators that the study of the properties of enzymically active xanthine oxidase from cow's milk is hampered by the presence in purified preparations of variable amounts of inactive flavoproteins. Progress in such studies would be greatly facilitated if the pure active enzyme, free from these contaminating flavoproteins, could be prepared.

4. Present investigations

A necessary preliminary to the preparation of pure enzymically active milk xanthine oxidase would be to determine the origins of the inactive flavoproteins found in the preparations of Bergel and Bray and their collaborators. The work presented in this thesis was therefore undertaken with the following objectives in view -

(a) To determine the contribution of the FAD of xanthine oxidase to the total bound riboflavin of cow's milk, and, if this enzyme was found not to account for all the bound riboflavin, to attempt to identify and to characterize the other flavin compound or compounds present.

(b) To determine the origins of the inactive flavoproteins occurring in purified preparations of xanthine oxidase from cow's milk, and to study the factors controlling their appearance in such purified preparations.

SECTION II

METHODS

1. Reagents and materials

(a) Water. Distilled water from an electrically heated still with a tinned copper boiler and resistance-glass condenser was used throughout the course of this work. The distilled water was stored in a Pyrex glass aspirator bottle fitted with a glass stopcock.

(b) Reagents for enzymic assays. Xanthine (laboratory reagent grade, not less than 97% pure) and DL-alanine (micro-analytical reagent grade) were obtained from British Drug Houses Ltd., Poole. Before use they were dried in a desiccator over dried magnesium perchlorate ("Anhydrone") at room temperature.

FAD used as an assay standard was Grade I obtained from the Sigma Chemical Company, St. Louis, U.S.A. It was stored at -20° in a brown glass bottle inside a light-tight, air-tight package containing silica-gel as desiccant.

Glycylglycine, L-cysteine hydrochloride monohydrate, orotic acid, crude D-amino acid oxidase and crystalline catalase were obtained from L. Light & Co. Ltd., Colnbrook.

Glycylglycine-sodium phosphate buffers were made by dissolving equimolar quantities of glycylglycine and Analar grade sodium dihydrogen phosphate in water and adjusting the solution to the desired pH with Analar grade sodium hydroxide. Glycine-sodium phosphate buffers were made in a similar way using Analar grade glycine.

Sodium pyrophosphate buffers were made either from Analar grade tetrasodium pyrophosphate adjusted with Analar grade hydrochloric acid, or from Analar grade tetrasodium pyrophosphate and disodium dihydrogen pyrophosphate (B.D.H. laboratory reagent grade).

All buffers were checked with a pH meter and glass electrode.

The ethanol and potassium hydroxide used in the enzymic reactions were of Analar grade.

(c) Reagents for molybdenum assays. Standard molybdenum solutions were prepared from Analar grade molybdenum trioxide. Before use, this was dried at 105° for 4 hr. and then stored in a desiccator over Anhydrone.

Toluene-3,4-dithiol (dithiol) (spot-test reagent grade) and thioglycollic acid (laboratory reagent grade) were obtained from British Drug Houses Ltd., Poole.

Thiourea (organic reagent for metals grade) and sodium iodide (B.P. grade) were obtained from Hopkin & Williams Ltd., Chadwell Heath.

All the other chemicals used for the molybdenum assays were of Analar grade.

2. The determination of the xanthine oxidase activity of milk

(a) Application of the manometric method of Rodkey & Ball (1946). In the initial stages of this work, the manometric method of Rodkey & Ball (1946), modified by the substitution of an equimolar amount of xanthine for the hypoxanthine originally specified, was used for the

determination of the xanthine oxidase activity of cow's milk. This modification was made in order to simplify the kinetics of the system since the oxidation of hypoxanthine to uric acid is a two-stage process with xanthine as the intermediate oxidation product. The modified method was as follows -

In the body of a Warburg flask with a single side arm were placed 2 ml. of milk and 1 ml. of 0.1M-sodium phosphate buffer (pH 7.2), and 0.2 ml. of 0.05M-xanthine dissolved in 0.07N-NaOH was placed in the side arm. The flask was then attached to its appropriate manometer, placed in the Warburg water bath at 37°, and allowed to equilibrate for 5 min., after which blank readings were taken every 6 min. for a 30-min. period. The reaction was then started by adding the xanthine from the side arm, and readings were taken every 6 min. for a period of 48 min. The concentration of phosphate ion in the reaction mixture was 0.031M, and the initial concentration of xanthine was 3.1mM. the initial rate of the reaction was determined from a plot of manometer readings against time, and this, expressed as microlitres (µl.) of oxygen per hr. per ml. of milk, was taken as the measure of the activity of the milk.

Determinations of the activity of cow's milk by this method were unsatisfactory. The agreement between replicates was poor, and the results were erratic and non-reproducible. On investigation it was found that the total oxygen uptake resulting from the complete oxidation of a given amount of xanthine was both variable and non-stoicheiometric. It followed from this that

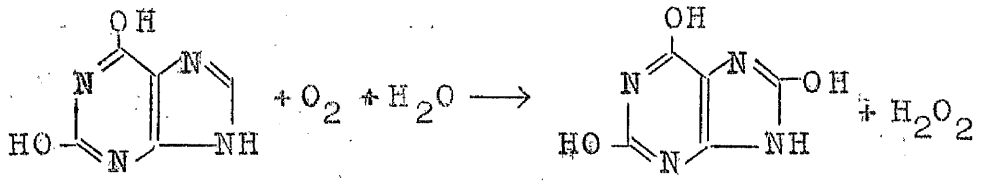
the rates of uptake of oxygen must have been subject to a variable and indeterminate error. Preliminary determinations of the variation of the activity of cow's milk with pH indicated that the optimum pH was between 8.5 and 9, but the graphs in which activity was plotted against pH were very variable both in the shapes of the curves and in the positions of the maximum values. It was concluded that this was due to the presence in cow's milk of a bound form of xanthine oxidase which, until released, was enzymically inactive (p. 27). It seemed possible that exposure to alkaline conditions was releasing this bound activity, but to a variable and erratic degree.

Before investigating these difficulties, the sensitivity of the method was increased approximately three-fold by increasing the pH from 7.2 to 8.5. The buffer used was an equimolar mixture of glycylglycine (pK_a 8.1) and sodium dihydrogen phosphate (pK_2 7.2), all adjusted to pH 8.5. Phosphate ion was included because it was found that in its presence the agreement between replicates was improved. At this higher pH it was found necessary to increase the buffer concentration in the reaction mixture from 0.031M to 0.16M in order to maintain the pH constant throughout the course of the reaction. This increase in buffer concentration was found to be without effect on the rate of the reaction. Potassium hydroxide (20% w/v), together with a roll of filter paper (Whatman no. 41), was added to the centre well of the Warburg flask to

absorb carbon dioxide.

The modified system then consisted of 1 ml. milk + 2 ml. 0.25M-glycylglycine-sodium phosphate buffer (pH 8.5 at 20°) + 0.2 ml. 0.05M-xanthine (10μmoles) at a reaction temperature of 37°. As before, the reaction was started by adding xanthine from the side arm. The first manometer reading was made 6 min. later and successive readings were made at 3-min. intervals for a total reaction period of at least 30 min.

(b) <u>Investigations into the non-stoicheiometric oxygen uptake of the manometric method.</u> The aerobic oxidation of xanthine to uric acid in the presence of xanthine oxidase takes place according to the equation



(1)

(xanthine) (uric acid)

Cow's milk always contains catalase (Jenness & Patton, 1959 a), so that, in the presence of cow's milk, the further reaction

$$H_2O_2 \longrightarrow H_2O + \tfrac{1}{2}O_2 \qquad (2)$$

should follow. If reaction (2) goes to completion, then the overall reaction will be

$$\underset{\text{(xanthine)}}{\text{-N=CH-NH-}} + \tfrac{1}{2}O_2 \longrightarrow \underset{\text{(uric acid)}}{\text{-N=C(OH)-NH-}} \qquad (3)$$

The complete oxidation of 0.2 ml. of 0.05M-xanthine (10μmoles) to uric acid according to equation (3) should be accompanied by the uptake of 112μl. of oxygen.

In one experiment the observed total uptakes of oxygen resulting from the oxidation of 10 µmoles of xanthine in the system described in the previous paragraph varied from 140 to 150 µl. In another experiment, given in detail in Table 1, the same system was used but the pH was varied from 7.2 to 9.2. The total uptakes of oxygen observed in this experiment varied from 128µl. (114% of theoretical) to 175µl. (156% of theoretical). It is obvious that positive errors of this magnitude must cause corresponding positive increases in the rates of oxygen uptake, and thus lead to false estimates of the activity of the enzyme.

This finding obtained here that the total uptakes in this system can be considerably in excess of the theoretical values is not in agreement with the observations of Morgan et al. (1922) or of Dixon (1925), who claimed that the uptakes were stoicheiometric. However, Morgan et al. (1922) recorded that occasionally the total uptake accompanying the oxidation of xanthine was 10 to 20% in excess of theoretical, but they could give no explanation for it. The explanation of the discrepancy between the observations made here and those of Morgan et al. (1922) and of Dixon (1925) probably lies in the fact that both these authors used the Barcroft type of manometer for their determinations of total uptake, whereas in the present work the Warburg type of manometer was used. The design of the Warburg manometer permits the exact determination of the pressure at the beginning of

Table 1. Effect of addition of a catalatic amount of catalase on the total oxygen uptake of cow's milk + xanthine at various pH values

Buffer pH at 20°	Total oxygen uptake (µl.)	
	No added catalase	3µg. crystalline catalase added
7.2	135	-
7.8	163	120
8.2	175	120
8.6	168	129
8.8	158	126
9.0	-	132
9.2	128	133

Reaction mixture - 1 ml. cow's milk + 2 ml. 0.25M-glycylglycine-sodium phosphate buffer + 10 µmoles xanthine in a reaction volume of 3.2 ml. at 37°. Gas phase, air. Filter paper + 0.2 ml. 20% w/v KOH in centre well.

Theoretical total uptake of oxygen in presence of catalase = 112 µl.

a reaction, but this is not possible with the Barcroft type, in which the reaction must be started before any pressure readings can be taken. This procedure would lead both Morgan *et al.* and Dixon to underestimate their total oxygen uptakes, which would thus be thought to be nearer to the theoretical figure than they actually were.

Harrison & Thurlow (1926) showed that hydrogen peroxide produced during the aerobic oxidation of hypoxanthine in the presence of xanthine oxidase could oxidize some unidentified milk constituent, provided that a peroxidase was also present. The lactoperoxidase of cow's milk was shown to be effective in this respect. This suggests that the non-stoicheiometric total oxygen uptakes observed in the present work are due to competition by the catalase and the lactoperoxidase of cow's milk for the hydrogen peroxide produced during the oxidation of xanthine. The relevant reactions are

$$H_2O_2 \longrightarrow H_2O + \tfrac{1}{2}O_2 \quad \text{(catalatic reaction)} \qquad (4)$$

$$H_2O_2 + H_2A \longrightarrow A + 2H_2O \quad \text{(peroxidatic reaction)} \qquad (5)$$

where H_2A represents the oxidizable milk constituent. If these two reactions are proceeding simultaneously, then since one mole of hydrogen peroxide is produced for every mole of xanthine oxidized to uric acid, the observed total oxygen uptake will lie between 0.5 mole and 1 mole of oxygen per mole of xanthine present in the reaction mixture.

From the foregoing it is clear that this simple method for the determination of xanthine oxidase activity

in milk is subject to serious errors due to the reactivity of the hydrogen peroxide produced. It may be noted that the method of Ball (1939), from which this method is derived, is still used, for example by Gudnason & Shipe (1962). At this stage it was felt that the manometric type of method was still the procedure of choice, so methods of removing the hydrogen peroxide formed were sought. Dixon (1925) observed that the addition of catalase to the system milk + buffer + hypoxanthine prevented the destruction of xanthine oxidase by hydrogen peroxide. The experimental evidence for this was that in the absence of added catalase the initial rate of the uptake of oxygen declined soon after the reaction began. In the presence of added catalase the initial rate of uptake, although less than the initial rate in the absence of added catalase, was maintained until all the hypoxanthine was oxidized to uric acid. The total uptakes were said to correspond to the oxidation of hypoxanthine according to the equation

$$1 \text{ mole hypoxanthine} + O_2 \rightarrow 1 \text{ mole uric acid} \qquad (6)$$

These observations, with the exception of the stoicheiometry of the total uptake of oxygen, were confirmed during the present work (Figs. 1 and 2 and Table 1). The decline in the initial rate of uptake in the absence of added catalase, and the constancy of the initial rate of uptake in the presence of added catalase, are shown in Fig. 1. The decrease in the rate of uptake caused by the addition of catalase is shown in Figs. 1 and 2. Table 1 and Fig. 1 show that the addition

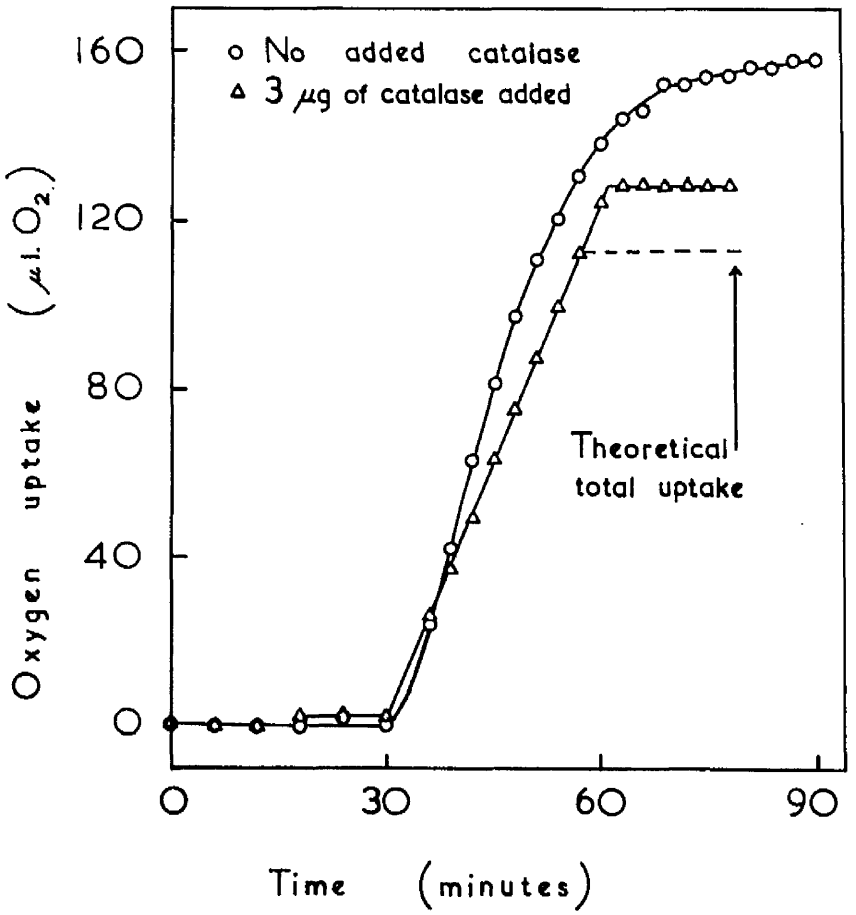


Fig. 1. Effect of addition of a catalatic amount of catalase on the oxygen uptake of cow's milk + xanthine.

Reaction mixture - 1 ml. cow's milk + 2 ml. 0.25M-glycylglycine-sodium phosphate buffer (pH 8.8) + 10 μmoles xanthine in a reaction volume of 3.2 ml. at 37°. Gas phase, air. Filter paper + 0.2 ml. 20% w/v KOH in centre well.

The reaction was started by adding the xanthine from the side arm at the 30-min. point.

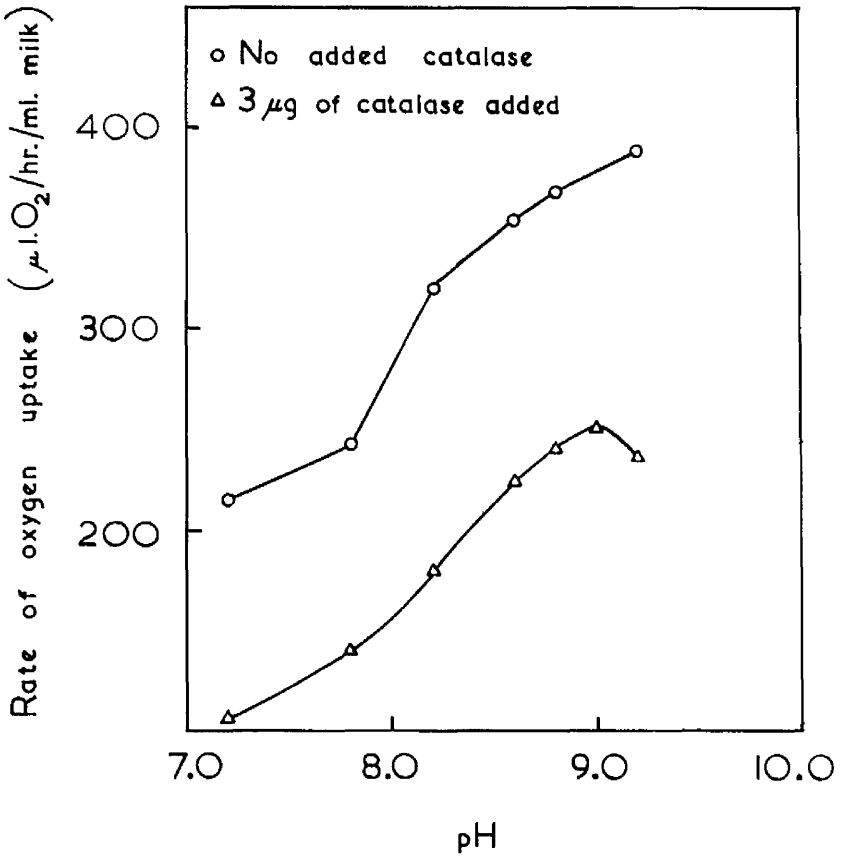


Fig. 2. Effect of addition of a catalatic amount of catalase on the rate of uptake of oxygen by cow's milk + xanthine at various pH values.

Reaction mixture - as for Fig. 1.

to the system of 3 μg. of catalase reduced the total uptakes to figures much nearer to, but still in excess of the theoretical figure. It was found that amounts of added catalase ranging from 0.5 μg. to 3 μg. were all equally effective in reducing the total uptakes to figures of the order of those shown in the second column of Table 1. Many determinations of total uptake were made subsequently using this system with 3 μg. of added catalase, but it was invariably found that the total uptake was in excess of theoretical to about the degree shown in Table 1. A possible explanation of this seemed to be that since lactoperoxidase is present in cow's milk in considerable amounts (Jenness & Patton, 1959 b), it was still able to compete with the increased concentration of catalase for the hydrogen peroxide (cf. Baldwin, 1959). A more efficient method for removing the hydrogen peroxide was therefore sought. It was thought that the addition to the system without added catalase of a substance capable of acting as an electron donor for lactoperoxidase might result in the peroxidatic reaction taking precedence over the catalatic reaction. The lactoperoxidase electron donors phenol, thymol, benzoic acid and salicylic acid were selected for testing on the basis of their unreactivity towards oxygen. Total oxygen uptakes of the system 1 ml. milk + 2 ml. 0.25M-glycylglycine-sodium phosphate buffer (pH 8.8) + 0.2 ml. 0.05 M-xanthine (10μmoles) in the presence of 20 μmoles of each of these were determined. For comparison, the effect of adding a catalatic amount of

catalase to the system was also determined. The results are shown in Table 2.

It is clear from these results that the addition of catalase had the usual effect of reducing the magnitude of the peroxidatic reaction (compare with Table 1). It is also seen that of the four electron donors for lactoperoxidase that were added, only phenol reacted under these conditions, and that the total uptake in its presence was in excess of theory. This may have been due to the occurrence of non-enzymic oxidation by oxygen of the oxidation product of the phenol in addition to the peroxidatic oxidation. The uptake of oxygen in blank determinations was negligible in all the reaction mixtures of this experiment. In view of these results this approach was not pursued further.

The next attempt to remove the interfering hydrogen peroxide was based on the observation of Keilin & Hartree (1936, 1945) that under suitable conditions catalase can function as a peroxidase. Keilin & Hartree found that concentrations of catalase of the order of 0.5 to 2μM, together with a suitable electron donor such as ethanol, added to a system generating hydrogen peroxide, caused the hydrogen peroxide to be quantitatively utilized as electron acceptor for the oxidation of the ethanol to acetaldehyde. In the system xanthine oxidase + xanthine + catalase (0.5 - 2μM) + ethanol, the consecutive reactions according to Keilin & Hartree are

$$\underset{\text{(xanthine)}}{\text{-N=CH-NH-}} + O_2 + H_2O \longrightarrow \underset{\text{(uric acid)}}{\text{-N=C(OH)-NH-}} + H_2O_2 \qquad (7)$$

Table 2. The effect on the total oxygen uptake of cow's milk + xanthine of addition of a catalatic amount of catalase, and of various electron donors for lactoperoxidase

Addition	Total oxygen uptake (µl.)
None	187
Catalase (3µg.)	134
Phenol (20µmoles)	254
Thymol (20µmoles)	182
Benzoic acid (20µmoles)	183
Salicylic acid (20µmoles)	193

Reaction mixture - 1 ml. cow's milk + 2 ml. 0.25M-glycylglycine-sodium phosphate buffer (pH 8.8) + 10µmoles xanthine in a reaction volume of 3.2 ml. at 37°. Gas phase, air. Filter paper + 0.2 ml. 20% w/v KOH in centre well.

Theoretical total oxygen uptake for the catalatic reaction = 112 µl.

Theoretical total oxygen uptake for the peroxidatic reaction = 224 µl.

$$H_2O_2 + CH_3CH_2OH \longrightarrow CH_3CHO + 2H_2O \qquad (8)$$

and the overall reaction is thus

$$-N{=}CH{-}NH- + O_2 + CH_3CH_2OH \longrightarrow -N{=}C(OH){-}NH- + CH_3CHO + H_2O \qquad (9)$$

A difficulty that arises in this particular system is that the acetaldehyde produced is itself a substrate of xanthine oxidase, and must therefore be removed if the stoicheiometry of the system is to be preserved. Keilin & Hartree (1945) have shown that this can be accomplished by adding strong potassium hydroxide solution together with filter paper to the centre well of the reaction flask. This causes the rapid condensation of the acetaldehyde to an inactive polymer, and thus effectively removes it from the reaction mixture.

In the present work, this system was tested by adding 1mg. of crystalline catalase (final concentration in reaction mixture 1.2μM) and 100 μmoles of ethanol to the system 1ml. milk + 2 ml. 0.25M-glycylglycine-sodium phosphate buffer (pH 8.8) + 0.2ml. 0.05M-xanthine (10μmoles) at 37°. Potassium hydroxide (0.2 ml. 20% w/v) together with filter paper was placed in the centre wells of the reaction flasks. Total uptakes and rates of uptake in the presence and absence of catalase + ethanol, when present together, were determined. The effect of adding a catalatic amount of catalase (3 μg.) to this system was also determined.

The results, recorded in Table 3, show that the addition of catalase + ethanol to the system brought the

Table 3. The effect on the total oxygen uptake and rate of oxygen uptake of cow's milk + xanthine of addition of a catalatic amount of catalase, and of a peroxidatic amount of catalase + excess ethanol

Addition	Total oxygen uptake (µl.)	Rate of oxygen uptake (µl./hr./ml. milk)
None	184, 190	424, 414
3µg. catalase	133, 135	326, 326
1 mg. catalase + 100 µmoles ethanol	230, 230, 229	491, 481, 485

Reaction mixture - 1 ml. cow's milk + 2 ml. 0.25M-glycylglycine-sodium phosphate buffer (pH 8.8) + 10 µmoles xanthine in a reaction volume of 3.4 ml. at 37°. Gas phase, air. Filter paper + 0.2 ml. 20% w/v KOH in centre well.

Theoretical total oxygen uptake for the peroxidatic reaction = 224 µl.

total uptake to the expected theoretical figure within the limits of experimental error. This result was confirmed by many subsequent determinations. In other experiments the minimum concentration of catalase necessary to maintain the total uptake at the theoretical figure was found to be 0.6μM; twice this concentration was adopted for routine determinations. In the presence of a concentration of catalase of at least 0.6μM the rate of uptake was constant throughout the course of the reaction as shown in Fig. 3.

Table 3 shows the considerable increase in the rate of uptake achieved with this system compared with the rate resulting from the addition of a catalatic amount of catalase only. It can be seen also that the total uptake in the presence of a peroxidatic amount of catalase and excess ethanol (the Keilin & Hartree system) was double that of the catalatic system for a given amount of xanthine. This permitted both a reduction in the volume of milk required for a determination of activity and a reduction in the concentration of xanthine in the reaction mixture. This last is desirable since xanthine oxidase is subject to inhibition by excess of substrate, the optimum concentration of xanthine for maximum activity being about 0.1 mM (Hofstee, 1955). The initial concentration of xanthine used in the present work up to this point was 10μmoles in 3.2 - 3.3ml., i.e. 3mM, which from the data of Hofstee (1955) and of Bray (1959) would be markedly inhibitory. With the adoption of

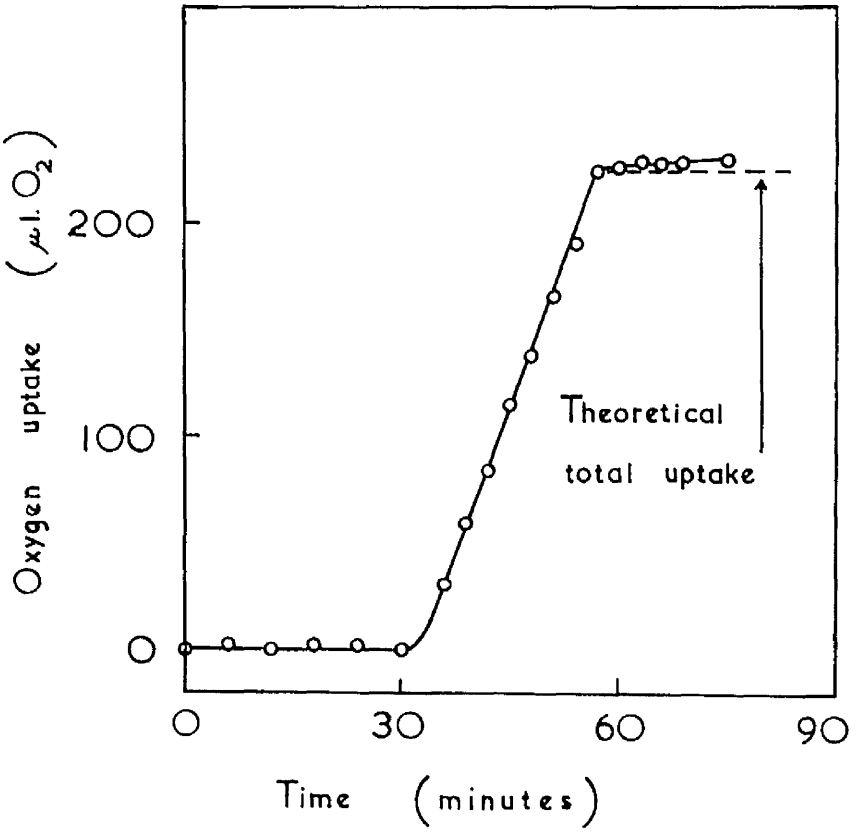


Fig. 3. The oxygen uptake of cow's milk + xanthine + excess ethanol in the presence of a peroxidatic amount of catalase.

Reaction mixture - 1 ml. cow's milk + 2 ml. 0.25M-glycylglycine-sodium phosphate buffer (pH 8.8 at 20°) + 0.5 mg. catalase + 100 µmoles ethanol + 10 µmoles xanthine in a reaction volume of 3.4 ml. at 37°. Gas phase, air. Filter paper + 0.2 ml. 20% w/v KOH in centre well.

The reaction was started by adding the xanthine from the side arm at the 30-min. point.

the Keilin & Hartree system it proved possible to reduce the xanthine concentration to 4μmoles in 3.4 - 3.5ml., i.e. about 1.2mM. This concentration was still inhibitory but considerably less so than the concentration of 3mM, as shown in Fig. 4. It is sufficient to saturate the amounts of enzyme normally present in this system, since the Michaelis constant for xanthine is of the order of 1μM (Bray, 1959). Four μmoles of xanthine were found to provide the lowest practicable amount which would allow a sufficient number of manometric readings to be taken during a fast reaction (rate of uptake >200μl. O_2/hour) before the reaction stopped owing to complete conversion of the xanthine to uric acid (Fig. 5).

(c) Determination of the optimum pH of activity of milk xanthine oxidase. After achieving stoicheiometry of reaction by use of the Keilin & Hartree system, attempts were made to determine the optimum pH of the complete system in order to obtain the maximum sensitivity possible for the determination of xanthine oxidase activity in milk.

Initially, the variation of activity of whole cow's milk in the pH range 8 - 10 was determined using glycylglycine (pK_a 8.1) and glycine (pK_a 9.8). As before, equimolar amounts of sodium dihydrogen phosphate were added to the buffers which were then adjusted with sodium hydroxide to their nominal pH values at 20°. The milk was treated to release bound xanthine oxidase by shaking it for 20 - 30 min. at room temperature

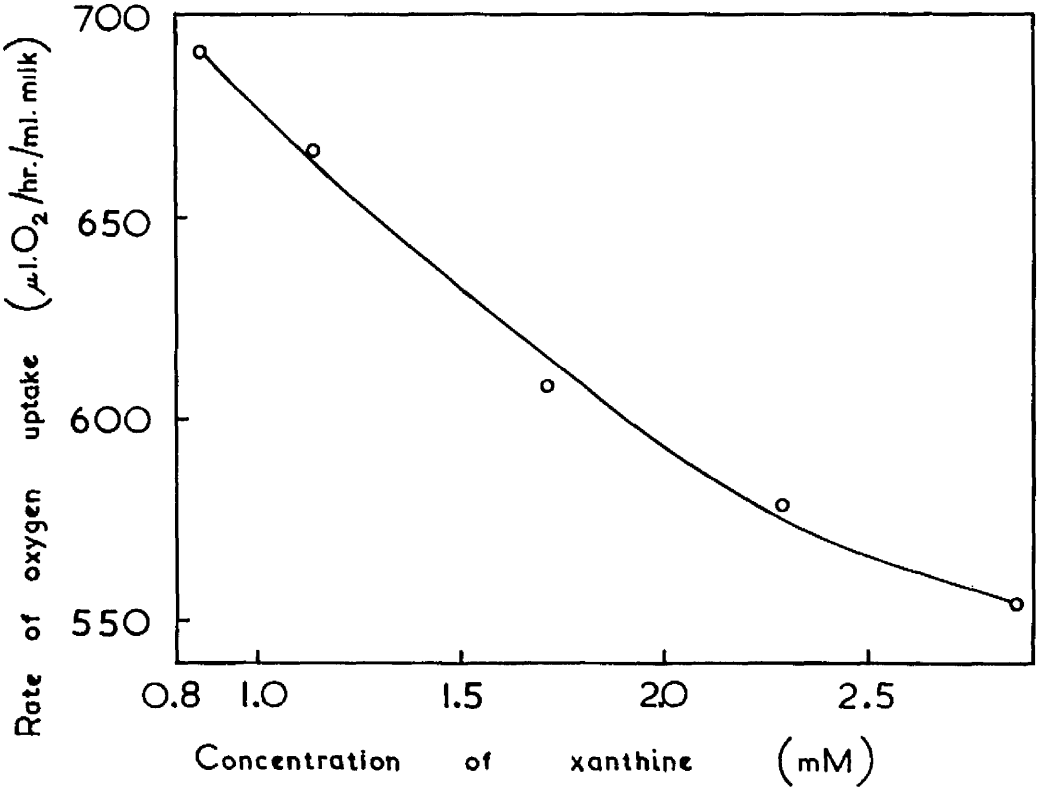


Fig. 4. Effect of xanthine concentration on the xanthine oxidase activity of cow's milk.

Reaction mixture - 0.2 ml. cow's milk + 2 ml. 0.25M-glycine-sodium phosphate buffer pH 9.2 at 20° (pH 8.8 at 37°) + 1 mg. catalase + 100 µmoles ethanol + xanthine in a reaction volume of 3.5 ml. at 37°. Gas phase, air. Filter paper + 0.2 ml. 20% w/v KOH in centre well.

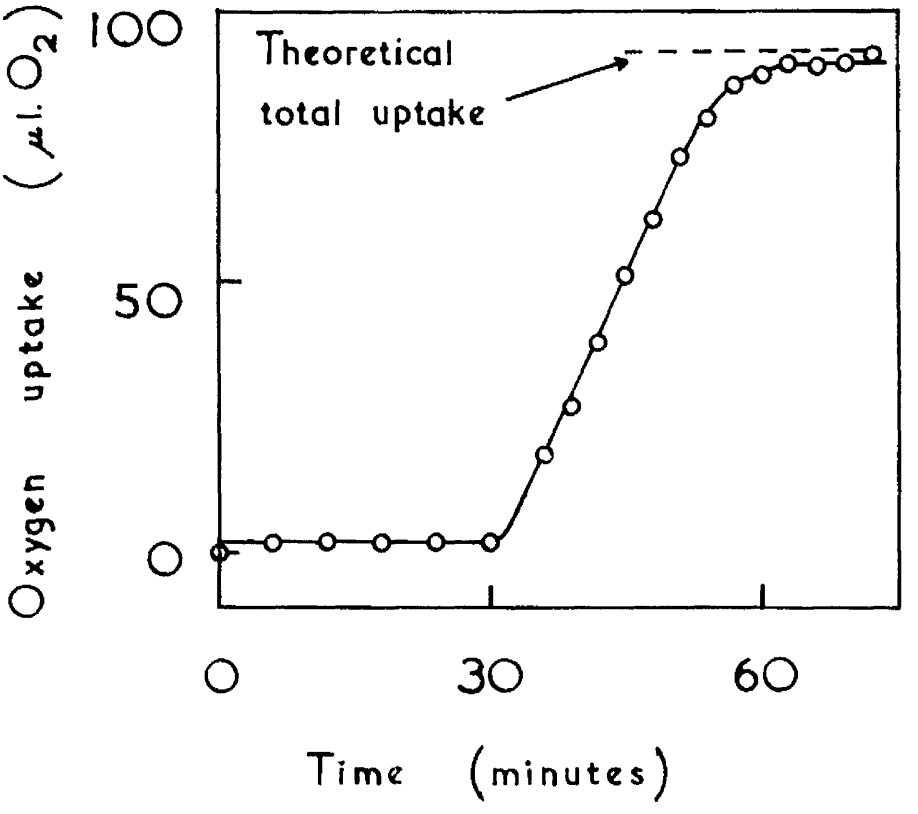


Fig. 5. The duration of the linear phase of oxygen uptake during the rapid oxidation of 4 µmoles of xanthine.

Reaction mixture - 0.5 ml. cow's milk + 2 ml. 0.25M-glycine-sodium phosphate buffer pH 9.4 at 20° (pH 9.0 at 37°) + 1 mg. catalase + 100 µmoles ethanol + 4 µmoles xanthine in a reaction volume of 3.4 ml. at 37°. Gas phase, air. Filter paper + 0.2 ml. 20% w/v KOH in centre well.

The reaction was started by adding the xanthine from the side arm at the 30-min. point.

in a reciprocating laboratory shaker (Worden, 1943). The results from these experiments were disappointing. The pH of maximum activity appeared to be at about 9.2 (buffer pH at 20°) but the shape of the curve was not reproducible. Some points showed a wide deviation from the general trend and the agreement between replicates was often poor.

A likely explanation of these difficulties seemed to be that release of the bound xanthine oxidase by the shaking treatment of Worden (1943) was incomplete, and that a further but erratic release of this bound enzyme was occurring under the alkaline conditions of the determination. Morton (1953a, 1954) showed that the alkaline phosphatase and xanthine oxidase of cow's milk were both associated with the fat phase in the form of a particulate lipoprotein complex, and that aggregation of the fat globules was accompanied by release of this particulate complex into colloidal suspension in the aqueous phase. Robert & Polonovski (1955) suggested that the increase in xanthine oxidase activity of whole milk resulting from cooling or other physical treatment accompanied the dissociation of the particulate enzyme complex from the fat globules. Therefore, it seemed likely that the difficulties associated with the erratic release, in alkaline conditions, of bound xanthine oxidase present in shaken whole milk might be avoided if skim milk separated from shaken whole milk were used in place of whole milk.

To test this, fresh whole milk from one cow was shaken for 30 min. at room temperature, and was then centrifuged at 2300xg to bring the fat to the surface. The skim milk layer was removed and the variation of its xanthine oxidase activity with pH was determined. The results are shown in Fig. 6, from which the optimum pH is seen to be about 9.2 (buffer pH measured at 20°). In subsequent determinations using skim milk from another cow it was found that the shape of this curve and the position of the peak were now reproducible. This confirmed the suggestion (see Section II, p. 9) that the partial release of bound xanthine oxidase in alkaline conditions was the cause of the erratic results obtained when using shaken whole milk. When purified xanthine oxidase, prepared from cow's milk by Dr. R.C. Bray of the Chester Beatty Research Institute, London, was used in place of skim milk, the shape of the curve and the position of the peak were the same as those obtained using skim milk.

Morton (1955) has pointed out that the pH values of alkaline glycine-sodium hydroxide buffers decrease markedly on raising the temperature from 20° to 37°. The variations of pH with temperature for the buffers used in this work were therefore determined. The buffers were made up at their stock concentrations of 0.25M-glycine or glycylglycine + 0.25M-sodium dihydrogen phosphate and were adjusted to the desired pH at a temperature of 20° with carbonate-free sodium hydroxide to the values shown in the first column of Table 4. Each buffer was then

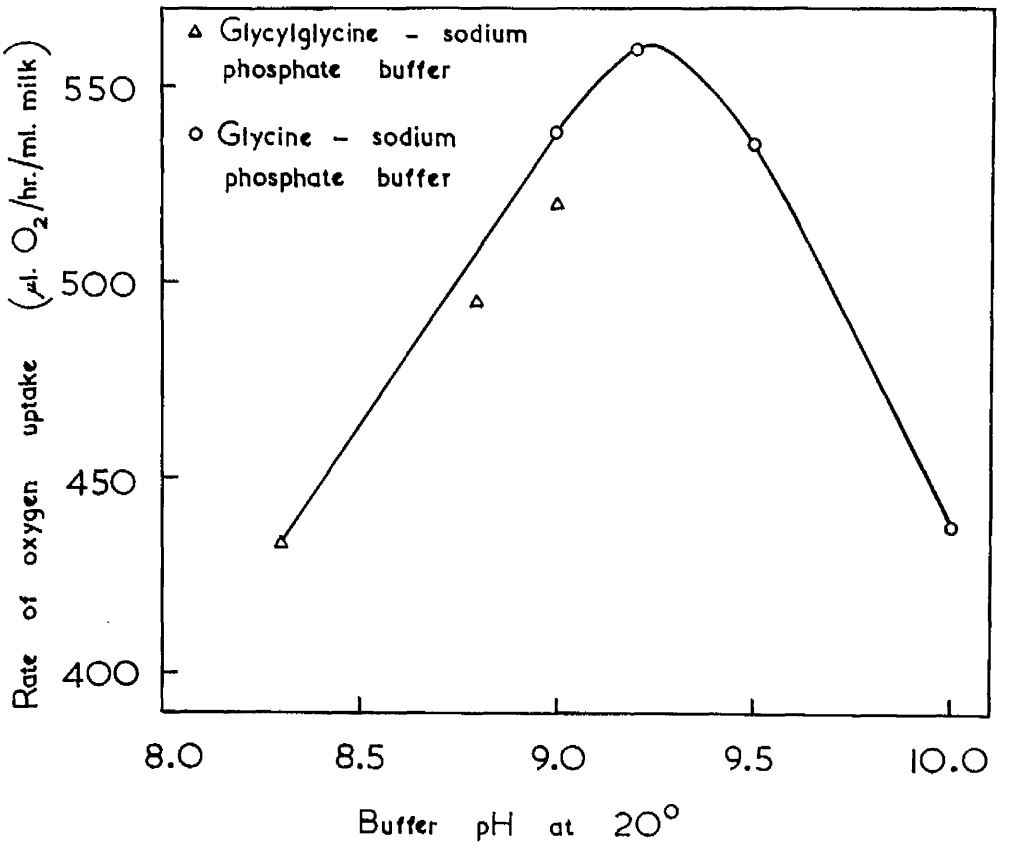


Fig. 6. Variation with pH of the xanthine oxidase activity of skim milk.

Reaction mixture - 0.25 ml. skim milk (see Section II, p. 23) + 2 ml. 0.25M-buffer + 4 µmoles xanthine + 1 mg. catalase + 100 µmoles ethanol in a reaction volume of 3.5 ml. at 37°. Gas phase, air. Filter paper + 0.2 ml. 20% w/v KOH in centre well.

diluted with freshly distilled water to a concentration of 0.15M (the final concentration in the reaction mixture) and its pH was measured at 20° and at 37°. The pH meter was standardized both at 20° and at 37° with 0.05M-sodium tetraborate, the pH of which was taken to be 9.22 at 20° and 9.08 at 37°. (These values are extrapolated and interpolated respectively from the values given in British Standard 1647: 1961 "Specification for pH Scale"). The results of these pH determinations are shown in the second and third columns of Table 4.

It is seen from this Table that the pH of glycylglycine-phosphate buffer was changed by dilution and by a change of temperature, whereas the pH of glycine-phosphate buffer was changed by a change of temperature but not by dilution except when the pH approached 10. The changes in pH with the temperature of glycine-sodium phosphate buffers are in agreement with those reported by Morton (1955) for glycine-sodium hydroxide buffers.

The variation of activity with pH of purified milk xanthine oxidase prepared by Dr. R.C. Bray was then redetermined using 0.25M-glycine-phosphate buffers standardized at 20°, but taking the pH values of the reaction mixtures from Table 4. The results are shown in Fig. 7. The optimum pH at 37° was found to lie between 8.8 and 9.0. This result is in agreement with the work of Palmer, Bray & Beinert (1964), who found the pH optimum to be at 8.9 under the conditions described by Bergel & Bray (1959).

Table 4. The variation of the pH values of glycylglycine-sodium phosphate buffers and of glycine-sodium phosphate buffers with concentration and with temperature

(1) Glycylglycine-sodium phosphate buffers

Concentration Temperature	0.25M 20°	0.15M 20°	0.15M 37°
pH	8.3	8.25*	7.9*
	8.8	8.7*	8.35*
	9.0	8.95*	8.55*

(2) Glycine-sodium phosphate buffers

Concentration Temperature	0.25M 20°	0.15M 20°	0.15M 37°
pH	8.8	8.8*	8.4*
	9.0	9.0*	8.6*
	9.2	9.2*	8.8*
	9.4	9.4*	9.0*
	9.5	9.5*	9.1*
	9.6	9.6*	9.2*
	9.8	9.85*	9.45*
	10.0	10.1*	9.65*

*Measured at the temperature shown after dilution of the 0.25M-buffer to 0.15M.

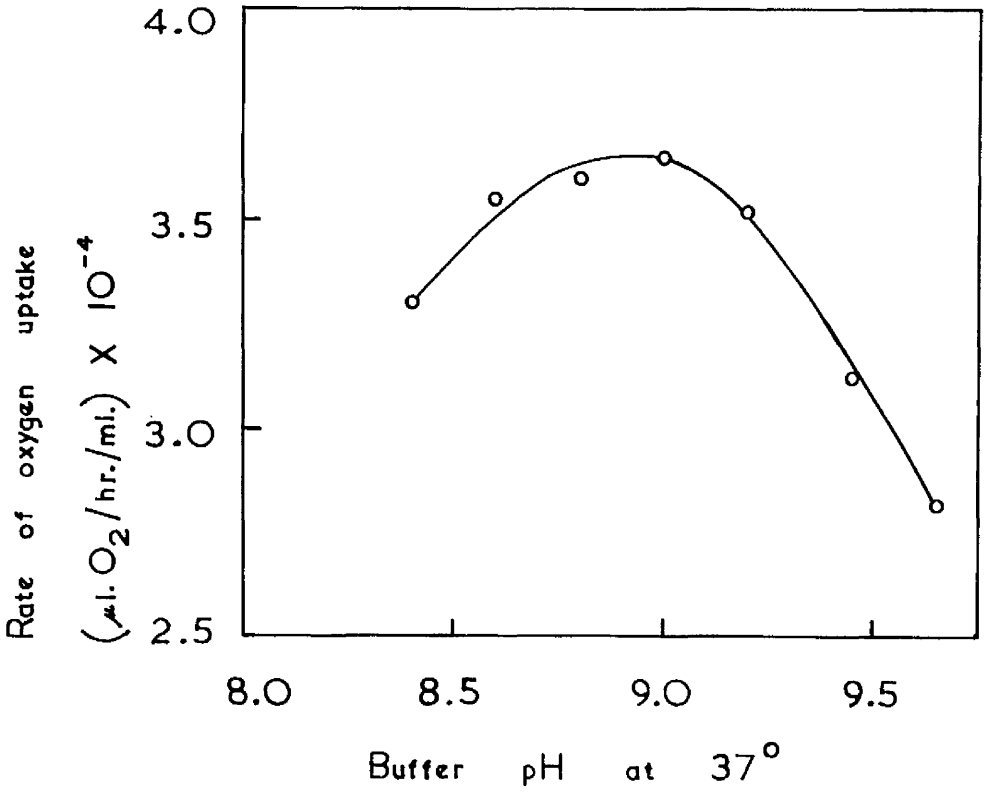


Fig. 7. Variation with pH of the activity of purified bovine milk xanthine oxidase. (prepared by Dr. R.C. Bray).

Reaction mixture - 0.005 ml. xanthine oxidase solution + 2 ml. 0.25M-glycine-sodium phosphate buffer + 4 µmoles xanthine + 1 mg. catalase + 100 µmoles ethanol in a reaction volume of 3.4 ml. at 37°. Gas phase, air. Filter paper + 0.2 ml. 20% w/v KOH in centre well.

(d) Final form of the manometric method for the determination of xanthine oxidase activity. It was observed that at pH values above 8.8 the initial rate of reaction tended to fall off, possibly due to inhibition or destruction of the catalase at these high pH values. A pH of 8.8 at 37° was therefore selected as the most suitable pH for the routine determination of xanthine oxidase activity. The conditions of the routine method were as follows.

The reaction mixture consisted of 0.1 to 1.0 ml. of milk (or 1 ml. of a suitable dilution of purified xanthine oxidase) + 2 ml. of 0.25M-glycine-sodium phosphate buffer pH 9.2 at 20° (pH 8.8 at 37°) + 0.2 ml. of 0.02M-xanthine (4 µmoles) + 1 mg. of crystalline catalase + 100 µmoles of ethanol in a reaction volume of 3.4 ml. at 37°. Potassium hydroxide (0.2 ml. 20%) and filter paper were placed in the centre well of the Warburg flask. After temperature equilibration, the reaction was started by adding the xanthine from the side arm, and was allowed to continue for at least 30 min. Pressure readings were taken every 3 min. after the first 6 min. of reaction. The initial rate of reaction was determined graphically and expressed as µl. of oxygen per hr. per ml. of milk or per ml. of purified xanthine oxidase solution. Under these conditions the rate of reaction remained constant until the xanthine was completely oxidized. Blank rates of uptake were always measured over a 15-min. period before starting the reactions and were always found to be negligible.

(e) <u>The development of a method for the quantitative release of the bound xanthine oxidase of cow's milk.</u>

When attempting to purify xanthine oxidase of cow's milk, Dixon & Kodama (1926) observed that the total activity of their final preparation was about four times the total activity of the milk from which the preparation was made. To account for this they suggested that the milk contained an inactive precursor of xanthine oxidase from which the active enzyme was liberated by the action of rennin used during their purification procedure.

Macrae (1930) observed that the xanthine oxidase activity of fresh cow's milk was low, but that on adding thymol as a preservative and allowing the milk to stand for up to several days at room temperature, a large increase in activity occurred. This increase in activity could be caused to occur in a much shorter time if the milk was subjected to any of the following treatments:-

(1) vigorous bubbling of air through the milk for 2 hr. at room temperature; (2) shaking the milk for 20 min. in an atmosphere of air or nitrogen; (3) cooling the milk (Macrae, 1930; Wieland & Macrae, 1930). These authors recognized that the xanthine oxidase was present in a bound form which was associated with the fat globules, and that the effect of all these treatments of the milk was to cause aggregation of the fat globules, which led to release of the bound enzyme. These observations were confirmed and extended by Worden (1943) who found that

shaking cow's milk in air for 20 min. was as effective in releasing activity as allowing the milk to stand in the presence of added thymol for 36 hr. at room temperature. The final activity was then 5 to 6 times the initial activity. Cooling the milk immediately after the milking released a smaller amount of activity, the final activity resulting from this treatment being 3 to 4 times the initial activity.

In the initial stages of the present work, Worden's (1943) procedure of shaking milk for 20 min. at room temperature was used to release bound xanthine oxidase activity. The milk (50-80 ml.) was placed in screw-capped cylindrical glass bottles of 150 ml. capacity. The screw caps were made of aluminium, and were fitted with rubber liners. The tightly closed bottles were placed in a reciprocating laboratory shaker and shaken gently for 20 min.

This method was used for preliminary determinations of the pH of maximum activity of xanthine oxidase in whole milk. As described on p. 22 the results indicated that the release of bound xanthine oxidase by this treatment was not complete. Methods of obtaining quantitative release of the enzyme were therefore sought.

It was found that refrigeration of the milk at +5° or -20° before it was shaken, shaking followed by homogenization of the milk in a commercial hand homogenizer, and a combination of refrigeration, shaking and homogenization were all more efficient than was shaking alone. However, these investigations showed also that

the essential factor upon which the extent of release of bound xanthine oxidase depended was the degree of visible clotting of the milk fat. Whatever treatment of the milk was used, a further increase in activity was observed when visible aggregation of the fat into a non-dispersible clot occurred. This could only be brought about by shaking the milk, but it was observed that shaking alone sometimes failed to bring about this phase inversion. It appeared that some form of pre-treatment of the milk was required to facilitate aggregation of the fat globules.

Since the separation of cream from milk in a mechanical separator is most efficient when the milk is at blood heat, and since the fat is fully liquid at this temperature (Ling, Kon & Porter, 1961a), it seemed possible that warming the milk to blood heat before it was shaken might facilitate aggregation of the fat globules during shaking. In practice, warming it to 37° followed by shaking at room temperature for 1 to 1½ hr. was found to bring about visually complete clotting of the fat of most of the samples of milk studied.

The following procedure was adopted at this stage to release bound xanthine oxidase activity. A sample of morning milk was collected and was immediately placed in a refrigerator at about 5° for a period of about 3 hr. It was then warmed to 37° in a water bath, placed in a reciprocating laboratory shaker and shaken gently at a rate of about 90 cycles/min. for a total

period of 1 to 1½ hr. The milk was inspected occasionally throughout the shaking period and the progress of clotting of the milk fat was observed. Shaking was stopped when clotting appeared to be complete.

The time required to achieve complete clotting in individual milk samples varied considerably, but few samples failed to clot after 1½ hr. of this treatment. It was observed that milks of low fat content tended to require longer periods of shaking, but many other factors undoubtedly influence this, as for example, the range and distribution of the diameters of the fat globules. It was observed that goat's milk, which contains about the same percentage of fat as cow's milk (Ling et al. 1961b), was often resistant to clotting of the fat by this treatment, which is in accord with the fact that the mean fat globule diameter of goat's milk is somewhat less than that of cow's milk (Ling et al. 1961a).

This method was used to release the bound xanthine oxidase activity of the milk samples obtained in the molybdenum-dosing experiment described later in Section III, p. 70 . During that experiment it was observed that the ratio of the xanthine oxidase activity to the molybdenum content of the milks of undosed animals tended towards a common constant value, in confirmation of the work of Kiermeier & Capellari (1958). The significance of this observation is discussed in Section IV, p. 91 , where the hypothesis is advanced, from this and from other evidence, that, given certain conditions,

all the molybdenum present in cow's milk is present in the enzymically active form of xanthine oxidase. For the purpose of assessing the completeness of release of bound xanthine oxidase it was assumed that this was so, and the efficiency of a particular treatment in accomplishing this release of bound activity was judged by whether or not it brought about a significant increase in the ratio of activity to molybdenum content of a given sample of milk.

The ratios of xanthine oxidase activity to molybdenum content were calculated for 27 milk samples from the molybdenum-dosing experiment described in Section III (p. 70), the samples being taken before molybdenum dosing was begun. The xanthine oxidase activities of this set of milks were all determined at the same milk dilution (cf. Section II, p. 37). The results from these milks are shown in Table 5, from which it can be seen that the mean value of the ratio of activity to molybdenum content, expressed as activity (µl. O_2/hr./ml.)/ Mo (µM), was 1810. The standard deviation of the group of ratios was 149, giving a coefficient of variation of 8.2%. The most likely explanation of this variation seemed to be that the warming-shaking treatment of the milk used to release bound activity was not always fully effective. Attempts were therefore made to find a more efficient method of releasing the bound activity.

Gilbert (1962) found that addition of 2mM-L-cysteine or of 3mM-2-mercaptoethanol to buttermilk caused a marked increase in its xanthine oxidase

Table 5. Xanthine oxidase activities, molybdenum contents, and activity/molybdenum ratios of milk samples obtained from undosed cows during the molybdenum-dosing experiment of Section III, (p. 70)

Date	Cow no.	XO (man.) µl. O_2/hr./ml.	Mo µM	XO (man.)/Mo
3.5.62	1	628	0.352	1780
"	2	465	0.276	1680
"	3	440	0.221	1990
"	4	372	0.201	1850
"	5	552	0.283	1950
"	6	454	0.304	1490
7.5.62	1	589	0.356	1650
"	4	295	0.150	1970
"	6	302	0.173	1750
9.5.62	1	609	0.333	1830
"	4	382	0.202	1890
"	6	327	0.185	1770
11.5.62	1	734	0.354	2070
"	4	504	0.263	1920
"	6	398	0.244	1630
13.5.62	1	616	0.383	1610
"	4	461	0.238	1940
"	6	466	0.283	1650
15.5.62	1	625	0.394	1590
"	4	381	0.215	1770
"	6	385	0.223	1730
17.5.62	1	674	0.342	1970
"	4	421	0.210	2000
"	6	458	0.233	1970
19.5.62	1	706	0.381	1850
"	4	388	0.213	1820
"	6	494	0.273	1810
				Mean 1810

The xanthine oxidase activities were determined by the manometric method described in Section II, p. 26, using 0.25 ml. of treated milk in a reaction volume of 3.4 ml.

activity (see also Gilbert & Bergel, 1964). Robert & Polonovski (1955) reported that the addition of 40 mg/ml. of sodium lauryl sulphate to milk caused an increase in the xanthine oxidase activity of the milk. Morton (1953 a, b) showed that treatment of milk fractions with n-butanol caused the alkaline phosphatase present in these fractions to dissociate from the particulate lipoprotein complex to which it and xanthine oxidase are attached.

These treatments were tested in conjunction with the warming-shaking method of releasing bound xanthine oxidase activity in the following way -

Milk samples from six cows fed on the diet described in Section III, p. 77 were treated to release bound xanthine oxidase activity by the warming-shaking procedure described above. These shaken milks were then subjected to the following treatments:-

(1) L-cysteine hydrochloride was added to give a concentration in the milk of 2mM, and the milks were allowed to stand at room temperature for 1 hr. before their xanthine oxidase activities were determined.

(2) 2-mercaptoethanol was added to give a concentration in the milk of 3mM, and the milks were allowed to stand at room temperature for 1 hr. before their xanthine oxidase activities were determined.

(3) Sodium lauryl sulphate was added to give a concentration in the milk of 40 mg/ml.

(4) The shaken milks were warmed to 38°, shaken with half their volume of n-butanol for 1 min., cooled to 20° and centrifuged. A sample of the lower aqueous layer was removed and its xanthine oxidase activity was determined.

The xanthine oxidase activities of the milk samples subjected to these treatments were determined by the method described in Section II (p. 26), and the molybdenum contents of the untreated milks by the method described in Section II (p. 57). As before, the ratio xanthine oxidase activity (µl. O_2/hr./ml. milk)/Mo (µM) was calculated for each milk. (For convenience, this ratio will be abbreviated to XO (man.)/Mo).

In agreement with the work of Gilbert (1962) it was found that treatment with both L-cysteine and 2-mercaptoethanol caused an increase in the XO (man.)/Mo ratio of shaken milk. On the other hand, ratios resulting from treatment with either sodium lauryl sulphate or with n-butanol were slightly lower than those obtained with the shaking treatment alone. Treatment with L-cysteine gave slightly higher ratios than treatment with 2-mercaptoethanol, again in agreement with the work of Gilbert (1962). The results from milks treated by shaking alone and by shaking followed by the addition of 2mM-L-cysteine are shown in the first two columns of Table 6. The mean of the differences between these two columns was found, by Student's t test, to be highly significant ($p < 0.01$). In a further series of tests, the effects of increasing the concentration of L-cysteine

Table 6. Effect of treating shaken cow's milk with various concentrations of L-cysteine on the value of the ratio (manometric xanthine oxidase activity)/Mo(μM) of the milk

Date	Cow no.	Concentration of added L-cysteine in undiluted milk			
		None	2mM	4mM	8mM
		XO(man.)/Mo	XO(man.)/Mo	XO(man.)/Mo	XO(man.)/Mo
26.7.62	1	1880	1970	-	-
26.7.62	5	2030	2160	-	-
30.7.62	2	1610	1920	-	-
1.8.62	3	1830	1930	-	-
8.8.62	2	1910	1950	-	-
8.8.62	6	1930	2050	-	-
10.8.62	3	2030	2060	-	-
10.8.62	4	1970	2060	-	-
14.8.62	2	1540	1560	1720	-
14.8.62	6	1580	1620	1850	-
16.8.62	6	1740	-	1870	1870
16.8.62	2	1700	-	1880	1950
21.8.62	5	-	-	1900	1910
21.8.62	3	-	-	2030	1950
		Mean 1810	Mean 1930	Mean 1880	Mean 1920

All cows were fed on the diet described in Section III, p. 77. The xanthine oxidase activities were determined by the manometric method described in Section II, p. 26 using 0.2 ml. of treated milk in a reaction volume of 3.4 ml.

from 2mM to 4mM and 8mM on the value of the XO (man.)/ Mo ratio were determined. These results are also shown in Table 6. There was little difference in the mean values of the ratios obtained from treatment with either 2mM, 4mM or 8mM-L-cysteine. Treatment of shaken milk with 8mM-L-cysteine was therefore adopted as the standard treatment for the release of bound xanthine oxidase activity.

(f) Final form of the method of treatment of milk to release bound xanthine oxidase activity. The handling and treatment of milk for the purpose of releasing bound xanthine oxidase activity that was finally adopted was as follows. Morning milk was collected between 6 a.m. and 6.30 a.m. from cows milked with a 'Gascoigne' milking machine. A sample of about 400ml. in a 450ml. glass bottle with aluminium screw cap and rubber liner was immediately placed in a refrigerator at about 5° for a period of about 3 hr. The contents were then mixed thoroughly by repeated inversion, and 50 - 80 ml. were placed in a 150ml. glass bottle with aluminium screw-cap and rubber liner. This was warmed to 38° in a water bath, placed in a reciprocating laboratory shaker and shaken gently at a rate of about 90 cycles/min. for a total period of 2 hr. Every half-hour the bottles were removed from the shaker and re-warmed to 38°, since this procedure was found to shorten the fat-clotting time and was effective in inducing clotting of the fat of all the samples of cow's milk encountered. After 2 hr. of this treatment, cow's milk took on the appearance of

skim milk with a layer of coagulated fat or of discrete butter granules floating on the surface. A sample (5.0ml.) of the lower skim milk layer was taken with a pipette from near the bottom of the bottle with as little disturbance of the fat layer as possible and was placed in a 25- or 50-ml. volumetric flask, according to the dilution required. To this was added 0.1 ml. of a solution, prepared immediately beforehand, of L-cysteine hydrochloride monohydrate containing 0.070 g/ml. (concentration in the milk 8mM). The contents of the flask were mixed thoroughly, allowed to stand for 1 hr. at room temperature, and finally made up to volume with distilled water. The xanthine oxidase activity was then determined immediately using the manometric method described in Section II (p. 26).

(g) <u>The presence of an inhibitor of xanthine oxidase in cow's milk.</u> In the early stages of the work on the development of the method for the release of the bound xanthine oxidase activity of cow's milk, it was observed that the apparent activity tended to increase with increasing dilution of the milk. The effect was not very marked in the milk sample used, and, owing to uncertainty at that time about the completeness of the release of the bound activity, it was not certain that the effect was real.

In the initial period of the molybdenum-dosing experiment, described in Section III (p. 70), and before actual molybdenum dosing was begun, it was observed that increasing the dilution of the milk used for the

determination of xanthine oxidase activity was accompanied by a noticeable increase in the xanthine oxidase activity / molybdenum ratios of the milks of all six cows used in this experiment (Table 7). This was taken as indirect evidence for the presence in the milks of all these cows of an endogenous inhibitor of xanthine oxidase. Direct evidence for the presence of such an inhibitor was obtained by determining the manometric activities of cow's milks at various dilutions, using the improved method of release of bound activity described in Section II (p. 36). As expected, the apparent activity increased with increasing dilution of the milk, as shown in Table 8. This effect was most undesirable in the present studies, since with milks with average activity, not less than 0.2 ml. of milk in a reaction volume of 3.4 ml. was required in order to obtain a reasonably rapid rate of oxygen uptake. From Table 8 it is clear that the activity determined at this dilution could be considerably less than the true activity. It was not practicable to use as little as 0.05 ml. of milk except with the most active milks, and even then the observed rates of uptake, which were of the order of 15 - 20 µl. of oxygen in 30 min., were near the limit of sensitivity of the method. Further, the trend of the results in Table 8 indicated that it was by no means certain that this dilution would be sufficient to nullify the effect of the inhibitor.

The presence of this inhibitor in varying concentration from milk to milk may have been the

Table 7. Effect of milk dilution on the value of the xanthine oxidase activity/molybdenum ratio of the milk

Cow no.	Date	Vol. of milk (ml.) used for determination of activity in a reaction vol. of 3.4 ml.	XO (man.) / Mo (μM)
1	1.5.62	0.50	1370
	3.5.62	0.25	1780
	5.5.62	0.20	1960
2	1.5.62	0.50	1360
	3.5.62	0.25	1680
3	1.5.62	0.50	1420
	3.5.62	0.25	1990
4	1.5.62	0.50	1470
	3.5.62	0.25	1850
	5.5.62	0.20	1980
5	1.5.62	0.50	1460
	3.5.62	0.25	1950
6	1.5.62	0.50	1370
	3.5.62	0.25	1490
	5.5.62	0.20	2060

These milk samples were from the initial period of the experiment described in Section III, p. 70 .

The milks were treated to release bound xanthine oxidase activity by the method described in Section II, p. 29 . The activities were determined by the manometric method described in Section II, p. 26.

Table 8. Effect of dilution of cow's milk on the xanthine oxidase activity of the milk

Cow no.	Vol. of milk (ml.) in a reaction vol. of 3.4 ml.	Manometric XO activity (μl.O_2/hr./ml.)
5	0.2	540
	0.1	590
	0.05	670
6	0.02	590
	0.1	600
	0.05	670

The milks were treated to release bound xanthine oxidase activity by the method described in Section II, p. 36. The activities were determined by the manometric method described in Section II, p. 26.

explanation for the rather wide range of values of the xanthine oxidase activity / molybdenum ratio obtained from milks treated to release bound xanthine oxidase activity by the method described in Section II (p. 36) (see Table 6). Since it was essential to obtain an accurate value for this ratio (see Section IV), it was decided to attempt to identify the inhibitor and if possible to remove it or to nullify its effect.

Since cow's milk may normally contain up to 0.3 p.p.m. of copper (Archibald, 1958), and since copper is known to be a powerful inhibitor of milk xanthine oxidase (Bergel & Bray, 1959), it seemed advisable to determine whether or not copper could be responsible for the inhibition observed during the present work. In the presence of sodium pyrophosphate, copper is without effect on the activity of milk xanthine oxidase (Bergel & Bray, 1959). The xanthine oxidase activities of a sample of cow's milk at various dilutions were therefore determined in the usual glycine-sodium phosphate buffer and in sodium pyrophosphate buffer at the same pH. The results are shown in Table 9. Since both the magnitude of the activity at a given dilution and the increase in activity with dilution were unaffected by this substitution, it is clear that the copper of milk was not responsible for the inhibition.

Dr. D.A. Gilbert suggested in a personal communication that the inhibitor might be a pyrimidine constituent of cow's milk. Since cow's milk contains

Table 9. The manometric xanthine oxidase activity of a sample of cow's milk at various dilutions in glycine-sodium phosphate buffer and in sodium pyrophosphate buffer

Buffer	Vol. of milk (ml.) in reaction vol. of 3.4ml.	Manometric XO activity $\mu l. O_2/hr./ml.$	Mo µM	XO(man.)/Mo
0.15M-glycine-sodium phosphate, pH 8.8 at 37°	0.5	587	0.365	1610
	0.2	660	"	1810
	0.1	692	"	1900
0.12M-sodium pyrophosphate, pH 8.8 at 37°	0.5	600	"	1650
	0.2	650	"	1780
	0.1	672	"	1840

The buffer concentrations shown were those present in the reaction mixture.

The milk was treated to release bound xanthine oxidase activity by the method described in Section II, p. 36 .

The activities were determined by the manometric method described in Section II, p. 26 , but using the buffer shown.

50 - 100 mg./litre of orotic acid (6-carboxy-2,4-dioxypyrimidine) (Hallanger, Laakso & Schultze, 1953), the possibility that this might be the inhibitory constituent was examined. Two milk samples were treated to release bound xanthine oxidase by the method described in Section II (p. 36). Orotic acid was added to these samples at levels of 200 mg./litre and 1000 mg./litre, and the manometric activities were determined at the highest dilution possible of 0.05 ml. of milk in a reaction volume of 3.4 ml. The results are given in Table 10, and show clearly that orotic acid had no appreciable inhibitory effect on milk xanthine oxidase.

Bray (1959) observed that a competitive inhibitor of milk xanthine oxidase was present in a sample of buttermilk, and in some, but not in all of his preparations of purified milk xanthine oxidase. He observed that it was diffusible, and therefore of low molecular weight, but obtained no other evidence of its nature. Its presence in a sample of buttermilk suggests that it could be the same substance as the endogenous milk inhibitor observed during the present work. Some evidence in favour of this suggestion was obtained during the preparation of Batch No.HXO-1 of purified xanthine oxidase (see Section IV, p. 96), when it was observed that the manometric xanthine oxidase activities of the cream, the buttermilk and the first and second butter washings obtained during this preparation increased markedly on dilution, as shown in Table 21 (p. 98). However, it is clear that further work is

Table 10. Effect of addition of orotic acid to cow's milk on the xanthine oxidase activity of the milk

Concentration of added orotic acid in undiluted milk	Manometric XO activity ($\mu l. O_2/hr./ml.$)	
	Milk no. 1	Milk no. 2
None	870	860
200 mg./litre	870	810
1000 mg./litre	800	800

The milks were treated to release bound xanthine oxidase activity by the method described in Section II, p. 36. The activities were determined by the manometric method described in Section II, p. 26, using 0.05 ml. of treated milk in a reaction volume of 3.4 ml.

required to establish with certainty that Bray's inhibitor and the endogenous milk inhibitor are one and the same compound.

The failures to identify the endogenous milk inhibitor with either copper or orotic acid led to a consideration of the possibility of removing its influence on the determination of the xanthine oxidase activity of milk by using a more sensitive method. The comparatively small increase of the apparent xanthine oxidase activity of cow's milk with dilution in Tables 8 and 9 suggests that the effect of the milk inhibitor is largely diluted out at these dilutions of the milk, and that its effect might be negligible if the activity could be determined with a more sensitive method at dilutions slightly higher than those possible with the manometric method. The spectrophotometric method of Avis et al. (1955) was examined for its suitability in this respect.

(h) Application of the spectrophotometric method of Avis et al. (1955) to the determination of the xanthine oxidase activity of milk. The method of Avis et al. (1955) is as follows. The reaction mixture consists of 0.1mM-xanthine + 0.05M-sodium pyrophosphate (final pH 8.2) + diluted xanthine oxidase solution in a reaction volume of 2.6 ml. in a 1cm. silica spectrophotometer cell at a reaction temperature of 23.5°. The course of the reaction is followed by observing the increase in extinction at 295 mμ, due to the production of uric acid, over a 10-min. period in an ultra-violet

spectrophotometer fitted with a constant-temperature cell compartment. The initial rate of the reaction (ΔE_{295}/min.) is determined graphically and the activity of the undiluted xanthine oxidase solution is then 26 x (the dilution factor) x ΔE_{295}/min. spectrophotometric units per litre.

When the method was applied to cow's milk it was found that 0.02 ml. of milk in a reaction volume of 2.6 ml., i.e. a total dilution of the milk of 1 in 130, gave readily measurable rates of reaction. The possibility that the milk inhibitor (Section II, p. 37) might still be interfering at this dilution was tested by determining the activities of milks at this dilution and at a higher total dilution of 1 in 208. At this higher dilution the rates of reaction were slow but were still measurable. Three milk samples were obtained from cows fed on the diet described in Section III, p. 77 and were treated to release bound xanthine oxidase activity by the method described in Section II, p. 36. The activities of these treated milks were then determined at total dilutions of 1 in 130 and 1 in 208 by the method of Avis _et al._ (1955). The results are shown in Table 11. It can be seen that the activities were only slightly higher at the greater dilution, indicating that the inhibitor was almost inoperative at the lesser dilution. As the lesser dilution gave reasonably rapid rates of reaction (ΔE_{295}/min. usually 0.01 - 0.02) it was adopted for routine use.

Table 11. Effect of dilution on the xanthine oxidase activity of cow's milk determined by the method of Avis *et al.* (1955)

Cow no.	Breed	XO activity (spectr. units/l.)		Mo (µM)	Activity (at 1 in 130) / Mo (µM)
		Overall milk diln. 1 in 130	Overall milk diln. 1 in 208		
8	Ayrshire	1.39	1.44	0.284	4.90
3	Ayrshire	1.51	1.60	0.313	4.83
7	Jersey	1.90	2.04	0.550	3.46

The activities shown in this Table were determined on the day of milking.

A disadvantage of this method when applied to the determination of the activity of milk, was that the reaction mixture was very turbid, which caused the initial extinction at 295 mμ to be excessively high. The initial extinctions were reduced by balancing the spectrophotometer with a reference cell containing a suitable solution of uric acid, but this procedure required the use of very wide slit settings which sometimes approached the maximum setting available with the spectrophotometer used (a Unicam SP500). A method of reducing the turbidity of the reaction mixture while retaining all the xanthine oxidase activity was therefore sought.

It was observed when using the manometric method for the determination of the xanthine oxidase activity of milk (Section II, p. 26) that the mixture in the main body of the Warburg flask, consisting of milk + glycine-sodium phosphate buffer (pH 9.2 at 20°) + catalase + ethanol, tended to separate after being shaken for about 30 min. in the Warburg bath at 37° into visible aggregates of milk protein suspended in an aqueous phase which was considerably less turbid than the original mixture. It seemed possible that the aqueous phase might contain all the xanthine oxidase activity, provided that the milk had first been subjected to the treatment described in Section II, p. 36 .

This possibility was tested as follows. Five milk samples from individual cows given the diet

described in Section III, p. 77 were treated to release bound xanthine oxidase activity on the day of milking by the method described in Section II, p. 36. These treated milks were then stored overnight in a refrigerator at 5°. On the following day they were brought to room temperature and subjected to the following additional treatments.

Treatment A. 2-ml. samples were diluted to 10 ml. with 0.11M-sodium pyrophosphate buffer pH 8.0 (Avis *et al.* (1955) and the xanthine oxidase activities were determined over the next 2 days by the spectrophotometric method of Avis *et al.* (1955). This was the treatment used to obtain the results recorded in Table 11. When not in use the diluted samples were stored in a refrigerator at 5°.

Treatment B. 2-ml. samples were diluted to 10 ml. with the buffer used for the manometric determination of xanthine oxidase activity (0.25M-glycine-sodium phosphate buffer pH 9.2 at 20°, Section II, p. 26). The resulting mixtures were put into the main compartments of Warburg flasks which were then attached to open manometers, placed in the Warburg bath at 37°, and shaken for 30 min. The contents were then filtered through coarse (Pyrex No. 1) sintered glass filters. The resulting filtrates were considerably less turbid than the diluted samples resulting from Treatment A. The xanthine oxidase activities of the filtrates were determined over the next 2 days by the method of Avis *et al.* (1955). When not in use, the filtrates were

stored in a refrigerator at 5°.

The results from these two treatments are shown in Table 12. For determinations of activity made 2 - 3 days after milking, both treatments gave identical results (Nos. 8, 7, 6, 3). For determinations of activity made 1 day after milking (No. 4), Treatment A gave a lower activity than Treatment B. On keeping the test preparation from cow No. 4 for a further period of 2 days, the activity of the test preparation from Treatment A rose to that of the test preparation from Treatment B, the activity of which remained constant over that period. This suggests that Treatment B immediately released any bound activity that escaped release by the treatment described in Section II, p. 36, whereas Treatment A was not always immediately effective in this respect.

The molybdenum contents of the milks used in the experiments summarized in Tables 11 and 12 were determined by the method described in Section II, p. 57, and are given in these two tables together with the activity/molybdenum ratios. The values of these ratios in Table 12, calculated from the activities obtained using Treatment B, approximated closely to the mean value of 5.17. Two of the values of this ratio in Table 11 (from cows Nos. 8 and 3) approached this mean value, but the third value (from cow No. 7) fell considerably short of it. Since the activities listed in Table 11 were obtained using Treatment A described above, and were determined on the day of

Table 12. Effect of the clarification treatment of Section II, p. 49 on the xanthine oxidase activity of cow's milk

Cow no.	Breed	Date of milking	Date of XO determination	XO activity* (spectr. units/l.)		Mo (μM)	Activity+ / Mo(μM)
				Treatment A**	Treatment B**		
4	Ayrshire	5.12.63	6.12.63 8.12.63	1.78 2.00	1.99 1.99	0.393	5.06
8	Ayrshire	5.12.63	7.12.63	1.43	1.38	0.265	5.21
7	Jersey	5.12.63	8.12.63	3.58	3.44	0.653	5.27
6	Ayrshire	5.12.63	8.12.63	1.40	1.43	0.280	5.11
3	Ayrshire	5.12.63	8.12.63	1.86	1.80	0.346	5.20
							Mean 5.17

* Overall milk dilution 1 in 130.

** See Section II, p. 49 for details of these treatments.

\+ Calculated from XO activities obtained using Treatment B.

milking, this is further evidence that the full activity of the milk may not be released immediately by this treatment.

Other evidence for the constancy of the activity/molybdenum ratio is presented later in Section IV, together with a discussion of the significance of the constancy of this ratio.

From the results of Table 12 it seems reasonable to conclude that Treatment B, in combination with the milk treatment described in Section II, p. 36, consistently released all the bound activity of cow's milk. Treatment B had the further advantage over Treatment A that the turbidity of the final reaction mixture was considerably reduced, thus permitting the use of narrower slit settings on the spectrophotometer.

3. The determination of molybdenum

(a) Method. The colorimetric dithiol method of Clark & Axley (1955), modified by the addition of ferrous iron to catalyse the formation of the molybdenum-dithiol complex (Gilbert & Sandell, 1960) was selected for the determination of molybdenum in milk and in feeding stuffs. The method was as follows:-

To the test solution, containing up to 10 μg. of molybdenum, in approximately 4N-hydrochloric acid were added the following, in order - 2 mg. of ferrous iron (as ferrous sulphate), 0.5 g. potassium iodide, sufficient sodium thiosulphate to decolorize any liberated iodine, 0.5 g. tartaric acid. Toluene-3, 4-

-dithiol (dithiol) reagent was then added to form the green molybdenum-dithiol complex. This was extracted with 5.0 ml. of isoamyl acetate and the extinction of the extract was read in a 1 cm. cuvette at 680 mμ.

In the present work the development of the molybdenum colour and its subsequent extraction with isoamyl acetate were carried out in glass-stoppered borosilicate glass test tubes measuring 200 x 25 mm. (Bingley, 1959). The extracts were centrifuged to remove suspended water droplets (Bingley, 1959) and their extinctions were read at 680 mμ in a Unicam SP500 spectrophotometer.

(b) Interference by copper. Clark & Axley (1955) claimed that 1.2 micrograms of molybdenum could be determined by this method in the presence of 50 milligrams of copper, and Bingley (1959) claimed that 30 micrograms of copper did not interfere in the determination of 5 micrograms of molybdenum. It was thought advisable to check this, since other workers (Piper & Beckwith, 1948; Williams, 1955) took care to remove copper before developing the molybdenum-dithiol colour.

Cow's milk may normally contain up to 300 μg. of copper per litre (Archibald, 1958), and it was estimated that for the determination of the molybdenum content of milk, a maximum volume of 100 ml. of milk might be required. The effect of adding up to 30 μg. of copper (as copper sulphate) on the determination of

up to 10 µg. of molybdenum by Clark & Axley's method was therefore determined. The results are shown in Fig. 8. It can be seen that the extinctions of the isoamyl acetate extracts were considerably depressed by the addition of these amounts of copper. It was observed that the extracts from molybdenum solutions with no added copper were pure grass-green in colour, while the extracts from molybdenum solutions containing added copper were yellow-green. However, the position of the extinction maximum of these yellow-green extracts remained unchanged at 680 mµ.

(c) Suppression of interference by copper. Haider & Khundkar (1954) found that interference by copper in the gravimetric determination of zinc with 8-hydroxyquinoline could be prevented by reducing the copper to the cuprous state with potassium iodide followed by sodium thiosulphate, and then adding thiourea ($CS(NH_2)_2$) which forms a very stable cuprous complex. The effect of adding thiourea to standard molybdenum solutions with and without added copper immediately before the addition of dithiol reagent was therefore examined. The results recorded in Table 13 show that interference by up to 100 µg. of added copper was effectively suppressed by the addition of 200 mg. of thiourea. The isoamyl acetate extracts from molybdenum solutions with added copper and thiourea had the pure grass-green colour typical of extracts from molybdenum solutions with no added copper.

After this method of suppressing interference

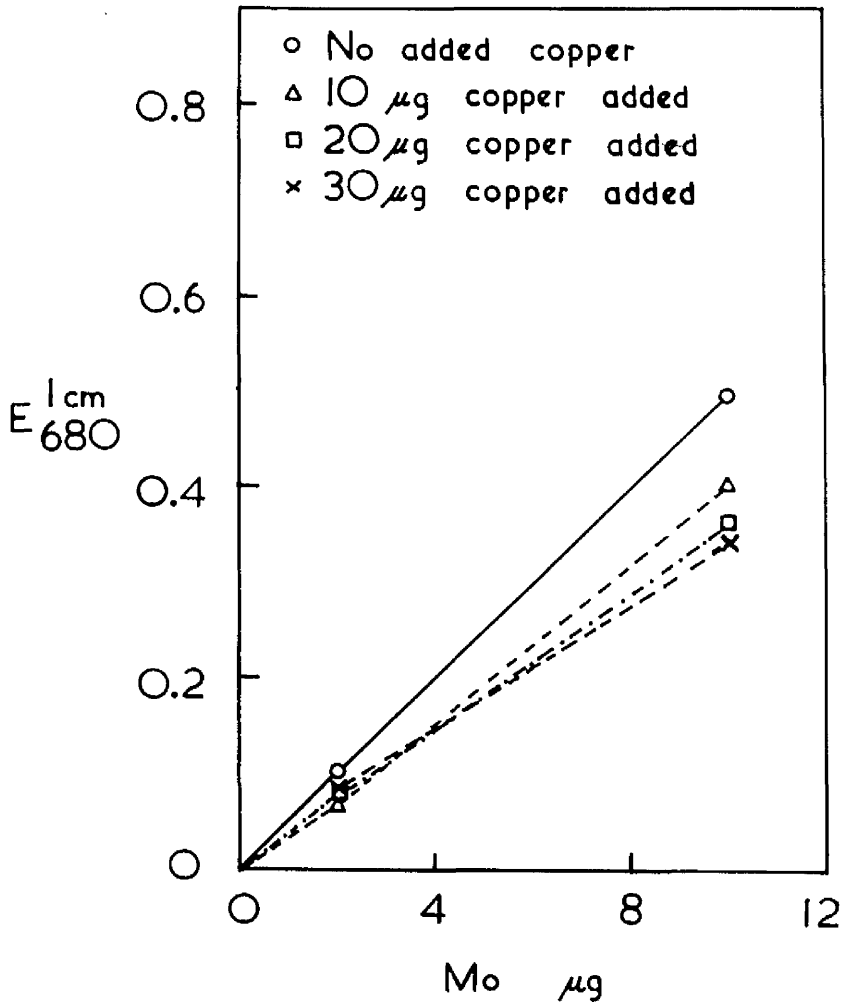


Fig. 8. Effect of added copper on the determination of molybdenum by the modified method of Clark & Axley (1955) described in Section II, p. 52 .

Table 13. Effect of thiourea on interference by copper in the determination of molybdenum by the method of Clark & Axley (1955)

Mo (µg.)	Cu (µg.)	Thiourea (mg.)	E^{1cm}_{680}
0	-	-	0.001
2	-	-	0.101
10	-	-	0.485
0	-	200	0.001
2	-	200	0.103
10	-	200	0.493
0	30	-	0.001
2	30	-	0.070
10	30	-	0.305
0	30	200	0.001
2	30	200	0.099
10	30	200	0.480
4	0	200	0.201
4	50	200	0.203
4	100	200	0.198

by copper had been developed, Bingley (1963) published a modification of his earlier dithiol method for the determination of molybdenum (Bingley, 1959) in which thiourea was used to suppress interference by up to 1000 µg. of copper. However, Bingley (1963) claims that up to 30 µg. of copper does not interfere with the determination of molybdenum by his unmodified method (Bingley, 1959). This is not in agreement with the results obtained during the present work (cf. Fig. 8).

(d) Preparation and storage of dithiol reagent.

The reagent was prepared as follows. The contents of a 1 g. ampoule of toluene-3, 4-dithiol (dithiol) were melted by immersing the unbroken ampoule in water at about 35°. The ampoule was then opened and its contents were quickly added to 500 ml. of 1% w/v sodium hydroxide and dissolved by vigorous agitation. When the dithiol had dissolved, 7.0 ml. of thioglycollic acid were added dropwise with vigorous stirring. The resulting solution was immediately transferred to glass-stoppered Pyrex reagent bottles of 60 ml. or 100 ml. capacity which were filled as completely as possible, and then stored in a refrigerator at about 5°. The contents of partly full bottles were discarded if not used with 1 day. When prepared in this way, the reagent kept well for at least 4 weeks and often for longer. The end of its useful life was marked by the gradual appearance of a white precipitate which was accompanied by a progressive decline in the slope of the standard curve and by poor agreement of replicate determinations. As soon as

this precipitate began to appear, the reagent was discarded.

This preparative procedure differed from that of Clark & Axley (1955) in that the volume of thioglycollic acid added was reduced from about 8 ml. to 7.0 ml. The keeping qualities of the reagent were found to be much improved by this modification.

(e) Preparation of standard molybdenum solutions. A stock standard solution containing 100 µg. of molybdenum per ml. was prepared according to the directions of Clark & Axley (1955) by dissolving 0.150g. of Analar molybdenum trioxide (dried at 105° for 4 hr.) in 20 ml. of 0.1N-sodium hydroxide. This solution was diluted with distilled water to about 100 ml., acidified with N-hydrochloric acid to about pH 5.5 (B.D.H. narrow-range indicator paper) and diluted to 1 litre with distilled water.

A working standard solution containing 0.500 µg. of molybdenum per ml. was prepared by diluting 5.0 ml. of this stock standard solution plus 1 ml. of N-hydrochloric acid to 1 litre with distilled water.

All standard molybdenum solutions were kept in the dark as recommended by Clark & Axley (1955).

(f) Working method. Milk and feed ingredients were wet ashed by the nitric acid method of Middleton & Stuckey (1954). For milk, the preliminary procedure was as follows. Duplicate samples of cow's milk (25 ml.) or goat's milk (50 ml.) were placed in 1-litre Pyrex beakers (height about 146 mm., diameter about 106 mm.)

and carefully evaporated to dryness at a low heat on an electrically heated hotplate fitted with a Simmerstat control. When just dry, the beakers were removed from the hotplate, allowed to cool, and 10 ml. of concentrated nitric acid (sp. gr. 1.4) were added, after which the mixture was again cautiously evaporated to dryness.

For feed ingredients, the preliminary procedure was as follows. As before, duplicate samples (1 - 2 g.) were placed in 1 litre beakers. To each beaker were added 10 ml. of distilled water and 5 ml. of concentrated nitric acid (sp. gr. 1.4), and the mixtures were carefully evaporated to dryness at a low heat.

From this point the following procedure was applied to the residues from both milk and feed samples. The hotplate temperature was raised to about 350° and the open beakers were heated at this temperature until there was no further change in the appearance of the contents. This usually took about 30 min. The beakers were allowed to cool, and the contents were moistened with nitric acid (sp. gr. 1.4). The beakers were covered with clock glasses and heated on the hotplate at about 350° for 15 min. after the residues had become dry. This procedure was repeated until the residues were whitish with dark patches. Fuming nitric acid (sp. gr. 1.5) was then used in place of ordinary nitric acid, and the procedure was continued until ashing was complete.

The beakers were then allowed to cool, and 15 ml. of concentrated hydrochloric acid and 3 ml. of 60%

perchloric acid were added. The beakers were left open and their contents were slowly evaporated to dryness at a low heat. When drying was complete, the temperature was increased to about 350° and heating was continued until fuming ceased. This procedure was found necessary to remove nitrate ion, which otherwise interfered seriously with the subsequent determination of molybdenum.

The beakers were allowed to cool, 15 ml. of 6N-hydrochloric acid were added, the clock glasses were placed in position and the beakers were heated gently until the ash dissolved. They were then allowed to cool, and the contents were transferred to glass-stoppered borosilicate glass test-tubes measuring 200 x 25 mm. A further 10 ml. of 6N-hydrochloric acid were added, the clockglasses were replaced, the beakers were warmed again, and the contents were transferred to the appropriate tubes. Finally, the beakers were rinsed with 10 ml. of distilled water and the rinsings were transferred to the appropriate tubes.

Throughout this procedure identical volumes of acids were added to all the beakers at each stage and to duplicate blank beakers (reagent blanks).

The volumes in the test tubes were made up to 35 ml. with distilled water, and the following were added, in order -

(1) 0.2 ml. of 5% w/v ferrous sulphate solution in 2% v/v sulphuric acid (= 2 mg. of ferrous iron).

(2) 0.5 ml. of freshly prepared 50% sodium iodide solution. The solutions were allowed to stand for 10 min. with occasional swirling to allow for complete liberation of iodine.

(3) 5% w/v sodium sulphite solution was added dropwise until the iodine colour was just discharged.

(4) 2 ml. of freshly prepared 10% w/v thiourea solution.

(5) 1 ml. of 50% w/v tartaric acid solution.

(6) 2 ml. of 0.2% w/v dithiol reagent (Section II, p. 56). The tubes were stoppered, shaken vigorously for 30 sec., and allowed to stand for 30 min.

(7) 2.5 ml. of isoamyl acetate. The tubes were stoppered, shaken vigorously for 30 sec., and allowed to stand until the two phases separated and cleared. The upper isoamyl acetate layers were then removed with a suction device attached to a graduated pipette, transferred to 15 ml. Pyrex glass centrifuge tubes and centrifuged for 5 min. to remove suspended water droplets. The clarified extracts were transferred to 4 cm. microcells of 1.6 - 1.7 ml. capacity (Unicam Instruments Ltd.) and their extinctions were read in a Unicam SP500 spectrophotometer at 680 mμ against a reference cell containing isoamyl acetate.

A standard curve was prepared for each assay by adding known volumes of the working standard

molybdenum solution containing 0.500 µg.Mo/ml. to duplicate stoppered borosilicate glass test tubes, adding 25 ml. of 6N-hydrochloric acid, making up the volumes to 35 ml. with distilled water, and applying procedures 1 - 7 listed above. A typical standard curve is shown in Fig. 9. The molar extinction coefficient at 680 mµ obtained from this curve was 24,700 which agreed well with the value of 24,400 (in benzene) reported by Gilbert & Sandell (1960). In five determinations, recoveries of 0.500 µg. of molybdenum added to 25 ml. of cow's milk which contained about 0.5 µg. of natural molybdenum, fell within the range of 95 - 103%.

4. The determination of FAD

(a) Enzymic method. The enzymic method used in this work for the determination of FAD was derived from the manometric method of Burton (1951) for the determination of D-amino acid oxidase. A standard Warburg apparatus was used, with air as the gas phase. The apo-protein of D-amino acid oxidase was prepared from commercial crude D-amino acid oxidase powder by the method of DeLuca, Weber & Kaplan (1956), and was stored at -20°.

For most of the determinations reported here, the reaction mixture consisted of 0.2 ml. of 0.68M-DL-alanine (68 µmoles of D-alanine) + 1.5 ml. of 0.20M-sodium pyrophosphate buffer (pH 8.3 at 20°) + 1 mg. of crystalline catalase + 0.25 ml. of 1M-ethanol + 0.1 - 0.4 ml. of a solution of the apo-protein of D-amino acid oxidase

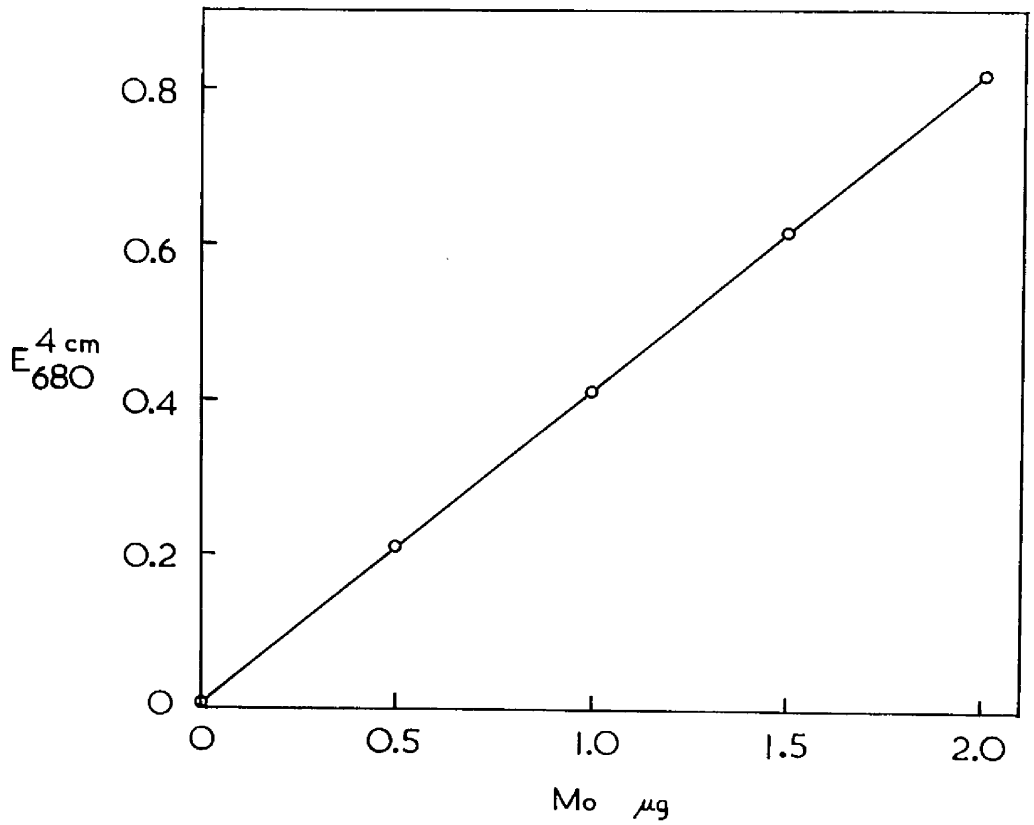


Fig. 9. Standard curve for the molybdenum assay of Section II, p. 57.

+ 0 - 0.5 μg. of FAD in a reaction volume of 3.4 ml. The reaction temperature was 37°, and the final pH at this temperature was 8.5. Potassium hydroxide (0.2 ml. 20% w/v) + filter paper was placed in the centre well of the Warburg flask. The concentrations of the constituents in this reaction mixture were, D-alanine, 0.020M; sodium pyrophosphate, 0.088M; ethanol, 0.07M; catalase, 1.2μM. Since the Michaelis constant for D-alanine in this system lies between 0.002M and 0.009M (Burton, 1955), and since an appreciable amount of D-alanine was oxidized in the 30 min. reaction period during a fast reaction(about 8 μmoles out of a total of 68 μmoles), the concentration of D-alanine was later increased to 0.025M to ensure that the apo-protein was saturated with D-alanine over the reaction period. At the same time, the reaction volume was decreased to 3.2 ml. The concentrations of the constituents in the reaction mixture were then - D-alanine, 0.025M; sodium pyrophosphate, 0.09M; ethanol, 0.078M; catalase, 1.3μM.

The DL-alanine was placed in the side-arm of the Warburg flask, and all the other components of the reaction mixture were placed in the main compartment. The flasks were allowed to equilibrate in the water bath at 37° for 5 min. Blank readings were then taken for 9 min. after which the reaction was started by adding the DL-alanine from the side-arm. After the first 6 min. of reaction, pressure readings were taken every 3 min. for a period of 30 min. The initial rate of reaction was determined graphically and expressed as μl. of oxygen per hr. Under these conditions the rate of

reaction remained constant over the 30-min. period.

The stoicheiometry of the reaction was checked by determining the total uptake of oxygen resulting from the complete oxidation of 0.2 ml. of 0.068M-DL-alanine (≡6.8 μmoles of D-alanine). This was found to be 147 μl. of oxygen (mean of four determinations). The theoretical total uptake predicted by the equation

$$CH_3CH(NH_2)COOH \text{ (D-form)} + O_2 + CH_3CH_2OH$$
$$\longrightarrow CH_3COCOOH + NH_3 + CH_3CHO + H_2O$$

is 152 μl. of O_2, and the reaction therefore proceeds according to this equation (cf. equation 9, Section II, p. 18).

A standard curve was set up with each determination by adding 0 - 0.5 μg. of standard FAD to duplicate Warburg flasks. The resulting rates of uptake were plotted as ordinates against the amounts of FAD in the reaction mixture, and the points were connected by a curve drawn freehand. A typical standard curve is shown in Fig. 10. The rates of uptake of suitably diluted unknowns were determined in duplicate and their contents of FAD were calculated by reference to the standard curve.

The method was tested in collaboration with Dr. R.C. Bray, as follows. A sample of purified milk xanthine oxidase (No. XO-421), prepared by Dr. Bray, was analyzed for FAD by the writer, using this method, and by Dr. Bray, using the fluorimetric method of Burch, Bessey & Lowry (1948). Before analysis by the enzymic method, the xanthine oxidase

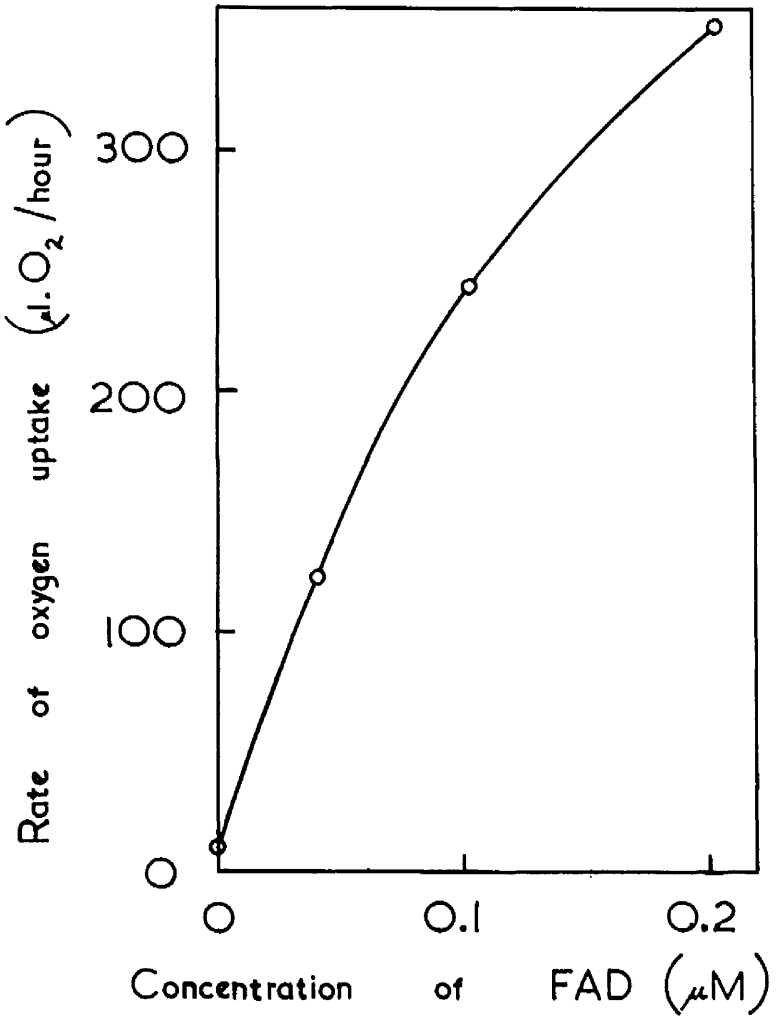


Fig. 10. The relationship between the concentration of FAD and the rate of uptake of oxygen by the D-amino acid oxidase system of Section II, p. 61 .

Reaction mixture - 0.2 ml. 0.80M-DL-alanine + 1.5 ml. 0.20M-sodium pyrophosphate buffer (pH 8.3 at 20°) + 1 mg. catalase + 0.25 ml. 1M-ethanol + 0.1 ml. D-amino acid oxidase apo-protein + FAD in a reaction volume of 3.2 ml. at 37°. Gas phase, air. Filter paper + 0.2 ml. 20% w/v KOH in centre well.

was heated in boiling water for 5 min. to release the bound FAD. The mean of three separate determinations of FAD in this sample by the enzymic method was 59 μM; the mean of three determinations by the fluorimetric method was 62 μM.

(b) Preparation and standardization of standard FAD solutions. Throughout this work a single batch of FAD (see Section II, p. 6) was used for the preparation of standard FAD solutions for use in the enzymic assay just described. This FAD was examined for the presence of other flavins by the paper chromatographic method of Crammer (1948). Samples of flavin mononucleotide (FMN) and of riboflavin were also placed on the chromatogram. Examination of the chromatogram under ultraviolet light revealed that the sample contained a barely detectable trace of FMN, and that free riboflavin was absent.

All standard FAD solutions were made up in "low actinic" brown glass standard flasks. A strong standard solution of FAD was prepared on the day of assay by dissolving approximately 1.5 mg. of FAD in 50 ml. of freshly prepared 0.01M-sodium phosphate buffer, pH 7.0. The extinctions at 450 mμ and 260 mμ of this solution were measured in a 1 cm. cell using a Unicam SP 500 spectrophotometer, and the concentration of FAD was calculated from the following equation.

Concentration of FAD (μg./ml.)

$$= E^{1cm.}_{450} \times 786/11.3 \text{ (Whitby, 1953).}$$

The ratio E (260 mμ)/E (450 mμ) was always calculated, and always fell within the range of 3.17 - 3.21. For pure FAD the value of this ratio is 3.28 (Cerletti & Siliprandi, 1958).

A working standard FAD solution containing approximately 0.5 μg. FAD/ml. was prepared immediately before use by diluting the strong standard solution 50-fold with distilled water. Additions of this solution to the Warburg flasks containing all the other constituents of the reaction mixture were made in a dark room illuminated by a single tungsten light. Potassium hydroxide and filter paper were placed in the centre wells, the flasks were attached to the manometers, and were then immediately placed in the Warburg bath at 37°.

(c) Non-linearity of the standard curve of the enzymic method. The standard curve for this assay system was invariably curvilinear, as shown in Fig. 10. Various workers who have used this method for the determination of FAD have stated that the relation between the concentration of FAD in the reaction mixture and the rate of oxidation of D-alanine was linear up to an FAD concentration of 0.3 - 0.4 μM (DeLuca, Weber & Kaplan, 1956; Huennekens & Felton, 1957; Manson & Modi, 1957). Ochoa & Rossiter (1939) stated that the relation was almost linear up to an FAD concentration of 0.6 μM. However, in the present work, using either of the systems described in Section II, p. 61, the relation was invariably found to be

markedly curvilinear over the FAD concentration range of 0 - 0.2 μM (Fig. 10). This finding is in agreement with the work of Warburg & Christian (1938), whose data are shown plotted in Fig. 11.

That the graph must be curvilinear in this concentration range is evident from the following considerations. The kinetics of the combination of FAD with the apo-protein of D-amino acid oxidase in the presence of a large excess of D-alanine are in accordance with the Briggs-Haldane equation

$$v = \frac{V_m \cdot s}{K_m + s}$$

where v is the rate of oxidation of D-alanine, s is the concentration of FAD, V_m is the rate of oxidation of D-alanine in the presence of a large excess of FAD, and K_m is the Michaelis constant for FAD in this system (Warburg & Christian, 1938; Burton, 1951). This is the equation of a rectangular hyperbola which passes through the origin, and approaches asymptotically the value $v = V_m$ as s approaches infinity. It is seen from this equation that v is approximately proportional to s only when s is much smaller than K_m, so that when in practice s approximates to K_m, the hyperbolic curvature cannot be ignored. From a plot of 1/v against 1/s of the data of Fig. 10, K_m (FAD) = 0.18 μM. From the complete data of Warburg & Christian (1938), K_m (FAD) = 0.25μM. Estimates of K_m (FAD) vary from 0.13μM to 0.25μM (Burton, 1955). Since the working range of the FAD assay is 0 - 0.2 μM (Figs. 10 and 11), it

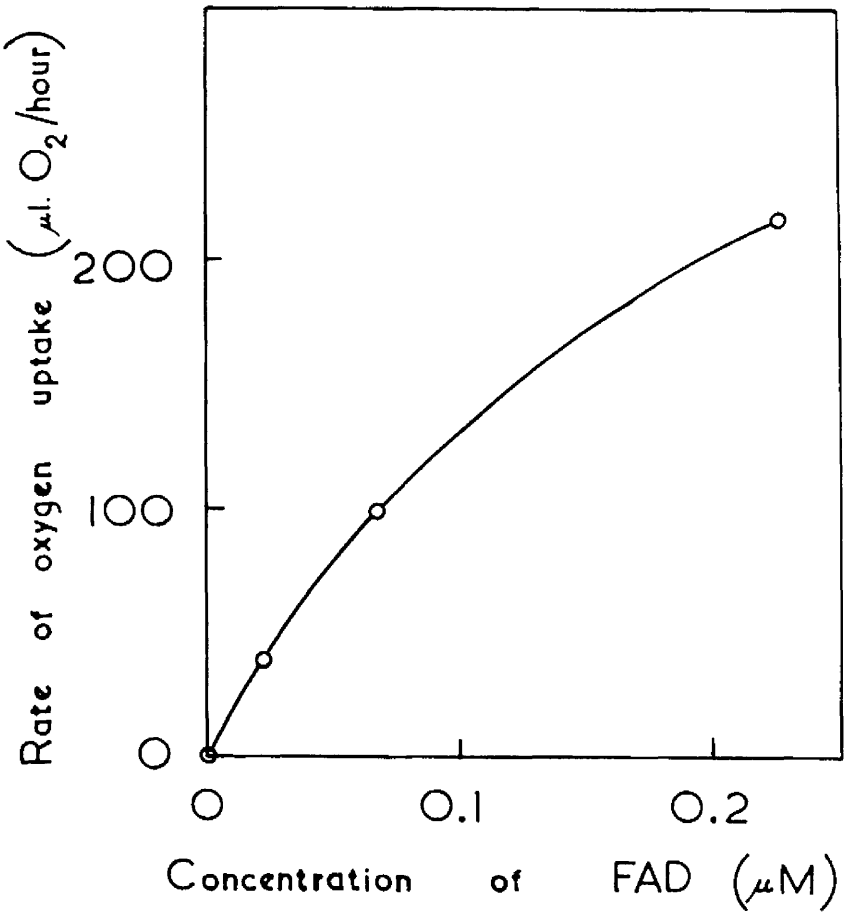


Fig. 11. The relationship between the concentration of FAD and the rate of uptake of oxygen by the D-amino acid oxidase system of Warburg & Christian (1938).

Reaction mixture - 0.2 ml. 0.51M-DL-alanine + 1 ml. 0.1M-sodium pyrophosphate buffer (pH 8.3) + 20 µg. D-amino acid oxidase apo-protein + FAD in a reaction volume of 2.4 ml. at 38°. Gas phase, oxygen. KOH in centre well.

Plotted from the data of Warburg & Christian (1938).

follows that the standard assay graph must exhibit hyperbolic curvature in this range of FAD concentration.

(d) Fluorimetric method. The method of Burch (1957) was used to determine the FAD contents of buttermilks prepared from cow's milk. In this method, flavins were extracted from buttermilk by adding cold trichloroacetic acid solution (TCA) to a final concentration of 10% w/v, and allowing the mixture to stand at 0° for 15 min. after which it was centrifuged. A portion of the clear supernatant liquid was removed and brought to pH 6.8 by the addition of dipotassium hydrogen phosphate. The fluorescence of this mixture was measured immediately. Another portion of the TCA extract was incubated overnight at 38°, brought to pH 6.8 as before, and the fluorescence determined. An increase in the fluorescence of the incubated extract relative to the fluorescence of the unincubated extract indicated the presence of FAD. The concentration of FAD in the original material was determined by reference to the fluorescence of known amounts of riboflavin which were carried through the entire procedure.

The fluorimeter used was a Locarte Model LFM/4 fitted with a mercury arc lamp (The Locarte Company, London, S.W.7). It was fitted with entrance filter No. LF/3, which isolated the mercury arc line at 436 mμ, and with exit filter No. LF/7, which transmitted at and above 510 mμ only.

The fluorescence of the solutions was measured in cylindrical quartz cuvettes which contained a working volume of approximately 1.2 ml.

SECTION III

THE INFLUENCE OF DIETARY MOLYBDENUM ON THE XANTHINE OXIDASE ACTIVITY OF COW'S AND OF GOAT'S MILK

1. Introduction

Kiermeier & Capellari (1958) investigated the relation between the xanthine oxidase activity and the molybdenum content of cow's milk and observed

(1) that the activity of xanthine oxidase in cow's milk was proportional to the molybdenum content of the milk,

(2) that the xanthine oxidase activity and molybdenum content of the milk depended on the amount of molybdenum ingested, and

(3) that administration of a soluble molybdate to cows caused an increase in the amount of molybdenum in the milk but did not change the xanthine oxidase activity of the milk if that activity had been relatively high before.

This work was repeated under more rigorously controlled conditions by the present author, using both cows and goats, in an attempt to confirm the observations of these workers and thereby to gain an insight into the factors controlling the output of xanthine oxidase and of molybdenum in the milk of ruminants.

2. <u>The effect of feeding sodium molybdate on the xanthine oxidase activity and molybdenum content of cow's milk</u>

(a) <u>Experimental</u>. Six stall-fed Ayrshire cows, all in early lactation, were divided into two groups (A and B) each containing three cows, so that the mean milk xanthine oxidase activity of the milk of group A was approximately equal to that of group B (determined over a period of 14 days). The cows were offered a low-molybdenum diet consisting of hay and concentrates. The hay and the ingredients of the concentrates were all selected for their low molybdenum content (determined by the method described in Section II, p. 57). The composition of the concentrates was - bruised oats, 6 parts; bean meal, 5 parts; cooked maize, 1 part. Molasses (5% by weight) was added as a binder to this mixture which was compressed into pellets ("cubes") by a local supplier of animal feeding stuffs.

Each cow was offered 8 lb. of hay and 8 lb. of concentrates twice daily. One cow in Group A consistently refused to eat more than 12 lb. of concentrates daily. Otherwise, with rare exceptions, all the ration offered was consumed by every cow throughout the experimental period. The diet conformed to accepted standards for the protein intake of lactating cows (Wood & Woodman, 1939). It was sufficient to provide for milk yields of some 35 - 40 lb. per day

while restricting the molybdenum intake to 5 mg. per cow per day for the duration of the experiment (4.5 mg. for the Group A animal which, as mentioned above, did not eat the full amount of concentrates). This was the lowest daily molybdenum intake that could be achieved with the feeding stuffs available locally.

On the 10th day following the introduction of this basal diet, the three cows of Group A were each given a daily dose of 45 mg. of molybdenum, thus raising their daily intake from 5 mg. to 50 mg. Mo/cow/day. The dose consisted of 57 ml. of a 0.20% (w/v) aqueous solution of Analar sodium molybdate, $Na_2MoO_4.2H_2O$. It was administered by means of a standard cattle drenching gun at about 11 a.m. each day. This dosing was continued for a total period of 10 days, and was then stopped. Six days later the cows of Group B were each given a daily dose of 95 mg. of molybdenum, thus raising their daily molybdenum intake from 5 mg. to 100 mg. Mo/cow/day. This dosing was continued for a total period of 10 days, and was then stopped. The cows were kept on the basal diet for a further 7 days and the experiment was then ended.

The duration of the experimental period, from the time that the cows were first offered the basal diet, was 43 days. Combined 24-hour milk samples were taken every second day from each cow throughout this period. A 24-hour milk sample was made by combining the milk from an afternoon milking (kept at 5° overnight) with the following morning's milk, the combination

being made in proportion to the respective yields. The daily milk yields from each cow on the day of sampling were recorded. The xanthine oxidase activities of the 24 hr. milk samples were determined immediately by the manometric method described in Section II, p. 26. The milks were treated to release bound xanthine oxidase activity by warming them to 37° and shaking them for 1 - 1¼ hr. (see Section II, p. 27). For the first 7 days of the experimental period, the xanthine oxidase activities of the milks were determined using 0.5 ml. of milk in a reaction volume of 3.4 ml. For the remainder of the experimental period, all the activities were determined using 0.25 ml. or 0.20 ml. of milk in a reaction volume of 3.4 ml. Throughout the experimental period, the activities of all the milk samples were determined at the same dilution on any one day.

The molybdenum contents of the milks were determined by the method described in Section II, p. 57. The fat contents of the milks were determined by Mr. R. Proudfoot of this Institute using the Gerber method. The molybdenum content of the basal diet was followed throughout the experimental period by taking daily samples of the hay and concentrates. Once a week these were combined into single samples of hay and of concentrates and the molybdenum and moisture contents of these samples were determined. The analyses showed that the molybdenum content of the basal diet did not vary appreciably throughout the experimental period.

(b) Results. The results are shown in Figs. 12 and 13. It is clear from Fig. 12 that the administration of sodium molybdate to either group of cows was followed by an immediate rise in the mean concentration of molybdenum in the milk of the dosed animals. It is apparent also that, by comparison with the control group, little or no change occurred in the mean xanthine oxidase activity of the milk of the dosed group on either occasion. Fig. 13 shows that no marked changes occurred either in the values for the mean fat percentages of the milk or in the mean milk yields of either group during the dosing periods.

It was observed that the ratios of xanthine oxidase activity to molybdenum content of the milk samples taken before molybdenum dosing began, tended towards a common constant value, in confirmation of the observation of Kiermeier & Capellari (1958). The experimental results from these milk samples are given in Table 5. The results of later investigations of the factors affecting the constancy of the ratio of activity to molybdenum content are described in Section II, pp. 27-52.

The apparent rise in the mean xanthine oxidase activities of the milks of both groups after day 7 (see Fig. 12) was probably due to increasing the dilution at which the xanthine oxidase activities were determined after that day (see Section III, p. 72 for details). The effect of this increase in dilution on the value of the activity/molybdenum ratio is shown in Table 7

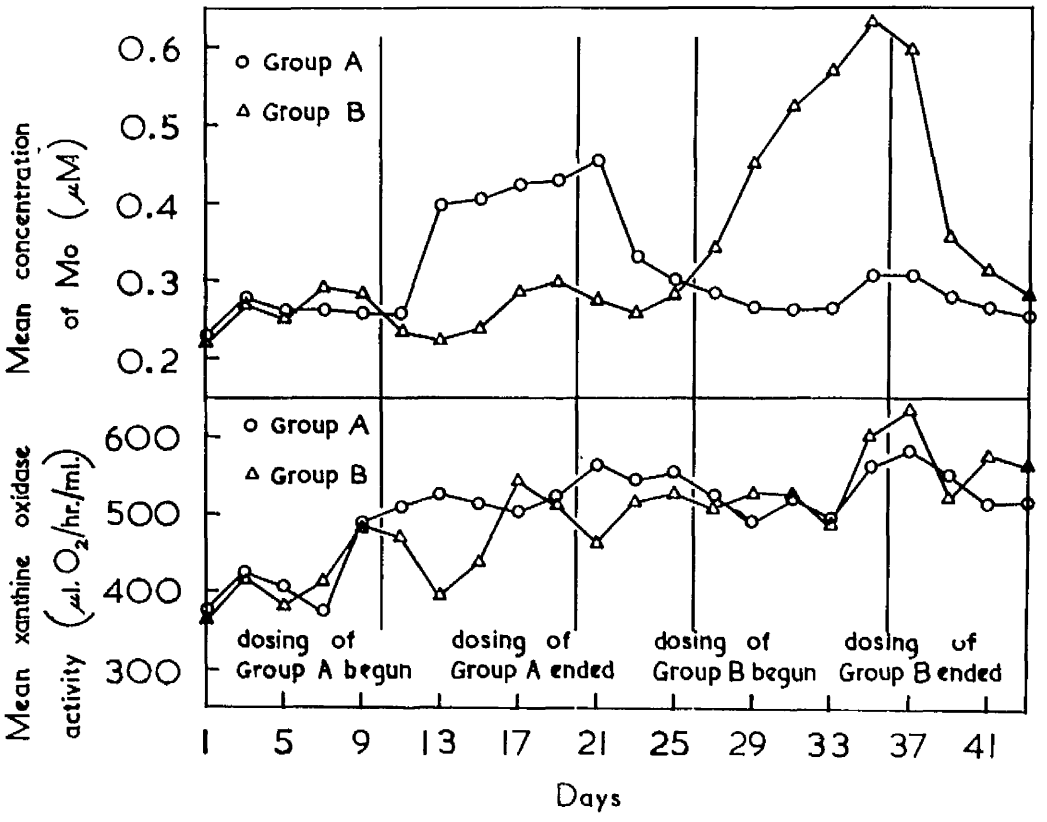


Fig. 12. Molybdenum contents and xanthine oxidase activities of cow's milks from the molybdenum dosing experiment of Section III, p. 70 .

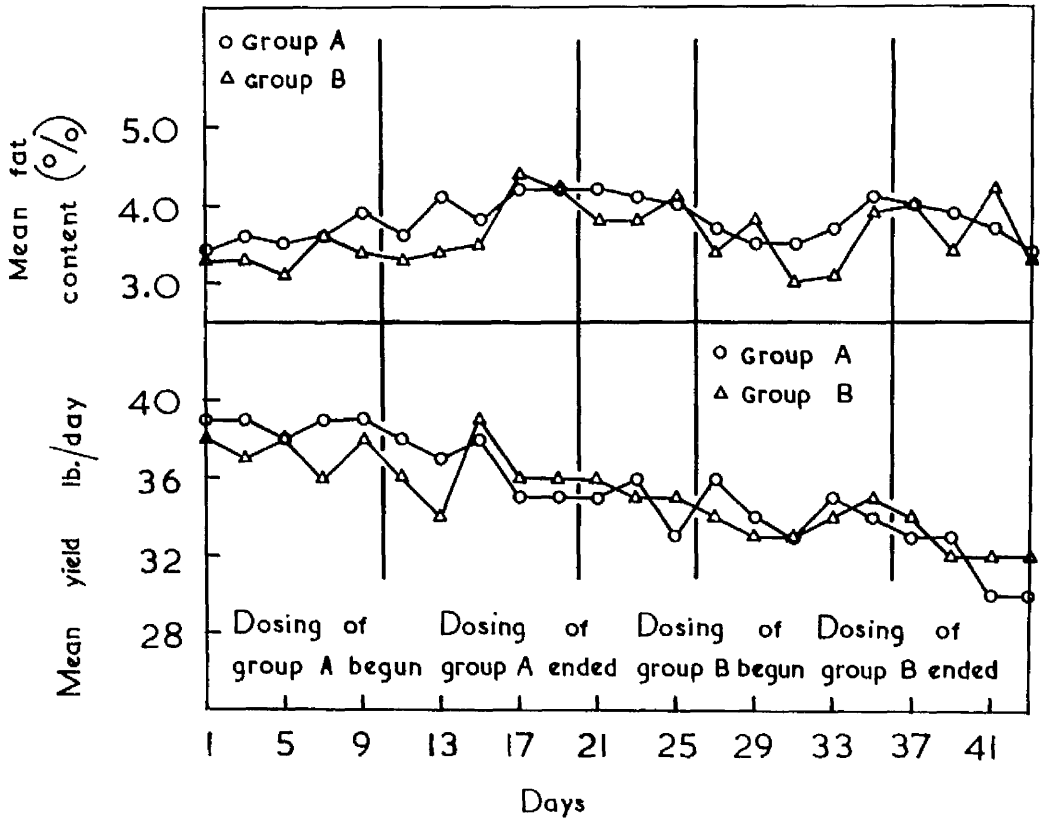


Fig. 13. Milk yields and fat contents of the cow's milks from the molybdenum dosing experiment of Section III, p. 70 .

(see also Section II, p. 37).

During and after the molybdenum dosing periods no signs indicative of excessive molybdenum intake were observed (Underwood, 1962).

(c) Discussion. The finding that the administration of a soluble molybdate to cows is followed by a rapid increase in the concentration of molybdenum in their milk is in agreement with the findings of Kiermeier & Capellari (1958) and of Archibald (1951). However, Kiermeier & Capellari's claim, that the xanthine oxidase activity of the milk of cows fed on a low-molybdenum diet could be increased by feeding a diet high in molybdenum, has not been confirmed during the present work. The reason for this discrepancy may lie in the fact that the design of the experiments of Kiermeier & Capellari (1958) differed from the design of the experiment in the present work, in that Kiermeier & Capellari used no control animals. In one of their experiments, a group of cows was fed for 5 days on hay which contained 0.16 p.p.m. of molybdenum. During this period the mean xanthine oxidase activity of the milk of this group fell from 10.5 units/2 ml. milk to 8.5 units/2 ml. milk. For the next 10 days, the cows were fed unspecified amounts of hay which contained 0.42 p.p.m. of molybdenum. Each cow also received a daily supplement of 1.5 kg. of a 1:1 mixture of soyabean meal and a milk production ration ("Milchviehfutter III"). The molybdenum content of this supplement was 3.54 p.p.m.

During this period, the mean milk xanthine oxidase activity of the group rose to a level of about 14 units/ 2 ml. milk.

It is possible that the variation in molybdenum intake of the cows was responsible for the observed variation in the mean milk xanthine oxidase activity of the group, but, since the gross composition of the low-molybdenum and high-molybdenum diets differed so markedly, it is equally likely that variation of some other constituent of these diets was responsible for the observed variation in the activity. In particular, the high-molybdenum diet must have contained considerably more protein, due to the inclusion of soyabean meal. It has been shown that the xanthine oxidase activity of rat liver is markedly dependent both on the amount and on the quality of the protein of the diet (Litwack, Williams, Feigelson & Elvehjem, 1950; Litwack et al. 1952, 1953). Therefore, variation of dietary protein intake cannot be excluded as a possible cause of the observed variation of milk xanthine oxidase activity in the experiments of Kiermeier & Capellari (1958).

In another experiment Kiermeier & Capellari (1958) obtained milks from two herds of cows grazing in separate localities. Each herd was of a different breed, and was grazing on fields of different soil type producing different types of pasture which were found to differ widely in molybdenum content. Kiermeier & Capellari observed that the mean xanthine oxidase

activity of the milk of the herd grazing on the low-molybdenum pasture was considerably less than the mean activity of the milk of the herd grazing on the high-molybdenum pasture. They claimed that this difference in milk xanthine oxidase activity was due to the difference in molybdenum content of the pastures, but it is clear that any one of the differences between the two groups listed above, i.e. breed of cow, soil type, and type of pasture, could have been responsible for this difference in milk activity.

In a third experiment Kiermeier & Capellari (1958) fed two cows on a diet of hay and roots which provided a molybdenum intake of about 2.6 mg. of molybdenum per cow per day, and observed that within 5 days, marked falls occurred in the xanthine oxidase activities of the milks of both these cows. They claimed that this result was indicative of exhaustion of the molybdenum reserves of the cows. By contrast with this result, in the present work, the cows of Group B (Section III, p. 70) received 5 mg. of molybdenum per cow per day for 25 days before being dosed with additional molybdenum. It is seen from Fig. 12 that the mean xanthine oxidase activity of the milk of this group fluctuated somewhat, but showed no sustained change over that period. This may indicate that the critical level of daily intake of molybdenum for maximum activity lies somewhere between 5 mg. and 2.6 mg. of molybdenum per cow per day.

(d) Diet of experimental cows subsequent to the molybdenum-dosing experiment. After the molybdenum-dosing experiment just described, the experimental cows were maintained on a modified form of the low-molybdenum diet used in that experiment. The modification consisted of replacing half of the concentrates in the ration by an equivalent weight of a commercial brand of concentrates for milk production, i.e. "H.Y. Dairy Nuts", manufactured by R. Silcock & Sons Ltd., Liverpool. The milk yields of the cows were considerably improved by this modification. The molybdenum content of the modified diet was about half as high again as that of the unmodified diet, so that the cows' intake of molybdenum was about 8 mg. Mo/cow/day. Most of the samples of cow's milk used for further investigations were taken from cows maintained on this modified diet. The magnitudes of the xanthine oxidase activity/molybdenum ratios of such samples were similar to those recorded in Tables 5 and 6.

3. The effect of feeding sodium molybdate on the xanthine oxidase activity and molybdenum content of goat's milk

(a) Experimental. Six British Saanen goats, all near the end of lactation, were arbitrarily divided into two groups of three, Groups A and B. Each goat was confined in a separate pen and was offered 3 lb. of hay and 1½ lb. of concentrates twice daily. The composition of the concentrates was - bruised oats, 6 parts; bean meal, 4 parts; decorticated groundnut meal, 1 part;

flaked maize, 1 part. This mixture was made up by a local supplier of animal feeding stuffs. The daily molybdenum intake provided by the complete diet was 1.1 mg. of molybdenum per goat per day.

The goats were fed on this basal diet for 10 days. At this point one goat in Group B ceased to lactate and it was replaced by another. All the results from the animal in which lactation stopped were discarded, so for the first 10 days of the experimental period, Group B comprised two goats only. For the next 7 days, the goats of Group A were each given a daily dose of 11.9 mg. of molybdenum, thus raising their daily intake from 1.1 mg. to 13.0 mg. Mo/goat/day. The dose was given as 30 ml. of a 0.10% (w/v) aqueous solution of Analar sodium molybdate. It was administered with a standard sheep drenching gun at about 11 a.m. each day.

After 7 days the dosing of Group A was stopped, and on the following day the goats of Group B were each given a similar daily dose, which was continued for a period of 7 days, and then stopped. The goats were kept on the basal diet for a further 3 days. The experiment then had to be terminated, since some of the goats were approaching the end of lactation.

Each goat was milked twice daily during the experimental period. Afternoon milk yields from each goat (kept at 5° overnight) were combined with the yields obtained the following morning, and the volumes of the combined yields were recorded. Samples for

analysis were taken from these combined yields every Monday, Wednesday and Friday morning. The manometric xanthine oxidase activities of these samples were determined immediately using the system 1 ml. milk (adjusted to pH 8.8 at 20°) + 2 ml. 0.25M-glycylglycine-sodium phosphate buffer pH 8.8 at 20° + 10 µmoles xanthine + 1 mg. crystalline catalase + 100 µmoles ethanol in a reaction volume of 3.4 ml. at 37°. Before determining the activities of these milks, they were shaken for 20 min. in a reciprocating laboratory shaker (Worden, 1943; see Section II, p. 28). The molybdenum contents of duplicate 100 ml. samples of the milks were determined by the method described in Section II, p. 57, except that the colour was extracted with 5.0 ml. of isoamyl acetate. The extinction of this extract was determined at 680 mµ in a 1 cm. cell.

Weekly samples of the hay and meal were taken and their molybdenum and moisture contents were determined. These analyses showed that the molybdenum content of the diet did not vary appreciably throughout the experimental period.

(b) Results. The results are shown in Figs. 14 and 15. As already explained, for the first 10 days of the experimental period, Group B comprised only two goats. A third goat was then added to this group and thereafter the experimental points for both groups in Figs. 14 and 15 each represent the means of the results for three goats in each group.

Fig. 14 shows that, as with cows, administration

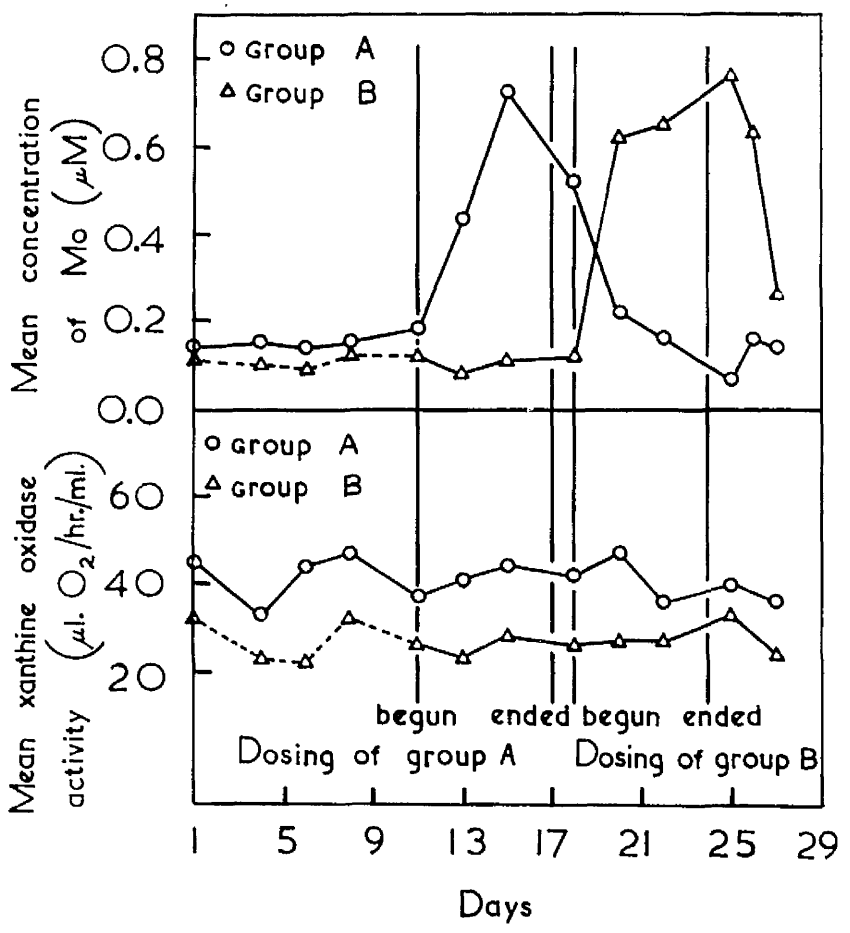


Fig. 14. Molybdenum contents and xanthine oxidase activities of goat's milks from the molybdenum dosing experiment of Section III, p. 77 .

○———○
△———△ } Mean of results from three animals.

△- - - -△ Mean of results from two animals.

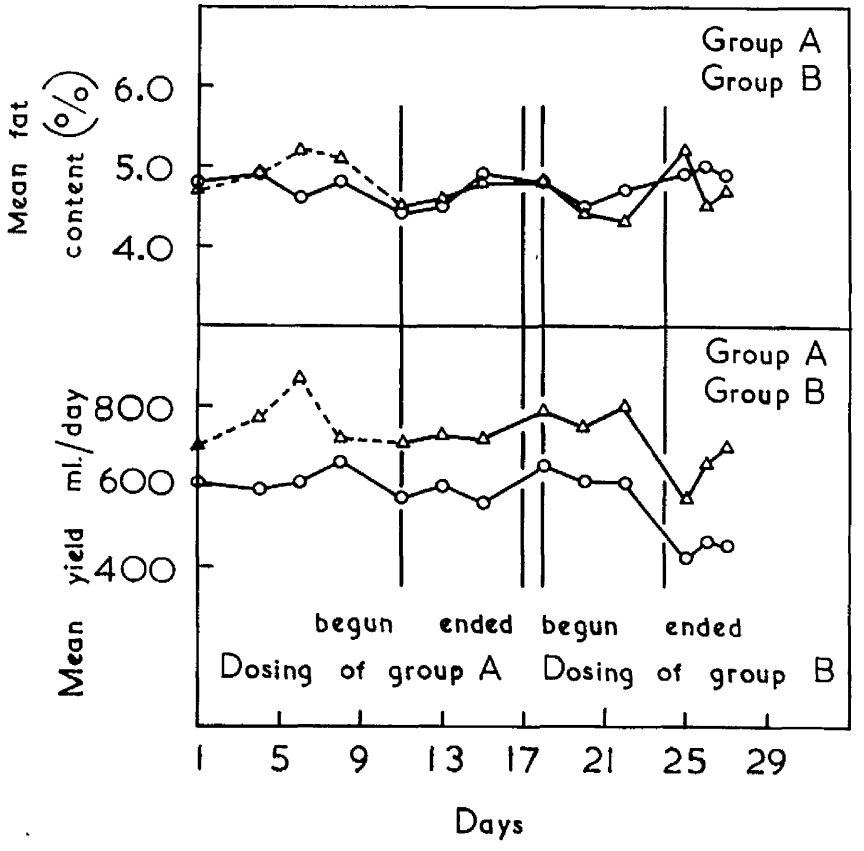


Fig. 15. Milk yields and fat contents of the goat's milks from the molybdenum dosing experiment of Section III, p. 77 .

○———○ △———△ } Mean of results from three animals.

△- - - -△ Mean of results from two animals.

of sodium molybdate was followed by an immediate rise in the mean molybdenum content of the milk, but that no change occurred in the mean xanthine oxidase activity. Fig. 15 shows that no marked changes occurred either in the mean values for the fat content of the milk or in the mean milk yields of either group during the dosing periods.

The xanthine oxidase activities of all the milk samples obtained during this experiment with goats were very low (20 - 50 µl. O_2/hr./ml.), compared with the corresponding activities of cow's milk (350 - 600 µl. O_2/hr./ml.) (Figs. 12 and 14). The manometric activities of some samples of goat's milk obtained during the experiment were so low as to be almost undetectable. The milk samples which were obtained from the goats before molybdenum dosing was begun, invariably contained molybdenum, although the average molybdenum content of these milks (about 0.1 µM) was slightly less than that of cow's milk (about 0.25 µM) (compare Figs. 12 and 14). In contrast to the findings for cow's milk, no correlation between xanthine oxidase activity and molybdenum content of milks from undosed goats was observed. The xanthine oxidase activities, molybdenum contents, and activity/molybdenum ratios of the milks of all six goats taken immediately before molybdenum dosing was begun (on Day 11, Fig. 14), are shown in Table 14. The results from successive samples of milk from a single goat are shown in Table 15. These results are typical of those obtained from the milks

Table 14. Xanthine oxidase activities, molybdenum contents and activity/molybdenum ratios of milk samples obtained from undosed goats during the molybdenum-dosing experiment of Section III, p. 77.

Goat no.	XO(man.) µl. O_2/hr./ml.	Mo µM	XO(man.)/Mo
1	19	0.11	170
2	56	0.14	400
3	28	0.14	200
4	43	0.18	240
5	15	0.07	210
6	26	0.25	100

Table 15. Xanthine oxidase activities, molybdenum contents, and activity/molybdenum ratios of successive milk samples from a single goat (No. 1, Group B) taken before molybdenum dosing was begun during the experiment described in Section III, p. 77.

Day	XO(man.) µl. O_2/hr./ml.	Mo µM	XO(man.)/Mo
1	42	0.14	300
4	25	0.11	230
6	25	0.11	230
8	41	0.16	260
11	19	0.11	170
13	19	0.07	270
15	34	0.15	230
18	40	0.19	210

of undosed goats. The activity/molybdenum ratio was quite variable from milk to milk, and also, by comparison with Table 5, it is clear that the magnitude of the ratio for goat's milk was much less than that of the corresponding ratio found in cow's milk.

During and after the molybdenum dosing periods no signs indicative of excessive molybdenum intake were observed (Underwood, 1962).

(c) Discussion. The finding that the administration of supplementary molybdenum to lactating goats was followed immediately by a rise in the concentration of molybdenum in their milk, but that the xanthine oxidase activity of the milk was unaffected, parallelled the results obtained from the molybdenum dosing experiment with cows described in Section III, p. 70. The finding in the present work that the xanthine oxidase activity of goat's milk was much less than that of cow's milk (Figs. 12 and 14) is in agreement with the findings of Morgan *et al.* (1922); likewise, the finding that the molybdenum content of goat's milk was less than that of cow's milk (Figs. 12 and 14) is in agreement with the findings of Teresi, Elvehjem & Hart (1942).

The low and variable ratios of activity to molybdenum content of goat's milk were in marked contrast to the much higher and constant value of the ratio found in cow's milk. It was thought that this finding might be due to the presence in goat's milk of very firmly bound xanthine oxidase which was not released

by the simple shaking treatment of Worden (1943). Also, the manometric method for the determination of xanthine oxidase activity used in this experiment employed a higher concentration of xanthine (10 μmoles/3.4 ml.) than that used in the corresponding experiment with cows (4μmoles/3.4 ml.). It was possible, therefore, that the observed activities of the goat's milks could have been low because of an increased degree of substrate inhibition relative to the activities found for the milks from the molybdenum-dosing experiment with cows (cf. Section II, p. 20 and Fig. 4). An alternative possibility was that the goat's milk contained a greater amount of the inhibitor discussed in Section II, p. 37 compared with cow's milk. These possibilities were now investigated.

4. <u>Further investigations of the relationship between the xanthine oxidase activity and the molybdenum content of goat's milk</u>

To test for the presence of bound xanthine oxidase in goat's milk, samples of milk from six goats fed on the diet described in Section III, p. 77 , were treated to release bound xanthine oxidase by the treatment described in Section II, p. 36 . The xanthine oxidase activities of the treated milks were determined by the improved manometric method of Section II, p. 26 , and the molybdenum contents by the method of Section II, p. 57 . The results are shown in Table 16. Comparison of this table with Tables 14 and 15 shows that the use of

Table 16. Xanthine oxidase activities, molybdenum contents, and activity/molybdenum ratios of goat's milks*

Goat no.	XO(man.) µl. O_2/hr./ml.	Mo µM	XO(man.)/Mo
1	66	0.130	510
3	38	0.090	420
4	69	0.142	490
5	45	0.117	380
7	23	0.096	240
8	55	0.125	440

* For details of these milks, see Section III, p. 84.

The milks were treated by the method described in Section II, p. 36 to release any bound xanthine oxidase activity that might have been present. The xanthine oxidase activities were determined by the manometric method described in Section II, p. 26.

improved methods for the release and determination of xanthine oxidase activity made no difference to the magnitudes of the ratios of activity to molybdenum content. From this, it seems unlikely that the goat's milk contained bound xanthine oxidase of the type found in cow's milk.

The presence in goat's milk of an inhibitor of xanthine oxidase similar to that of Section II, p. 37 was tested for by determining the xanthine oxidase activities of samples of cow's milk and goat's milk, and of a mixture of the two. The molybdenum contents of the milks were also determined. The results recorded in Table 17 show that, within the limits of experimental error, the activity of a mixture of equal volumes of the two milks was equal to the sum of the separate activities. This suggests either that there was no inhibitor present in the goat's milk or that if an inhibitor of xanthine oxidase was present in the goat's milk, there was insufficient of it to affect the activity of the xanthine oxidase added with the cow's milk.

5. Conclusions

The results obtained from these investigations of the relationship between the xanthine oxidase activity and molybdenum content of goat's milk are consistent with the hypothesis that the average xanthine oxidase content of goat's milk is much lower than that of cow's milk, and that most of the molybdenum found in goat's milk is

Table 17. Xanthine oxidase activities of cow's milk, of goat's milk, and of a mixture of the two

Source of milk	Vol. of milk (ml.) used for determination of activity in a reaction vol. of 3.4 ml.	XO(man.) µl. O_2/hr./ml.	Mo µM	XO(man.)/Mo
Cow no. 4	0.20	308	0.129	2390
Goat no. 9	0.20	37	0.118	310
Cow no. 4 + Goat no. 9	0.20 + 0.20	323	-	-

The milks were treated to release bound xanthine oxidase activity by the method of Section II, p. 36. The activities were determined by the manometric method of Section II, p. 26.

not associated with xanthine oxidase activity. However, this hypothesis is in no sense proved, and it is clear that further work is required to establish it with certainty.

SECTION IV

THE PRESENCE OF ENZYMICALLY INACTIVE FLAVOPROTEINS IN COW'S MILK AND IN XANTHINE OXIDASE ISOLATED FROM COW'S MILK

1. Introduction

Avis, Bergel & Bray (1956) and Bergel & Bray (1958) studied the properties and composition of many highly purified preparations of xanthine oxidase isolated from cow's milk. Their purest preparations contained 8 atoms of iron, 2 molecules of FAD, and 1.3 - 1.5 atoms of molybdenum, per molecule of protein. The specific activities of these preparations (defined as xanthine oxidase activity per μmole of FAD) varied from 3.7 to 1.6, and there was no correlation between the specific activity of a given preparation and its molybdenum content. To account for these observations, and others, Bray, Pettersson & Ehrenberg (1961) postulated that these highly purified preparations consisted of mixtures of the active enzyme with two other closely related but enzymically inactive flavoproteins, designated "xanthine oxidase-i_1" and "xanthine oxidase-i_2", with the compositions shown in Table 18. Bray *et al.* (1961) could not separate these inactive forms from the active enzyme or from one another, and could not distinguish them spectrophotometrically from one another. No investigation of the origins of these inactive forms appears to have been made. The failure of all attempts

Table 18. The composition and activity of the components postulated by Bray et al. (1961) to occur in their purified preparations of milk xanthine oxidase

Component	Fe : FAD : Mo*	Xanthine oxidase activity
Xanthine oxidase-a	8 : 2 : 2	Active
Xanthine oxidase-i_1	8 : 2 : 2	Inactive
Xanthine oxidase-i_2	8 : 2 : 0	Inactive

* g. atoms or moles / mole of enzyme.

to separate them from the active enzyme has led to doubts that they exist (Ackerman & Brill, 1962). However, if they do exist, it is clear that they must either be present in cow's milk together with the active enzyme and must accompany that enzyme throughout the purification procedure, or alternatively, they must arise by degradation of the active enzyme during the purification procedure.

The experimental findings presented here in Section IV are consistent with the following hypotheses-

(1) that the inactive forms postulated by Bray *et al.* (1961) do exist;

(2) that xanthine oxidase-i_1 is absent from cow's milk and arises by inactivation of the active enzyme during the purification procedure;

(3) that xanthine oxidase-i_2 is present in cow's milk and accompanies the active enzyme throughout the purification procedure employed by Bergel and Bray and their collaborators.

2. The origin of xanthine oxidase-i_1

(a) The relationship between xanthine oxidase activity and molybdenum content of cow's milk. The observation, that the ratio of xanthine oxidase activity to molybdenum content of cow's milk tended towards a constant value provided that the cows had not been dosed with a soluble molybdate, was first made by Kiermeier & Capellari (1958). This observation was confirmed during the molybdenum-dosing experiment with cows described in Section III,

p. 70, using the method of release of bound xanthine oxidase activity described in Section II, p. 29, and the manometric method (Section II, p. 26) for the determination of that activity. With the aid of the improved method for the release of bound xanthine oxidase activity described in Section II, p. 36, and the use of the spectrophotometric method of Section II, p. 45 to determine that activity, it has been found that the ratio of the xanthine oxidase activity to molybdenum content of cow's milk tends very closely towards a common constant value. Preliminary determinations of the value of this ratio in cow's milk made with these improved methods and expressed as (spectrophotometric activity)/Mo (μM), are recorded in Table 19, together with the corresponding activities given by the spectrophotometric and manometric methods. The most recent determinations of the value of the ratio of activity to molybdenum content have already been recorded in Table 12, p. 51. It will be observed that these values deviate very little from the mean value of 5.17.

The constancy of this ratio implies that all the molybdenum present in normal cow's milk must be bound to active xanthine oxidase. It follows from this that the molybdenum-containing but inactive xanthine oxidase-i_1 of Bray *et al.* (1961) (see Table 18) must be absent from normal cow's milk. This conclusion is supported by the finding (see below) that the ratio of xanthine oxidase activity to molybdenum content of two

Table 19. Xanthine oxidase activities, molybdenum contents and activity/molybdenum ratios of the milks of five Ayrshire cows given the diet described in Section III, p. 77 .

Cow no.	Date of milking	Manom. XO activity * µl. O_2/hr./ml.	Spectr. XO activity** units/l.	Mo µM	XO(man.)/Mo	XO(spectr.)/Mo	XO(man.)/XO(spectr.)
2	14.12.62	613	1.34	0.275	2230	4.87	457
1	14.12.62	795	1.94	0.388	2050	5.00	410
5	14.12.62	822	1.96	0.421	1950	4.66	419
3	23. 1.63	549	1.33	0.277	1980	4.80	413
6	23. 1.63	596	1.51	0.298	2000	5.07	395
2	23. 1.63	645	1.52	0.304	2120	5.00	424

* Determined by the method of Section II, p. 26, after treatment of the milks by the method of Section II, p. 36 .

** Determined by the method of Section II, p. 45, after treatment of the milks by Treatment B (Section II, p. 49).

purified preparations of bovine milk xanthine oxidase were almost identical with the mean value of the ratio for cow's milk recorded in Table 12.

(b) The relationship between xanthine oxidase activity and molybdenum content of purified preparations of bovine milk xanthine oxidase. The activities and molybdenum and FAD contents of two samples of purified bovine milk xanthine oxidase are shown in Table 20. Sample no. XO-421 was prepared by Dr. R.C. Bray by the method of Gilbert & Bergel (1964), and was homogeneous in the ultracentrifuge (R.C. Bray, personal communication). Sample no. MXO-1 was prepared by the present writer by a modification of the method of Gilbert & Bergel (1964). This modified method is fully described in (c) below. The xanthine oxidase activities of these samples were determined by the spectrophotometric method of Avis et al. (1955). The molybdenum contents were determined by the method described in Section II, p. 57, after wet oxidation of the samples with a mixture of perchloric and sulphuric acids. The FAD contents were determined by the enzymic method described in Section II, p. 61.

The finding, that the activity/molybdenum ratios of these samples were almost identical with the mean value of the ratio found for cow's milk, is strong supporting evidence for the conclusion that xanthine oxidase-i_1 is absent from normal cow's milk. The mean of all the values for this ratio, recorded in Tables 12 and 20, is 5.2. It follows that a sample of purified

Table 20. Properties of preparations of xanthine oxidase from cow's milk

Sample no.	XO activity (spectr. units/l.)	E_{450}	Mo content µM	FAD content µM+++	Specific activity (Activity/E_{450})	Activity / Mo (µM)	FAD(µM) / Mo (µM)
XO-421*	207+ } Mean 185++ } 196	1.95+	37.4++	59	101	5.24	1.6
HXO-1**	38.5	0.585	7.48	20	66	5.15	2.7

* Prepared by Dr. R.C. Bray by the method of Gilbert & Bergel (1964).

** Prepared in the present work by the method described in Section IV, p. 96 .

\+ Determined by Dr. R.C. Bray.

++ Determined in the present work.

+++ Determined by the enzymic method of Section II, p. 61 .

bovine milk xanthine oxidase with an activity/ molybdenum ratio of less than this value will contain xanthine oxidase-i_1 which would arise by inactivation of active xanthine oxidase (the xanthine oxidase-a of Bray *et al.* 1961).

(c) Preparation of xanthine oxidase sample no. HXO-1.

In the method of Gilbert & Bergel (1964) for the isolation and purification of xanthine oxidase from cow's milk, the removal of casein is accomplished by digestion with pancreatin for $3\frac{1}{2}$ hr. at a pH of 7.5 and a temperature of 37°. Morton (1953a, b) considered that the use of proteolytic digestion to remove casein, when isolating alkaline phosphatase from cow's milk, was undesirable, and instead precipitated the casein by adjusting the pH to 4.95 with acetic acid. Carey, Fridovich & Handler (1961) have obtained evidence that in the isolation of xanthine oxidase from cow's milk, the use of proteolytic digestion to remove casein was responsible for the production of several forms of the enzyme which could be distinguished chromatographically. In view of this finding, it was thought worthwhile in the present work to investigate the possibility that the pancreatic digestion step of Gilbert & Bergel (1964) might be responsible for the production of xanthine oxidase-i_1 and xanthine oxidase-i_2 from xanthine oxidase-a. A sample of partially purified xanthine oxidase (no. HXO-1, Table 20) was therefore prepared by the method of Gilbert & Bergel (1964), except that casein was removed by the method of Morton

(1953 b) referred to above. The modified method was as follows.

One litre of cream (fat content 59%) was obtained by separating 8.4 litres of milk in a conventional cream separator, immediately after milking. The milk was a bulk sample of morning milk from three Ayrshire cows which were all near the end of their lactation period and which were given the diet described in Section III, p. 77 .

The cream was diluted with distilled water to a fat content of 50%, and was then churned by hand in a small glass churn fitted with a wooden paddle. Churning was continued until the separation of butter and buttermilk appeared to be complete. The buttermilk (300 ml.) was removed by decantation. The butter was washed twice with distilled water (300 ml. on each occasion). The volumes of the various fractions and their manometric xanthine oxidase activities are given in Table 21. The marked increase in activity with dilution of the cream and cream fractions has already been discussed in Section II, p. 43 .

The buttermilk fraction was treated as follows.

(i). To the buttermilk were added, to give the concentrations shown in brackets, 20% (w/v) sodium salicylate (1 ml./l.), L-cysteine hydrochloride (0.3 g./l.) and disodium ethylene-diamine tetra-acetate (0.4 g./l.).

(ii). The buttermilk so treated was warmed to 32°, and n-butanol (400 ml./l.) was added. The mixture was

Table 21. Xanthine oxidase activities of milk and milk fractions obtained during the preparation of xanthine oxidase

sample no. HXO-1

Fraction	Volume (ml.)	Volume (ml.) used for determination of activity in a reaction volume of 3.4 ml.	Manometric XO activity* (µl. O_2/hr./ml.)	Total manometric units**	% of total activity in whole milk
Whole milk	8400	0.1	720	60	100
Skim milk	7000	0.5 0.2	120 125	8.8+	15
Cream	1000	0.1 0.05	3920 5260	53+	88
Buttermilk	300	0.05 0.02	6550 9180	28+	47
1st butter wash	300	0.1 0.05	3070 3870	12+	20
2nd butter wash	300	0.5 0.1	890 1540	4.5+	7.5
HXO-1	26	0.005	16600	4.3	7.2

* Determined by the method of Section II, p. 26 after treatment by the method of Section II, p. 36.

** One manometric unit ≡ 1×10^5 µl. O_2/hr.

\+ Calculated using the activity obtained at the higher dilution.

stirred vigorously and was heated to 35° for about 10 min. The casein was then precipitated by adjusting the pH to 4.95 (glass electrode) with 0.25N-acetic acid (Morton, 1953 b). The mixture was centrifuged, when it separated into three layers, an upper butanol layer, a brownish-green aqueous layer, and a layer of precipitated casein.

(iii). The upper butanol layer was removed by suction and was discarded. The aqueous layer was also removed by suction and was filtered through a layer of Hyflo Super Cel (Johns-Manville Co., Ltd.) supported on No. 1 Whatman filter paper in a Büchner funnel. The pH of the filtrate was adjusted to 7.5 with sodium hydroxide, causing a white precipitate to appear, and the mixture was allowed to stand overnight at 5°. On the following morning, as much as possible of the supernatant liquid was decanted. The remainder was centrifuged and the precipitate was discarded.

(iv). The supernatant liquid was cooled to -2°, and ammonium sulphate (190 g./l.) was added. The mixture was allowed to stand at 5° for 21 hr.

(v). The turbid mixture was centrifuged at 1150 x g for 45 min., but it remained turbid. It was then filtered through Hyflo Super Cel. The filtrate was still turbid but began to clear on standing, so it was left overnight at 5°.

(vi). Ammonium sulphate (110 g./l.) was added to the cold filtrate with stirring. The mixture was allowed

to stand at 5° for 3 hr., when it separated into a brown gelatinous layer floating on a layer of yellow-green serum. The serum was removed as far as possible by suction and was discarded. The brown material was centrifuged to remove further serum. The brown gelatinous clumps were removed, suspended in 0.01M-sodium-potassium phosphate buffer (pH 6) containing 0.2 mg. sodium salicylate per ml., and dialysed overnight against this suspension medium. The resulting solution was brown and rather turbid. The turbidity was not removed by centrifuging for 15 min. at about 1000 x g.

(vii). The phosphate concentration of the solution was increased from 0.01M to 0.2M, still at pH 6. The solution was then filtered through Hyflo Super Cel which removed some, but not all, of the suspended turbidity. No further purification was attempted. The resulting pale-brown solution, designated no. HXO-1, was stored at 5° in a glass-stoppered Pyrex flask inside a light-tight container.

The activity of this preparation in relation to its molybdenum content has been discussed in (b) above. The specific activity of this preparation, and of various preparations of Bergel & Bray and their collaborators, are discussed in the following section.

3. <u>The specific activity of xanthine oxidase-a.</u>

Bray <u>et al</u>. (1961) proposed a value for the specific activity (expressed as the ratio of spectrophotometric xanthine oxidase activity to extinction

at 450 mμ) for pure active bovine milk xanthine oxidase (xanthine oxidase-a) of 117. This value was derived from the analytical data for a crystalline sample of xanthine oxidase (prepared by the method of Avis et al. 1955) which had a specific activity (defined as above) of 82 and a molar Mo/FAD ratio of 0.70, corresponding to a molybdenum content of 1.40 g. atoms per mole of protein. It was assumed that the sample contained no xanthine oxidase-i_1, i.e. that it was a mixture of xanthine oxidase-a and xanthine oxidase-i_2 only. The value for the specific activity of pure xanthine oxidase-a was then calculated as 82 x 2/1.40 = 117.

That this extrapolated value for the specific activity of xanthine oxidase-a is low, is evident from the following considerations. The molar extinction coefficient at 450 mμ (ε_{450}) of xanthine oxidase is 70,000 (Bray et al. 1961), and all the forms of xanthine oxidase listed in Table 18 contain 2 moles of FAD per mole of protein. It follows that the ratio of activity to molar molybdenum content of the crystalline preparation of Bray et al. (1961), from which the extrapolated specific activity of 117 was derived, was

$$\frac{\text{Activity}}{\text{Mo}} = \frac{\text{Activity}}{E_{450}} \times \frac{\text{FAD}}{\text{Mo}} \times \frac{E_{450}}{\text{FAD}}$$

$$= \frac{\text{Activity}}{E_{450}} \times \frac{\text{FAD}}{\text{Mo}} \times \frac{\varepsilon_{450}}{2}$$

$$= 82 \times \frac{1}{0.70} \times \frac{70000}{2}$$

$$= 4.1 \times 10^6$$

$$\text{i.e. } \frac{\text{Activity}}{\text{Mo}(\mu\text{M})} = 4.1.$$

But the value of this ratio found in the present work is 5.2 (Tables 12 and 20, and p. 50). It follows that this crystalline preparation of Bray et al. (1961) must have contained a considerable amount of xanthine oxidase-i_1, despite the assumption to the contrary. However, in a recent personal communication, Dr. Bray has stated that it is possible that the value for the molybdenum content of this preparation may have been as much as 20% too high. If so, the true activity/Mo (μM) ratio of this preparation would have been 4.9 instead of 4.1. It would then follow that it contained only a relatively small proportion of xanthine oxidase-i_1.

The value for the activity/Mo (μM) ratio of xanthine oxidase of 5.2, found in the present work, leads to a minimum value for the specific activity of pure xanthine oxidase-a of 150, obtained as follows.

$$\frac{\text{Activity}}{E_{450}} = \frac{\text{Activity}}{\text{Mo}} \times \frac{\text{Mo}}{E_{450}}$$

$$= \frac{\text{Activity}}{\text{Mo}} \times \frac{\text{FAD}}{E_{450}}$$

$$= \frac{\text{Activity}}{\text{Mo}} \times \frac{2}{\varepsilon_{450}}$$

$$= 5.2 \times 10^6 \times \frac{2}{70000}$$

$$= 150.$$

The assumptions made in deriving this value are that the composition of xanthine oxidase-a is as postulated by Bray et al. (1961) (see Table 18), and that the molar

extinction coefficient (ε_{450}) for xanthine oxidase-a is 70,000 (Bray et al. 1961).

At the present time the highest specific activity that has been observed in a preparation of purified bovine milk xanthine oxidase, is 120 (Gilbert & Bergel, 1964).

4. Investigations into the origin of xanthine oxidase-i_2

(a) The occurrence of xanthine oxidase-i_2 in purified preparations of bovine milk xanthine oxidase. Since the molar ratios of FAD to molybdenum in the purified preparations of xanthine oxidase recorded in Table 20 are considerably greater than unity, it follows that both of these preparations must have contained considerable amounts of xanthine oxidase-i_2. The most highly purified preparations of Bergel and Bray and their collaborators had FAD/Mo ratios of 1.3 - 1.5, indicating that xanthine oxidase-i_2 was present in all of these preparations (Avis, Bergel & Bray, 1956; Bergel & Bray, 1958). This raises the question of the origin of xanthine oxidase-i_2.

It is possible that xanthine oxidase-i_2 arises by degradation of xanthine oxidase-a, with concomitant loss of molybdenum, during the isolation procedure of Gilbert & Bergel (1964), or during the modified version of this procedure used to prepare sample no. HXO-1 (Section IV, p. 96). However, the molybdenum of xanthine oxidase-a appears to be very firmly bound, since both the activity and molybdenum content of a preparation of purified bovine milk xanthine oxidase were unaffected by

dialysis of the preparation against 0.01M-ammonia for a period of 10 days (Avis, Bergel & Bray, 1956). In view of this finding, it does not seem probable that the mild conditions of the isolation procedure of Gilbert & Bergel (1964), or of the author's modification of this procedure (Section IV, p. 96) could be responsible for degrading xanthine oxidase-a to xanthine oxidase-i_2.

A more likely explanation of the occurrence of xanthine oxidase-i_2 in purified preparations of bovine milk xanthine oxidase is that it is present in the milk, and accompanies xanthine oxidase-a throughout the purification procedure. If so, then the ratios of activity : Mo : FAD in a preparation of purified xanthine oxidase should be identical with those of the milk from which it is prepared, and with those of all the intermediate milk fractions. The results of some preliminary attempts to verify this hypothesis are given in the following sections.

(b) <u>Investigations into the occurrence of xanthine oxidase-i_2 in cow's milk</u>. As a preliminary to the comparison of the activity : Mo : FAD ratios of purified bovine milk xanthine oxidase with those of the milk from which the enzyme was prepared, the values of these ratios were determined in cow's milk. It was expected that the value of the FAD/Mo ratio would give an indication of the presence (FAD/Mo>1) or absence (FAD/Mo=1) of xanthine oxidase-i_2 in cow's milk.

Milks were obtained from Ayrshire cows which were receiving the diet of Section III, p. 77, and from Ayrshire cows of the Institute herd which were being given a normal milk production ration. After treatment of the milks to release bound FAD (see below), the FAD contents were determined by the enzymic method of Section II, p. 61. The FAD contents were compared with the molybdenum contents of the milks determined by the method of Section II, p. 57.

In confirmation of the observations of Manson & Modi (1957), cow's milk was found not to contain free FAD. However, on heating the milks for 5 min. at 95 - 100°, which is a treatment commonly used to release bound FAD in biological material (DeLuca et al. 1956), free FAD appeared. The apparent FAD content of cow's milk remained unaltered on heating the milk for periods of up to 30 min., and on heating it when diluted 3-fold with distilled water. Besides releasing bound FAD, the heat treatment serves also to inactivate the nucleotide pyrophosphatase activity of cow's milk (Corran & Green, 1938 b; Manson & Modi, 1957), which would otherwise split the FAD as it was released (Manson & Modi, 1957).

The FAD contents of cow's milks, treated to release bound FAD by heating at 95 - 100° for 5 - 15 min., are shown in Table 22, together with manometric xanthine oxidase activities, molybdenum contents, ratios of xanthine oxidase activity to molybdenum content, and molar ratios of FAD to molybdenum. Percentage recoveries of known amounts

Table 22. The relationship between the FAD and molybdenum contents of cow's milk

Cow* no.	Date of milking	FAD** µM	Mo µM	Manom. XO activity(+) (µl. O_2/hr./ml.)	XO(man.)/Mo(µM)	FAD(µM)/Mo(µM)	Recovery of added FAD++ (%)
2	23.1.63	0.11	0.304	645	2120	0.4	-
3	"	0.06	0.278	549	1980	0.2	-
6	"	0.11	0.298	596	2020	0.4	-
9	4.3.63	0.20	0.388	687	1770	0.5	-
10	"	0.19	0.171	374	2190	1.1	-
11	"	0.25	0.428	931	2170	0.6	-
12	"	0.26	0.475	975	2050	0.5	-
13	"	0.20	0.428	927	2160	0.5	-
Herd bulk milk	"	0.22	0.367	763	2080	0.6	-
4	26.4.63	0.13	0.100	-	-	1.3	67
4	30.4.63	0.13	0.130	-	-	1.0	49
5	"	0.23	0.206	-	-	1.1	-
4	1.5.63	0.15	0.133	-	-	1.1	-
5	"	0.21	0.261	-	-	0.8	38

* Cows nos. 2 - 6 were given the diet of Section III, p. 77; cows 9 - 13 were cows of the Institute herd receiving a normal milk production ration.

** Determined by the enzymic method of Section II, p. 61 after heating the milks to 95 - 100° for 5-15 min. to release bound FAD.

(+) Determined by the manometric method of Section II, p. 26, after treatment by the method of Section II, p. 36.

++ Added after heating the milk to release bound FAD.

of FAD added to the milks after the heating treatment, are also shown.

From this Table, it is seen that the molar ratio of FAD to molybdenum was usually considerably less than unity, although for a few milks, the ratio was equal to, or slightly greater than unity. From Table 18, the value of this ratio in cow's milk should be at least equal to unity, corresponding to the presence in cow's milk of xanthine oxidase-a only. The fact that the ratios of xanthine oxidase activities to molybdenum content were normal (cf. Table 19), indicates that all the molybdenum present was in the form of xanthine oxidase-a. It follows that the apparent FAD contents of the milks, as determined by the enzymic method, were much too low. This conclusion is substantiated by the low recoveries of added FAD which were obtained. The numerical values of the FAD/Mo ratios obtained from these experiments were therefore of no assistance in deciding whether or not xanthine oxidase-i_2 was present in these milks.

A possible explanation of the poor recoveries of FAD added to cow's milk seemed to be that some free FAD was adsorbed by a constituent of the milk, and was thereby prevented from combining with the apo-protein of D-amino acid oxidase. Since buttermilk from cow's milk contains about 10 times as much xanthine oxidase as the whole milk (cf. Table 21), but is otherwise similar in gross composition to whole milk (except that it has a much lower fat content), it seemed possible that the analysis of buttermilk for FAD and molybdenum might

give a clearer indication of the presence or absence of xanthine oxidase-i_2 in cow's milk. It was expected that the higher xanthine oxidase content of buttermilk relative to whole milk might permit the "diluting out" of the interfering factor responsible for the low recovery of added FAD from cow's milk. Samples of buttermilk were therefore prepared and analysed for FAD and molybdenum as described in (c) below.

(c) Investigations into the occurrence of xanthine oxidase-i_2 in buttermilk. From the bulked milk of five cows given the diet of Section III, p. 77 , two samples of buttermilk were prepared by separating the cream, and converting it into butter and buttermilk in a small glass hand churn fitted with a wooden paddle. The buttermilk was decanted from the butter and was used without further treatment. When not in use, it was stored at -20°.

In order to release bound FAD in these samples and to inactivate the nucleotide pyrophosphatase of cow's milk, both the simple treatment used for whole milk, and various modifications of it, were employed. The modifications of the simple treatment (Nos. 2 - 7 below) represented attempts to prevent or to minimize the suspected adsorption of some of the free FAD by milk protein. The treatments were as follows.

(1) The undiluted buttermilk was heated at 95 - 100° for 5 min., cooled, and diluted 10-fold with distilled water.

(2) The buttermilk was diluted 4.5-fold with distilled

water, heated at 95 - 100° for 5 min., and cooled. Hydrochloric acid was then added to a concentration of 0.1N. The mixture was centrifuged, but remained turbid. A portion of the turbid supernatant layer was neutralized with sodium hydroxide, and was made up with distilled water to give an overall dilution of the original buttermilk of 12.5-fold.

(3) The buttermilk was diluted 18-fold with distilled water and then treated as in (2) above, the overall dilution resulting from this treatment being 20.2-fold.

(4) The buttermilk was diluted 9.9-fold with distilled water, and calcium chloride was added to a concentration of 0.01% (w/v), making a 10-fold dilution of the buttermilk. The mixture was heated at 95 - 100° for 5 min. It was then cooled and centrifuged, but remained turbid.

(5) The buttermilk was diluted 10-fold with distilled water, heated at 95 - 100° for 5 min. and cooled. The pH was then adjusted to 4.6 (the isoelectric point of casein) with 0.25N-acetic acid, and the precipitated casein was filtered off and discarded. A portion of the filtrate was neutralized with sodium hydroxide, and was made up with distilled water to give an overall dilution of the buttermilk of 18.8-fold.

(6) The buttermilk was diluted 10-fold with distilled water, heated at 95 - 100° for 5 min. and cooled. The pH was then adjusted to 4.6 with 0.25N-acetic acid, and the mixture was allowed to stand for 1 hr. at room

temperature. The pH was then adjusted to 1.5 with N-hydrochloric acid, and the mixture centrifuged. The pH was immediately re-adjusted to 4.6 with sodium hydroxide, and the mixture was again centrifuged. The supernatant liquid was decanted from the precipitated casein, and the precipitate was washed twice with 0.1N-hydrochloric acid. The supernatant liquid and washings were combined and neutralized with sodium hydroxide. The overall dilution resulting from this treatment was 25-fold.

(7) The buttermilk was diluted 5-fold with 0.25M-glycine-sodium phosphate buffer pH 9.2 at 20°, and the mixture was incubated with shaking in a Warburg flask at 37°. The mixture was then filtered through a coarse (Pyrex No. 1) sintered glass filter. The filtration removed some clotted material, but the filtrate was still somewhat turbid. A portion of the filtrate was adjusted to pH 7.0 with hydrochloric acid, heated at 95 - 100° for 10 min. and cooled. The pH was then adjusted to 8.5 with sodium hydroxide, and distilled water was added to give an overall dilution of the original buttermilk of 25-fold. This treatment is based on Treatment B of Section II, p. 49 for the partial removal of milk protein.

Recoveries of known amounts of FAD added to the buttermilks were determined in conjunction with all these treatments. The additions were made to separate portions of the buttermilks immediately after the heating step common to all these treatments, in order to avoid

possible breakdown of the added FAD by nucleotide pyrophosphatase. The amounts of FAD added were roughly equal to the estimated FAD contents of the portions taken for analysis. The molybdenum contents of the two buttermilk samples were determined by the method of Section II, p. 57 .

The results of these determinations, recorded in Table 23, show that low recoveries of added FAD were associated with all of these treatments for the release of bound FAD. They show also that the molar ratios of apparent FAD content to molybdenum content were similar to those observed in whole milk (Table 22). However, by correcting the apparent FAD contents given by each treatment for the concomitant loss of added FAD (assuming that the loss of native FAD was of the same magnitude as the loss of the added FAD), the corrected FAD contents shown in the last column of Table 23 were obtained. The fact that these figures are fairly consistent suggests that they may have approximated to the true FAD contents of the buttermilks. The mean of the values for the corrected FAD content of sample A is 4.4 μM, and the ratio of this to the molybdenum content of the sample is 1.4. The corresponding mean corrected FAD content of sample B is 11 μM, and the ratio of this to the molybdenum content of this sample is 2.3. Both these ratios are considerably greater than unity, which is consistent with the presence in these samples of xanthine oxidase-i_2 in addition to xanthine oxidase-a. However, the expedient used to obtain these ratios, i.e. the correction of the apparent FAD contents of the

Table 23. FAD and molybdenum in buttermilk prepared from cow's milk

Buttermilk sample and date of preparation	Mo* µM	Date of FAD determination	Sample treatment to release FAD	Method of determination of FAD	Apparent FAD content (µM)	Recovery of added FAD** (%)	Apparent FAD(µM) / Mo(µM)	Corrected FAD content(+) (µM)
A (28.5.63)	3.2	28.5.63	1 (p.108)	Enzymic	1.9	48	0.6	4.0
		29.5.63	1 (p.108)	Enzymic	2.2	50	0.7	4.4
		30.5.63	2 (p.108)	Enzymic	2.7	63	0.8	4.3
		3.6.63	4 (p.109)	Enzymic	2.4	48	0.8	5.0
		4.6.63	3 (p.109)	Enzymic	2.6	59	0.8	4.4
		4.3.64	10% TCA	Fluorimetric	2.3	99	0.7	-
		10.3.64	10% TCA	Fluorimetric	2.3	99	0.7	-
B (3.6.63)	4.8	6.6.63	5 (p.109)	Enzymic	5.5	44	1.1	12
		7.6.63	6 (p.109)	Enzymic	5.1	44	1.1	12
		26.6.63	7 (p.110)	Enzymic	5.3	64	1.1	8.3
		4.3.64	10% TCA	Fluorimetric	3.3	95	0.7	-
		10.3.64	10% TCA	Fluorimetric	3.8	96	0.8	-

* Determined by the method of Section II, p. 57.

** Added immediately after the FAD-releasing step.

(+) (Apparent FAD content) x 100/(% recovery).

buttermilks for the low recoveries of added FAD, is of doubtful validity when the recoveries concerned are so low. It would be much more satisfactory to determine the true FAD content of the milk and buttermilk by some method that would give reasonably good recoveries of added FAD. Unequivocal determinations of the molar ratios of FAD to molybdenum in cow's milk and buttermilk would then be possible, and the magnitudes of these ratios would indicate whether or not xanthine oxidase-i_2 was present in these materials.

Some time after these enzymic determinations of FAD were made, a sensitive fluorimeter became available which permitted the determination of FAD by the fluorimetric method of Burch (1957) (see Section II, p. 67). The FAD contents of these buttermilk samples, which had been stored meanwhile at -20°, were therefore determined by this fluorimetric method. Recoveries of added FAD were also determined. As in the enzymic determinations, these additions were made immediately after the treatment to release bound FAD (addition of cold trichloroacetic acid to the diluted buttermilks) in order to avoid possible breakdown of the added FAD by nucleotide pyrophosphatase. The amounts of FAD added were roughly equal to the estimated FAD contents of the portions taken for analysis. The results of these determinations, recorded in Table 23 together with the results from the enzymic determinations, show that almost quantitative recoveries of added FAD were obtained in conjunction with the fluorimetric method. Unexpectedly,

however, the method indicated that the apparent content of sample A was about the same as that indicated by the enzymic method, and that the apparent FAD content of sample B was rather less than the content indicated by the enzymic method.

The most likely explanation of these fluorimetric results, considered in conjunction with the excellent recoveries of added FAD, is that the FAD contents of both buttermilks declined during the period of about 9 months (see Table 23) that elapsed between the determinations of FAD content by the two methods. For the whole of that period, the buttermilks were stored at -20°, and their molybdenum contents remained unchanged, indicating that no changes in volume had occurred. An alternative explanation of the fluorimetric results is that the extraction of the bound FAD from the buttermilks by trichloroacetic acid was incomplete. However, this seems unlikely, for the reason that this method of extraction of FAD has been widely used since its development by Bessey and collaborators (Burch, Bessey & Lowry, 1948; Bessey, Lowry & Love, 1949), and so far its efficiency and reliability for the quantitative extraction of FAD from biological material have not been questioned. Therefore, it seems probable that the apparent contents of the buttermilks given by the fluorimetric method may represent their true FAD contents at the time that the fluorimetric determinations were made.

(d) Discussion. The results presented in the preceding sections do not give a clear indication of the

presence or absence of xanthine oxidase-i_2 in cow's milk, due to the unexpected difficulties encountered while attempting to determine the FAD contents of these materials. The hypothesis, that xanthine oxidase-i_2 is present in cow's milk, is supported by the findings that a molar excess of FAD over molybdenum was present in some samples of cow's milk (Table 22) and in one sample of buttermilk (Table 23). If the corrected FAD contents of the buttermilks given in Table 23 are accepted as approximations to the true FAD contents of these samples at the time that they were prepared, then the resulting large molar excesses of FAD over molybdenum (p. 111) in both of these samples provide further evidence in favour of this hypothesis. However, it is clear that definite proof of this will not be forthcoming until a reliable method for the determination of FAD in cow's milk and in buttermilk is available. Such a method must simultaneously (1) release bound FAD efficiently; (2) inactivate the nucleotide pyrophosphatase activity of cow's milk, and (3) permit a reasonable recovery of added FAD to be made.

It is possible that the trypsin-chymotrypsin digestion method developed by Cerletti and collaborators for the release of bound FAD in tissues (Cerletti & Ipata, 1960; Cerletti, Strom & Giordano, 1963), combined with heating to inactivate nucleotide pyrophosphatase, might fulfil these requirements. Alternatively, extraction of FAD with trichloroacetic acid, followed by removal of the trichloroacetic acid by the trioctylamine procedure

of Hughes & Williamson (1951) might be effective. It would be essential to remove the trichloroacetic acid if it were to be used in conjunction with the enzymic method for the determination of FAD, since it was found by Palmer (1962), and confirmed during the present work, that trichloroacetic acid is a strong inhibitor of D-amino acid oxidase.

The fluorimetric method of Burch (1957) is less suitable than the enzymic method for the determination of FAD in whole cow's milk because of the large proportion of free riboflavin relative to FAD that is present in the milk (see Section I, p. 3). The application of this method would result in the FAD content of cow's milk being estimated as a small difference between two large numbers, to both of which the fluorescence of the free riboflavin would contribute the greater part. This difficulty could, of course, be overcome by dialysing the milk to remove the free riboflavin. However, the sensitivity of the fluorimetric method (see Burch, 1957) is such that the procedure would have to be applied to undiluted or to very slightly diluted whole milk, which could lead to low recoveries of added FAD (Burch et al. 1948; Bessey et al. 1949).

In connection with the hypothesis that xanthine oxidase-i_2 is present in cow's milk together with xanthine oxidase-a, it is of interest that Pateman, Cove, Rever & Roberts (1964) have found that the enzymes nitrate reductase and xanthine dehydrogenase (both molybdoflavoproteins), occurring in mutants of the fungus Aspergillus

nidulans, possessed a common co-factor which also regulated the synthesis of nitrate reductase in that organism. It was found that the synthesis of this common co-factor was under genetic control. The composition of the co-factor was not determined, and there was no evidence that it regulated the synthesis of xanthine dehydrogenase. However, from a consideration of the characteristics of the mutants of *Aspergillus nidulans*, it was suggested by Pateman *et al.* (1964) that "if [the co-factor] is one of the known prosthetic groups [of these enzymes] it is more likely to be associated with the molybdenum than with the iron or flavin". Since xanthine oxidase-i_2 lacks molybdenum (Table 18), this suggests that xanthine oxidase-i_2 may be an intermediate in the synthesis of xanthine oxidase-a, and that this synthesis is completed by the addition of a molybdenum-containing co-factor which may be similar to that of Pateman *et al.* (1964). If this is so, then it is possible that the relative proportion of xanthine oxidase-i_2 to xanthine oxidase-a in cow's milk may be related to the stage of lactation of the cow. It may be significant in this respect that xanthine oxidase sample no. HXO-1 (Table 20), which was prepared from the combined milk of three cows all near the end of their lactation periods, contained a high proportion of xanthine oxidase-i_2, as shown by the high molar ratio of FAD to molybdenum (Table 20). This suggests that, once a reliable method for the estimation of FAD in whole milk is available, it would be worthwhile to determine whether a relationship exists

between the stage of lactation of a cow and the ratio of xanthine oxidase-i_2 to xanthine oxidase-a in its milk.

5. <u>The activity of purified bovine milk xanthine oxidase as coenzyme for the apo-protein of D-amino acid oxidase</u>

(a) <u>Experimental</u>. It has recently been claimed that bound FAD of purified bovine milk xanthine oxidase can take the place of free FAD as the coenzyme for the apo-protein of D-amino acid oxidase, and that the Michaelis constant for the bound FAD of xanthine oxidase in the system apo-D-amino acid oxidase + D-alanine is equal to the Michaelis constant for free FAD in the same system (Yamano, Miyazaki, Niki, Shiraishi & Sawada, 1956; Horiuchi, 1958; Matsukawa, 1958). A preliminary experiment to investigate these claims was made by adding varying amounts of a preparation of purified milk xanthine oxidase (No. XO-421, prepared by Dr. R.C. Bray; see Section IV, p. 94 and Table 20) to the assay system of Section II, p. 61 . This sample of xanthine oxidase was prepared about 10 months before this particular experiment was made. During that period it was stored under the conditions recommended by Bergel & Bray (1959). The preparation was found to possess appreciable coenzyme activity in the D-amino acid oxidase assay system. The observed rates of oxygen uptake were converted to apparent concentrations of free FAD in the original preparation by reference to the usual standard curve

determined at the same time. The total FAD content of the preparation, after heating at 100° for 5 min., was determined in the same D-amino acid oxidase assay system. The results of these determinations, recorded in Table 24, show that almost exactly one-third of the total FAD of this preparation of xanthine oxidase reacted as though it were free FAD, and that the amount of "apparent free FAD" found was proportional to the amount of the preparation that was added to the D-amino acid oxidase assay system.

(b) Discussion. There are two possible explanations of these results, (1) that this preparation of xanthine oxidase actually contained this concentration of free FAD, and (2) that some constituent of the preparation was capable of reacting as free FAD in the D-amino acid oxidase assay system.

The first possibility cannot be ruled out entirely, since the preparation was 10 months old at the time of this experiment, and had not been dialysed or treated in any way to remove free FAD immediately before the experiment. However, it was unlikely that the sample would have decomposed to that extent with the liberation of free FAD, since the stability of preparations stored under the conditions of Bergel & Bray (1959) is usually very good. Support for the second possibility comes from the analytical results for the composition of the preparation, given in Table 20 (p. 95). From this Table, the molar excess of FAD over molybdenum of sample no. XO-421 was 59μM-37μM = 22μM. From Table 24 (p. 120), the apparent content of free FAD of

Table 24. The activity of a preparation of purified milk xanthine oxidase in the enzymic assay system for FAD of Section II, p. 61.

Xanthine oxidase sample no.	Concn. of total FAD (μM)	Vol. of XO-421 added to assay system (μl.)	Apparent free FAD (μm-moles)	Concn. of apparent free FAD in XO-421 (μM)
XO-421*	59**	2	0.043	22
		5	0.090	18
		10	0.210	21
				Mean 20

* Prepared by Dr. R.C. Bray; see Section IV, p. 94.

** Determined by the enzymic method of Section II, p. 61, after heating at 100° for 5 min.

the preparation was 20μM. It was found in the present work that this preparation consisted of a mixture of xanthine oxidase-a and xanthine oxidase-i_2 only (see Section IV, pp. 94 and 103). Therefore, the molar excess of FAD over molybdenum represented the FAD content of the xanthine oxidase-i_2 present in the preparation. The fact that this was very nearly equal to the apparent content of free FAD suggested that it might be the FAD of xanthine oxidase-i_2 that was acting as a coenzyme for the apo-protein of D-amino acid oxidase. However, until other, freshly prepared samples of purified milk xanthine oxidase are examined for coenzyme activity in this system, this explanation must be regarded as tentative only.

Whatever may be the correct explanation of these results, it is clear that they do not support the claims of Yamano et al. (1956) and of Horiuchi (1958) that the Michaelis constants of xanthine oxidase-bound FAD and free FAD in the D-amino acid oxidase system are equal. If this were so, then all the FAD of XO-421 should have reacted as free FAD.

6. The problem of preparing pure xanthine oxidase-a

(a) Introduction. The desirability of preparing pure xanthine oxidase-a, free from inactive flavoproteins, is emphasized by the recent work of Bray and collaborators on the sequence of electron transfer within the molecule of active xanthine oxidase (Palmer, Bray & Beinert, 1963; Palmer, Bray & Beinert, 1964; Bray, Palmer & Beinert, 1964). During the aerobic oxidation of xanthine to uric acid, catalysed by milk xanthine oxidase, these authors

obtained electron-spin resonance signals which were attributed to two chemically distinct forms of pentavalent molybdenum. It was suggested (Bray et al. 1964) that both these types of molybdenum were present in the enzymically active form of xanthine oxidase, but there was some doubt about this because the variable specific activities of the preparations of xanthine oxidase used in this work indicated that one or more inactive forms of xanthine oxidase were also present (Palmer et al. 1964). One of the preparations of purified xanthine oxidase used by Bray and collaborators in this work was No. XO-421 (R.C. Bray, personal communication), the composition of which is given in Table 20 of this thesis. It has been shown in the present work that this particular preparation contained considerable amounts of xanthine oxidase-i_2, but did not contain xanthine oxidase-i_1 (Section IV, pp. 94 and 103). This strengthens the suggestion of Bray et al. (1964) that the two types of electron-spin resonance signal attributed to molybdenum were both due to molybdenum present in the enzymically active form of xanthine oxidase. However, it is clear that it would be more satisfactory to apply this technique to pure xanthine oxidase-a which could be demonstrated to contain a stoicheiometric amount of molybdenum and to be free of inactive metalloflavoproteins.

(b) A possible approach to the problem of separating xanthine oxidase-a and xanthine oxidase-i_2. The finding that the apparent free FAD content of a preparation of purified xanthine oxidase (determined by the D-amino acid

oxidase method) was equal to the FAD content of the xanthine oxidase-i_2 present in that preparation (Section IV, p. 119) suggests a possible approach to the problem of separating xanthine oxidase-a and xanthine oxidase-i_2. If the FAD of xanthine oxidase-i_2 were found to be responsible for the coenzyme activity of purified preparations of xanthine oxidase, then it would follow that this FAD must be bound by the apo-protein of D-amino acid oxidase. A Michaelis constant of about 0.2 μM (Burton, 1955) for the combination of free FAD with apo-D-amino acid oxidase indicates that the affinity of the apo-protein for free FAD is very strong. If a similar affinity exists between the apo-protein and the bound FAD of xanthine oxidase-i_2, then it is possible that the properties of the complex formed by these two might differ sufficiently from the properties of xanthine oxidase-a to permit their separation by one or more of the usual physico-chemical methods, e.g. chromatography, electrophoresis, or ultra-centrifugation. However, it would first be necessary to establish with certainty that the coenzyme activity of purified preparations of bovine milk xanthine oxidase towards apo-D-amino acid oxidase was indeed due to their content of xanthine oxidase-i_2.

SECTION V

SUMMARY

1. The relationships between the xanthine oxidase activity, molybdenum content, and flavin-adenine dinucleotide (FAD) content of cow's milk, and between the xanthine oxidase activity and molybdenum content of goat's milk, have been investigated.

2. A method for effecting quantitative release of the bound xanthine oxidase activity of cow's milk has been developed, and existing manometric and spectrophotometric methods for the determination of xanthine oxidase activity in milk have been modified and improved. With the aid of these improved methods it has been shown that, provided the diet of the cow is not supplemented with a soluble molybdate, a constant ratio of xanthine oxidase activity to molybdenum content is observed both in cow's milk and in purified xanthine oxidase isolated from cow's milk by the method of Gilbert & Bergel (1964). It follows from this observation that the inactive, molybdenum-containing "xanthine oxidase-i_1" of Bray *et al.* (1961) must arise by inactivation of active xanthine oxidase during the isolation of this enzyme from cow's milk. It is shown that the estimated value of Bray *et al.* (1961) of 117 for the specific activity (activity/E_{450}^{1cm}) of pure active bovine milk xanthine oxidase, is low. A new value of 150 is proposed for the specific activity of the pure enzyme.

3. The xanthine oxidase activity of goat's milk has been found to be very low compared with the activity of cow's milk. Molybdenum was found in all the samples of goat's milk that were examined, usually in concentrations slightly less than those found in normal cow's milk, but there was no correlation between the xanthine oxidase activities and molybdenum contents in the goat's milks.

4. An inhibitor of bovine milk xanthine oxidase of unknown composition was found to be present in cow's milk. Neither the copper nor the orotic acid present in cow's milk were responsible for the inhibition.

5. The influence of dietary molybdenum intake on the xanthine oxidase activities of cow's and of goat's milks was investigated. It was found that with daily intakes of 5 mg. Mo/cow/day and 1.1 mg. Mo/goat/day, supplementary molybdenum (as sodium molybdate) was without effect on the xanthine oxidase activities of the milks of either cows or goats.

6. The manometric D-amino acid oxidase method for the determination of FAD has been modified and improved. It gave satisfactory results when applied to the determination of the FAD content of purified bovine milk xanthine oxidase, but gave less satisfactory results when applied to the determination of the FAD content of cow's milk and of buttermilk. However, on the basis of the results obtained by the use of this method, it is suggested that cow's milk contains an enzymically inactive

ferroflavoprotein (the "xanthine oxidase-i_2" of Bray *et al.* 1961) which accompanies active xanthine oxidase throughout present procedures for the isolation of this enzyme from cow's milk.

7. In confirmation of earlier reports in the literature, a sample of purified bovine milk xanthine oxidase was found to possess some activity as coenzyme for the apo-protein of D-amino acid oxidase. The implications of this finding are discussed.

REFERENCES

Ackerman, E. & Brill, A.S. (1962). Biochim. biophys. Acta, 56, 397.

Archibald, J.G. (1951). J. Dairy Sci. 34, 1026.

Archibald, J.G. (1958). Dairy Sci. Abstr. 20, 712.

Avis, P.G., Bergel, F. & Bray, R.C. (1955). J. chem. Soc. p. 1100.

Avis, P.G., Bergel, F. & Bray, R.C. (1956). J. chem. Soc. p. 1219.

Avis, P.G., Bergel, F., Bray, R.C., James, D.W.F. & Shooter, K.V. (1956). J. chem. Soc. p. 1212.

Baldwin, E. (1959). Dynamic Aspects of Biochemistry, 3rd ed., p. 158. Cambridge : The University Press.

Ball, E.G. (1938). Science, 88, 131.

Ball, E.G. (1939). J. biol. Chem. 128, 51.

Bergel, F. & Bray, R.C. (1958). Symp. biochem. Soc. 15, 64.

Bergel, F. & Bray, R.C. (1959). Biochem. J. 73, 182.

Bessey, O.A., Lowry, O.H. & Love, R.H. (1949). J. biol. Chem. 180, 755.

Bingley, J.B. (1959). J. agric. Fd. Chem. 7, 269.

Bingley, J.B. (1963). J. agric. Fd. Chem. 11, 130.

Bourquin, A. & Sherman, H.C. (1931). J. Amer. chem. Soc. 53, 3501.

Bray, R.C. (1959). Biochem. J. 73, 690.

Bray, R.C., Malmström, B.G. & Vänngård, T. (1959). Biochem. J. 73, 193.

Bray, R.C., Palmer, G. & Beinert, H. (1964). J. biol. Chem. (in press).

Bray, R.C., Pettersson, R. & Ehrenberg, A. (1961). Biochem. J. 81, 178.

Burch, H.B. (1957). In Methods in Enzymology, vol. 3, p. 960. Ed. by Colowick, S.P. & Kaplan, N.O. New York : Academic Press Inc.

Burch, H.B., Bessey, O.A. & Lowry, O.H. (1948). J. biol. Chem. 175, 457.

Burton, K. (1951). Biochem. J. 48, 458.

Burton, K. (1955). In Methods in Enzymology, vol. 2, p. 199. Ed. by Colowick, S.P. & Kaplan, N.O. New York : Academic Press Inc.

Carey, F.G., Fridovich, I. & Handler, P. (1961). Biochim. biophys. Acta, 53, 440.

Cerletti, P. & Ipata, P. (1960). Biochem. J. 75, 119.

Cerletti, P. & Siliprandi, N. (1958). Arch. Biochem. Biophys. 76, 214.

Cerletti, P., Strom, R. & Giordano, M.G. (1963). Arch. Biochem. Biophys. 101, 423.

Clark, L.J. & Axley, J.H. (1955). Anal. Chem. 27, 2000.

Corran, H.S., Dewan, J.G., Gordon, A.H. & Green, D.E. (1939). Biochem. J. 33, 1694.

Corran, H.S. & Green, D.E. (1938 a). Nature, 142, 149.

Corran, H.S. & Green, D.E. (1938 b). Biochem. J. 32, 2231.

Crammer, J.L. (1948). Nature, 161, 349.

DeLuca, C., Weber, M.M. & Kaplan, N.O. (1956). J. biol. Chem. 223, 559.

Dixon, M. (1925). Biochem. J. 19, 507.

Dixon, M. & Kodama, K. (1926). Biochem. J. 20, 1104.

Euler, H. v. & Adler, E. (1934). Ark. Kemi Min. Geol. 11B, No. 28, pp. 6.

Gilbert, D.A. (1962). Ph.D. Thesis, University of London.

Gilbert, D.A. & Bergel, F. (1964). Biochem. J. 90, 350.

Gilbert, T.W. jun., & Sandell, E.B. (1960). J. Amer. chem. Soc. 82, 1087.

Green, D.E. & Beinert, H. (1953). Biochim. biophys. Acta, 11, 599.

Gudnason, G.V. & Shipe, W.F. (1962). J. Dairy Sci. 45, 1440.

György, P., Kuhn, R. & Wagner-Jauregg, T. (1934). Hoppe-Seyl. Z. 223, 241.

Haider, S.Z. & Khundkar, M.H. (1954). Analyst, 79, 783.

Hallanger, L.E., Laakso, J.W., & Schultze, M.O. (1953). J. biol. Chem. 202, 83.

Harrison, D.C. & Thurlow, S. (1926). Biochem. J. 20, 217.

Hoeflake, H. (1953). Neth. Milk & Dairy J. 7, 227.

Hofstee, B.H.J. (1955). J. biol. Chem. 216, 235.

Horiuchi, N. (1958). Osaka Daigaku Igaku Zassi, 10, 331; cited from Chem. Abstr. (1958), 52, 12031.

Houston, J., Kon, S.K. & Thompson, S.Y. (1940 a). J. Dairy Res. 11, 67.

Houston, J., Kon, S.K. & Thompson, S.Y. (1940 b). J. Dairy Res. 11, 155.

Huennekens, F.M. & Felton, S.P. (1957). In Methods in Enzymology, vol. 3, p. 950. Ed. by Colowick, S.P. & Kaplan, N.O. New York : Academic Press Inc.

Hughes, D.E. & Williamson, D.H. (1951). Biochem. J. 48, 487.

Jenness, R. & Patton, S. (1959 a). Principles of Dairy Chemistry, p. 183. London : Chapman & Hall Ltd.

Jenness, R. & Patton, S. (1959 b). Principles of Dairy Chemistry, p. 185. London : Chapman & Hall Ltd.

Karrer, P., Schöpp, K. & Benz, F. (1935). Helv. chim. acta 18, 426.

Keilin, D. & Hartree, E.F. (1936). Proc. roy. Soc. B, 119, 141.

Keilin, D. & Hartree, E.F. (1945). Biochem. J. 39, 293.

Kiermeier, F. & Capellari, K. (1958). Biochem. Z. 330, 160.

Kuhn, R., György, P. & Wagner-Jauregg, T. (1933). Ber. 66B, 1034.

Ling, E.R., Kon, S.K., & Porter, J.W.G. (1961 a). In Milk: the Mammary Gland and Its Secretion, vol. 2, p. 220. Ed. by Kon, S.K. & Cowie, A.T. New York and London : Academic Press Inc.

Ling, E.R., Kon, S.K. & Porter, J.W.G. (1961 b). In Milk: the Mammary Gland and Its Secretion, vol. 2, p. 198. Ed. by Kon, S.K. & Cowie, A.T. New York and London : Academic Press Inc.

Litwack, G., Williams, jun., J.N., Chen, L. & Elvehjem, C.A. (1952). J. Nutr. 47, 299.

Litwack, G., Williams, jun., J.N., Fatterpaker, P., Chen, L. & Elvehjem, C.A. (1953). J. Nutr. 49, 579.

Litwack, G., Williams, jun., J.N., Feigelson, P. & Elvehjem, C.A. (1950). J. biol. Chem. 187, 605.

Macrae, T.F. (1930). Ph.D. Thesis, University of Glasgow.

Manson, W. & Modi, V.V. (1957). Biochim. biophys. Acta, 24, 423.

Matsukawa, H. (1958). Osaka Daigaku Igaku Zassi, 10, 543; cited from Chem. Abstr. (1958), 52, 13832.

Middleton, G. & Stuckey, R.E. (1954). Analyst, 79, 138.

Milk Composition in the United Kingdom (1960). Report of an Interdepartmental Committee, p. 39. London : H.M. Stationery Office (Cmnd. 1147).

Modi, V.V. & Owen, E.C. (1956). Nature 178, 1120.

Modi, V.V., Owen, E.C. & Darroch, R.A. (1959). J. Dairy Res. 26, 277.

Morgan, E.J., Stewart, C.P. & Hopkins, F.G. (1922). Proc. roy. Soc. B, 94, 109.

Morton, R.K. (1953 a). Biochem. J. 55, 786.

Morton, R.K. (1953 b). Biochem. J. 55, 795.

Morton, R.K. (1954). Biochem. J. 57, 231.

Morton, R.K. (1955). Biochem. J. 60, 573.

Ochoa, S. & Rossiter, R.J. (1939). Biochem. J. 33, 2008.

Palmer, G. (1962). Biochim. biophys. Acta, 56, 444.

Palmer, G., Bray, R.C. & Beinert, H. (1963). Fed. Proc. 22, 466.

Palmer, G., Bray, R.C. & Beinert, H. (1964). J. biol. Chem. (in press).

Pateman, J.A., Cove, D.J., Rever, B.M. & Roberts, D.B. (1964). Nature, 201, 58.

Piper, C.S. & Beckwith, R.S. (1948). J. Soc. chem. Ind. Lond. 67, 374.

Richert, D.A. & Westerfeld, W.W. (1954). J. biol. Chem. 209, 179.

Robert, L. & Polonovski, J. (1955). Disc. Farday Soc. 20, 54.

Rodkey, F.L. & Ball, E.G. (1946). J. Lab. clin. Med. 31, 354.

Teresi, J.D., Elvehjem, C.A. & Hart, E.B. (1942). Amer. J. Physiol. 137, 504.

Totter, J.R., Burnett, jun., W.T., Monroe, R.A., Whitney, I.B. & Comar, C.L. (1953). Science 118, 555.

Underwood, E.J. (1962). Trace Elements in Human and Animal Nutrition, 2nd ed., p. 109. New York and London : Academic Press Inc.

Warburg, O. & Christian, W. (1938). Biochem. Z. 298, 150.

Whitby, L.G. (1953). Biochem. J. 54, 437.

Wieland, H. & Macrae, T.F. (1930). Liebigs Ann. 483, 217.

Williams, C.H. (1955). J. Sci. Fd Agric. 6, 104.

Wood, T.B. & Woodman, H.E. (1939). Rations for Livestock. Bulletin No. 48, Ministry of Agriculture and Fisheries. London : H.M. Stationery Office.

Worden, A.N. (1943). Nature, 152, 505.

Yamano, T., Miyazaki, M., Niki, H., Shiraishi, J. & Sawada, S. (1956). Osaka Daigaku Igaku Zassi, 8, 1357; cited from Chem. Abstr. (1957), 51, 9762.