RESAZURIN REDUCTION IN MILK

by

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ABSTRACT

Methods were developed to quantitatively measure resazurin reduction and the reducing capacity of milk. The method for resazurin determination involved butanol extraction of the dye from milk and measurement of the optical density of extracts at 582 mμ and 615 mμ after saturation with sodium bicarbonate; that for reducing capacity was based on reaction of 2,6-dichlorophenolindophenol with milk at its normal pH. For the latter determination excess indophenol was added and the quantity of dye remaining estimated spectrophotometrically at 660 mμ in butanol extracts saturated with sodium bicarbonate. This assay could be applied in the presence of resazurin or resorufin, since these compounds had negligible absorbance at 660 mμ. Both of these methods were reproducible in milk and their respective accuracies estimated at greater than 90 percent.

The quantitative assay for resazurin was employed in order to study the behavior of resazurin in milk and the systems in fresh milk responsible for reduction of the dye. Results obtained on aging whole and skim milk were used to demonstrate that the resazurin reduction dealt with in the present investigation was due to the reducing systems of milk rather than to bacterial activity. These results showed that the reducing system was less stable and more sensitive to temperature of aging in skim than in whole milk. Measurement of rates of resazurin reduction by various fractions of normal milk showed that the major portion of the reducing ability of whole milk was associated with the cream. The aqueous phase from centrifuged

warm gravity cream had a greater ability to reduce resazurin than did the whole milk from which the fractions were derived. The rate of resazurin reduction by milk decreased with incubation at 37°C, however, in the presence of sufficient numbers of bacteria, an acceleration of rate with incubation was noted. The point at which washed suspensions of added bacteria became significant in reduction was demonstrated as a change in slope of logarithmic plots of dye reduction rates.

Resazurin was shown to have a destabilizing influence on the reducing capacity of milk. This influence was catalytic and dependent on total concentration of dye; rate of inactivation being constant for a given dye concentration. Evidence was presented to show that the component of the reducing system that was inactivated was ascorbic acid. The influence of fractions, obtained from passage of resazurin through a silicic acid column, suggested that this catalytic effect was probably due to the dye as such, rather than to artifacts in the commercial dye preparation used. Examination of the reducing capacity of milk fractions before and after treatment with ascorbic acid oxidase indicated that resazurin reduction was brought about by that part of the reducing capacity that could not be accounted for as free ascorbic acid. In mastitic samples, this element of the reducing capacity was concentrated completely in the fat and centrifuged sediment.

It was concluded from these investigations that the reducing system of milk existed as a measurable entity at any given time rather than as a continuous evolution of electrons from

some slow enzymatic reaction. This system consisted of the measurable ascorbic acid of the milk, which occurred in the plasma, and some reducing agent bound to structural components of the cream and sediment. The measurable ascorbic acid accounted for approximately 80 percent of the reducing capacity but was concluded to have little influence on resazurin reduction. It was concluded that the bound reducing agent depended on structural elements in the milk for its ability to reduce resazurin, and that it lost this ability on dissociation from whatever particle it occurred on. It was postulated that this reducing agent was ascorbate and that it occurred bound to leucocytes and other cellular debris in the milk in situations analogous to its reported occurrence in blood. Attempts to identify this reducing agent as ascorbate were unsuccessful in this investigation, but the techniques employed were probably inadequate.

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INTRODUCTION AND HISTORICAL ASPECTS

The present investigation was concerned mainly with resazurin reduction in the absence of significant bacterial activity and was undertaken in an effort to determine mechanism of reduction by the naturally occurring reducing systems of milk. Since interest in resazurin reduction in milk has been focused on use of the dye as an indicator of sanitary condition, the bulk of the research on this topic has been centred on assessing milk produced under abnormal physiological or pathological udder conditions and on subsequent bacterial contamination. A great deal of the work has involved comparison of resazurin reduction with other tests such as bacterial and cell counts, catalase, methylene blue reduction, and potentiometric measurements. In the following summary, no effort will be made to detail this work since it has recently been reviewed (1). The reducing systems operative in normal and mastitic milks have also been the subject of a recent review (2). Those observations having a direct bearing on the present investigation will be considered briefly. Usually, these were passing observations made in the course of some study related to assessing hygenic quality of milk and were not investigated beyond their first recording. As a result, the following summary of these observations is brief and discontinuous with respect to time and context.

The use of resazurin as an indicator for determining the sanitary condition of milk was first studied in North America by Ramsdell et al. (3) in 1935, though the indicator had received

some attention in this regard in Europe prior to that time. These investigators observed that resazurin offered several advantages over the more electronegative methylene blue used in the well established reductase test. Resazurin became reduced much more readily than did methylene blue thus giving a more rapid assessment of bacterial activity. One of the more important observations made, however, was that resazurin was very sensitive to physiologically abnormal and pathological conditions of milk; rapid reduction correlating well with high catalase content and high cell count. Samples of this type showed rapid initial dye reduction after which little change took place until bacterial activity was evident. In attempting to correlate potentiometrically measured Eh values with degree of resazurin reduction, these workers found that there was a wide range of Eh values corresponding to a single shade of resazurin color in normal skim milk. If abnormal milk was added, the zones of reduction were shifted to more electropositive values, resulting in more rapid reduction of the dye. In these initial investigations, the observation was made that the yellow carotenoid pigments of the emulsion phase of the milk decreased the intensity of the blue color of the dye, making visual observation of color change difficult in some whole milk samples.

Strynadka and Thornton, in 1938 (4), using leucocytes obtained from blood and repeatedly washed in physiological saline, could obtain no significant alteration in reduction time of methylene blue when suspensions of these were added to raw

milk. In addition, they could not obtain good correlations between leucocyte counts of milk samples and ability to reduce methylene blue and concluded that leucocytes were not of particular importance in the reductase test.

The influence of resazurin on the Eh of milk was reported by Johns and Howson in 1940 (5). They found that the time-potential curves for milk containing resazurin differed from those of plain milk or milk containing methylene blue, the curves for resazurin-containing milk showing a sharper initial decrease in Eh followed by a flattening of the curves after the dye had reached the pink stage. Decreasing dye concentrations resulted in curves of the same shape but showing the decrease in Eh at an earlier time. They also noted that resazurin reduction occurred in a shorter time and at a higher Eh with lower dye concentrations. In addition, hourly inversion of tubes during incubation shortened reduction time, the incorporation of oxygen by this practice having no effect on the time-potential curves in the early stages and only a transient effect later.

In further studies concerning the behavior of resazurin in milk in 1941, Johns (6) found that resazurin could show marked changes in color with no decrease in potential of the milk. Using continuous aeration to maintain a high Eh in one series of samples, he showed that rate of resazurin reduction was the same for comparable unaerated samples in which the Eh decreased by as much as 0.2 volt. This observation, along with data on the influence of dilution of normal milk with leucocytes and centrifuge sediment from abnormal milk enabled him to suggest

that resazurin reduction depended on substances present in abnormal milk which had no influence on oxidation reduction potential. In connection with this observation, he pointed out that washing leucocytes in physiological saline definitely decreased their reducing activity, while washing in normal milk had less effect. In the same series of experiments, Johns observed an increase in potential of approximately 0.06 volt on 5 hours incubation of normal milk with added resazurin. Since control samples failed to show this trend, the author concluded that the increase was due to the presence of resazurin.

McBride and Golding (7), in 1951, found that if the resazurin test was conducted on quarter samples of milk within four hours of milking it measured leucocyte activity only. They also observed that mastitic milk could reduce the dye to the colorless form without the lag period found by earlier workers (3,5).

The reducing properties of normal and abnormal milk were reported by Nilsson in the years 1950 to 1957 and were reviewed by the same author in 1959 (2). She found that mastitic milk contained a reducing system associated with the fat, which was destroyed by heating to 85°C for 5 minutes. In testing substrates for the reducing enzymes of milk, she found that only those for xanthine oxidase produced a fall in potential comparable with that characteristic of mastitic milk. The difference between normal and mastitic milk with regard to this enzyme system was found to be the presence of guanase and higher levels of xanthine oxidase activity in mastitic samples.

In analyses for the products of this enzyme system, she noted an increase of 0.10 mg per ml in the uric acid content after drop in potential of mastitic milk. In normal milk there was no fall in potential and no increase in uric acid. She also found that the level of other acids was the same in both types of milk and concluded that the substrates could not have consisted of aldehydes but were probably precursors of xanthine or hypoxanthine. In this series of investigations, the observation was also made that leucocytes from mastitic milk did not, in themselves, have any reducing ability when added to pasteurized milk. The author concluded that the reducing activity found by earlier workers to be associated with high leucocyte counts was an indirect relationship and that high leucocyte count reflected an udder condition that permitted escape of blood substances into the milk.

The resazurin reducing ability of leucocytes was re-examined in 1960 by Campbell and Phelps (8). They found that removal of bacteria and leucocytes from milk by centrifuging had little influence on rate of resazurin reduction if the fat was reincorporated. Leucocytes obtained from bovine blood and washed in physiological saline had to be added to milk in concentrations of 1×10^6 per ml to obtain rapid resazurin reduction. When leucocytes freshly isolated from bovine blood were held in milk at 37°C for 4 hours before addition of resazurin, they lost most of their ability to reduce the dye. If obtained from milk and washed in physiological saline or in normal milk, addition of 4×10^6 leucocytes per ml of milk

produced only a slight acceleration of resazurin reduction rate. These authors also observed that when leucocytes isolated from milk were suspended in fresh bovine plasma for 2 hours they regained ability to reduce resazurin, their activity being comparable with that of those freshly isolated from bovine blood. These revitalized leucocytes did not lose reducing activity on repeated washing. Disruption of freshly isolated leucocytes by sonic oscillation, followed by treatments with ribonuclease or desoxyribonuclease destroyed a large part of their ability to reduce resazurin. From this, the authors concluded that leucocytes, even when disrupted and degraded, did not supply substrates to the xanthine oxidase system in sufficient quantity to account for rapid resazurin reduction.

The influence of the xanthine oxidase system on resazurin reduction by normal milk was reported by Campbell and Keur in 1961 (9). They could find no correlation between the concentration of xanthine oxidase in milk and rate of resazurin reduction. Pasteurization eliminated all resazurin reducing activity but had no effect on xanthine oxidase, while added xanthine oxidase inhibitors had no effect on rate of resazurin reduction.

This brief historical summary contains several salient points concerning the mechanism of resazurin reduction in milk in the absence of significant bacterial activity. Resazurin reduction was virtually independent of the aerobic electrode potential of milk, though the dye had some influence on measured potential (5,6). Rate of resazurin reduction was very sensitive to abnormal physiological and pathological conditions

of milk (3,7). The reducing agent appearing in these abnormal milks appeared to be associated with, and possibly bound loosely to, the leucocytes present (6), though it was not the leucocytes themselves (2,4,8). The xanthine oxidase was present in high concentration but lacked a substrate in normal milk (9). In mastitic milk, there appeared to be substrates for this enzyme (10).

It is evident from the foregoing that a resazurin reducing system exists in normal milk. Under abnormal udder conditions, either this system becomes more active, or a new reducing system appears. The present investigation was concerned with determining the nature of these reducing systems and whether or not they were separate systems.

MATERIALS AND METHODS

I MILK

Machine-drawn samples of milk from individual cows of the University herd, predominantly Ayrshire, were used for most of the work described. No effort was made to differentiate between milk from different quarters of the udder or different stages of the milking procedure. Any sample represented a complete milking, including strippings. If there was evidence of clinical mastitis, the affected quarter was hand-milked and the milk either discarded or used separately. It was found that there was little day to day variation in the reducing properties of milks from individual cows, any significant changes in the fresh milk occurring gradually over an interval of several weeks. There was, however, considerable variation between milks from different cows and mixed samples drawn from randomly selected ten-gallon cans often differed significantly from one another. Random mixed samples were often used as controls for a series of individual samples as well as to show that a given observation was not peculiar to a particular cow but applied more generally.

II RESAZURIN REDUCTION

Resazurin reduction was estimated visually according to the procedure in Standard Methods for the Examination of Dairy Products (11). Incubation was at 37°C and samples were kept under observation for the first 30 minutes. They were re-examined at 30 minute intervals with inversion of tubes at each examination. The time required for the color of the sample to

reach that of the Munsell 5P7/4 standard was recorded as being the reduction time. All samples were generally left at 37°C for 2 to 3 hours regardless of reduction time.

III REDUCED ASCORBIC ACID

A. Titration

The reagents and standards for the procedure listed in Official Methods of Analysis of the Association of Official Agricultural Chemists (12) were used. The titration procedure was according to Sharp (13), except that metaphosphoric acid-acetic acid solution was used in place of N/10 sulfuric acid to precipitate proteins prior to titration.

B. Paper Chromatography

The method advocated by Block, Durram, and Zweig (14) was used. The solvent system was phenol, acetic acid, water. The 2,6-dichlorophenolindophenol spray was used to locate ascorbic acid and was followed by the ammoniacal silver nitrate spray, which developed a new series of spots on chromatograms of milk extracts.

C. Oxidation to Dehydroascorbic Acid

Solid ascorbic acid was oxidized to dehydroascorbic acid with 0.01 N iodine according to the method outlined by Kohman and Sanborn (15). The dehydroascorbic acid was diluted and used within 10 minutes of its preparation.

D. Ascorbic Acid Oxidase

Concentrated cucumber juice was prepared according to Sharp et al. (16). This crude enzyme preparation was not purified further and was found to be sufficiently active for

removal of ascorbic acid from milk.

IV FREE SULFHYDRYL GROUPS

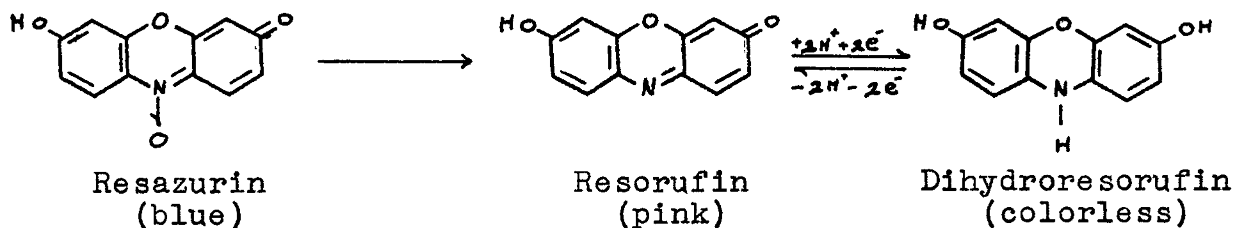
Free sulfhydryl groups were estimated using the nitroprusside procedure of Patton and Josephson (17). The standards and milk samples were kept in ice until reagents had been added to all tubes. They were then placed at room temperature for 10 minutes before comparison. It was found that maximum color developed in 10 minutes but faded rapidly after approximately 15 minutes at room temperature.

PRELIMINARY EXPERIMENTS

I RESAZURIN REDUCTION

A. Oxidation-Reduction Aspects

Resazurin reduction takes place in two stages:



Resazurin (blue) — Resorufin (pink) — Dihydroresorufin (colorless)

Only the second stage, the reduction of resorufin to dihydroresorufin, is reversible at an inert electrode or under physiological conditions. Twigg (18) showed that on titration of resazurin with leuco-rosinduline at pH values between 6 and 7 in the absence of oxygen, approximately 15 percent of the resorufin formed was simultaneously reduced to dihydroresorufin. This reduction occurred at Eh values higher than that of a resorufin solution at the same concentration. Although this simultaneous reduction has not been demonstrated in a physiological system, it presumably occurs to some extent depending on the nature of the system.

At pH values greater than 3.0 two electrons per molecule are transferred in the reaction, resorufin $\rightleftharpoons$ dihydroresorufin. This is typical of nearly all reversible dyes. The calculated value of pK_o for the system is 6.93 and of E_o is +0.380 volts. Twigg (18) found fairly good agreement between calculated and observed values of E_o' except at pH 6.583, 6.867 and 7.431. He attributed the deviation to low solubility characteristics, when the dye concentration was 1.5 X 10^{-5}M. Since the first of

these pH values is in the range of normal milk, and since the concentration of resazurin recommended for milk testing is 2.4×10^{-5}M, it is possible that there is some depression of the E_0' value of this stage of the reduction. This is probably of little practical importance because the variation observed by Twigg was slight and because the critical concentration of resorufin would not be reached until about half the added resazurin had been reduced. The E_0' of the dye system at this pH is -0.016 volt.

Since the reduction of resazurin to resorufin is irreversible in physiological systems, it is impossible to determine an E_0' value for the reaction. It has, however, been estimated at +0.050 volt at pH 7.0 (19). This value has no significance in an irreversible system and reduction is a normal first order decay reaction, the rate depending on Eh, if the system is homogeneous and constant with respect to Eh.

B. Limitations of Visual Estimates

The visual methods employed to determine resazurin reduction may be satisfactory for estimating the bacteriological quality of commercial samples of milk but they are not adequate for a detailed study of reduction rates. The color that is observed visually is a function of the ratio of the blue resazurin and pink resorufin. In some milks, particularly those having a high fat content, the color is obscured and all that is observed is a gray color having shades of blue and pink. Visual estimation of extent of reduction in these milks is very difficult and, in some cases, impossible. This is not a

major problem in estimating dye reduction in mixed milks but becomes quite important when dealing with milk from individual cows, where the intensity of dye color varies with the sample and often differs greatly from that of the color standard.

In addition to the uncertainty of visual color estimation, there is some uncertainty regarding the amount of colored dye present in solution. The 15 percent simultaneous conversion of resorufin to dihydroresorufin found by Twigg (18), during resazurin reduction in a relatively uncomplicated system, indicates that this conversion proceeds readily and is not solely a function of the Eh of the system. It is possible that this effect also occurs in milk, and equally possible that its magnitude varies from one sample to the next. This would change the ratio of resazurin to resorufin and give a false estimate of amount of reduction based on a visual observation. However, due to the ease of reoxidation of dihydroresorufin by atmospheric oxygen, inversion of tubes immediately prior to color estimation would minimize this effect. Another factor which could decrease the effective concentration of resorufin in the system is its low solubility in aqueous media in the pH range of milk. Twigg (18) attributed the deviation observed for the E_0' value of the resorufin/dihydroresorufin system at pH 6.6 to low solubility. His dye concentration was 1.5×10^{-5}M while that in milk is 2.4×10^{-5}M. If one considers that the volume occupied by fat globules and colloidal particles in milk is excluded to the dye, the actual dye concentration approaches 3.0×10^{-5}M, depending largely on the fat content of the milk.

Once the system is saturated with resorufin, the color change of the sample depends on the decrease of resazurin concentration alone, since only that resorufin which is in solution contributes to the pink color. During the early stages of reduction, the color change depends on increase of resorufin as well. This complicates the study of reduction rates, particularly since the magnitude of the effect is probably variable and dependent on the fat content of a given sample.

For detailed study of rates of reduction, an accurate, quantitative method permitting assay of up to twenty samples at one time is needed. Methods such as the iodometric method outlined by Twigg (18) were designed to determine the amount of resorufin present in a given batch of resazurin, and require fairly large quantities of dye. In milk, a small quantity of dye is used as an indicator and quantitative separation from interfering substances, as well as the assay itself, would become impractical, if not impossible. De Baun and de Stevens (20) have developed a spectrophotometric method for the simultaneous estimation of resazurin and resorufin in studies with erythrocytes and succinic dehydrogenase of rat liver. The method, as presented, requires some modification before it can be applied to milk. An extraction step has to be added because milk, unlike erythrocyte or enzyme preparations, cannot be clarified by centrifuging or filtering. If the dye can be extracted from the milk quantitatively, and if the absorption spectra of the two colored forms are sufficiently different and distinct in

the solvent, it should be possible to devise a method for accurately estimating degree of resazurin reduction in milk.

C. Spectrophotometric Estimation

Commercial resazurin tablets, certified by the Biological Stain Commission to contain approximately 11 mg dye and to be sufficiently pure and uniform for milk testing (11,21), were used. According to Standard Methods (11), each tablet makes 200 ml of the solution to be employed in a 1 plus 10 dilution with milk for visual estimation of resazurin reduction. This makes the final concentration in milk approximately 2.4×10^{-5}M. The concentration varies very slightly from one batch of stock solution to the next as does the initial concentration of resorufin (18,21). If the stock solution from one randomly chosen tablet were used as an arbitrary standard for calibration of the method, subsequent batches, made from different tablets could be standardized against the original calibration data. This would permit direct comparison of data obtained at different times and the use of different dye sources. Although the coefficients calculated from figure 2 are based on the assumption that the dye tablet used contained 11 mg resazurin, which is an admitted approximation, they serve to standardize the method and place the measurement of reducing rates on a quantitative basis.

Preliminary tests of a number of solvents revealed that n-butanol extracted the dye fairly efficiently and separated from the milk on centrifuging. The absorption spectra of resazurin and resorufin in butanol are shown in figure 1. The curves were obtained by adding standard resazurin solution to

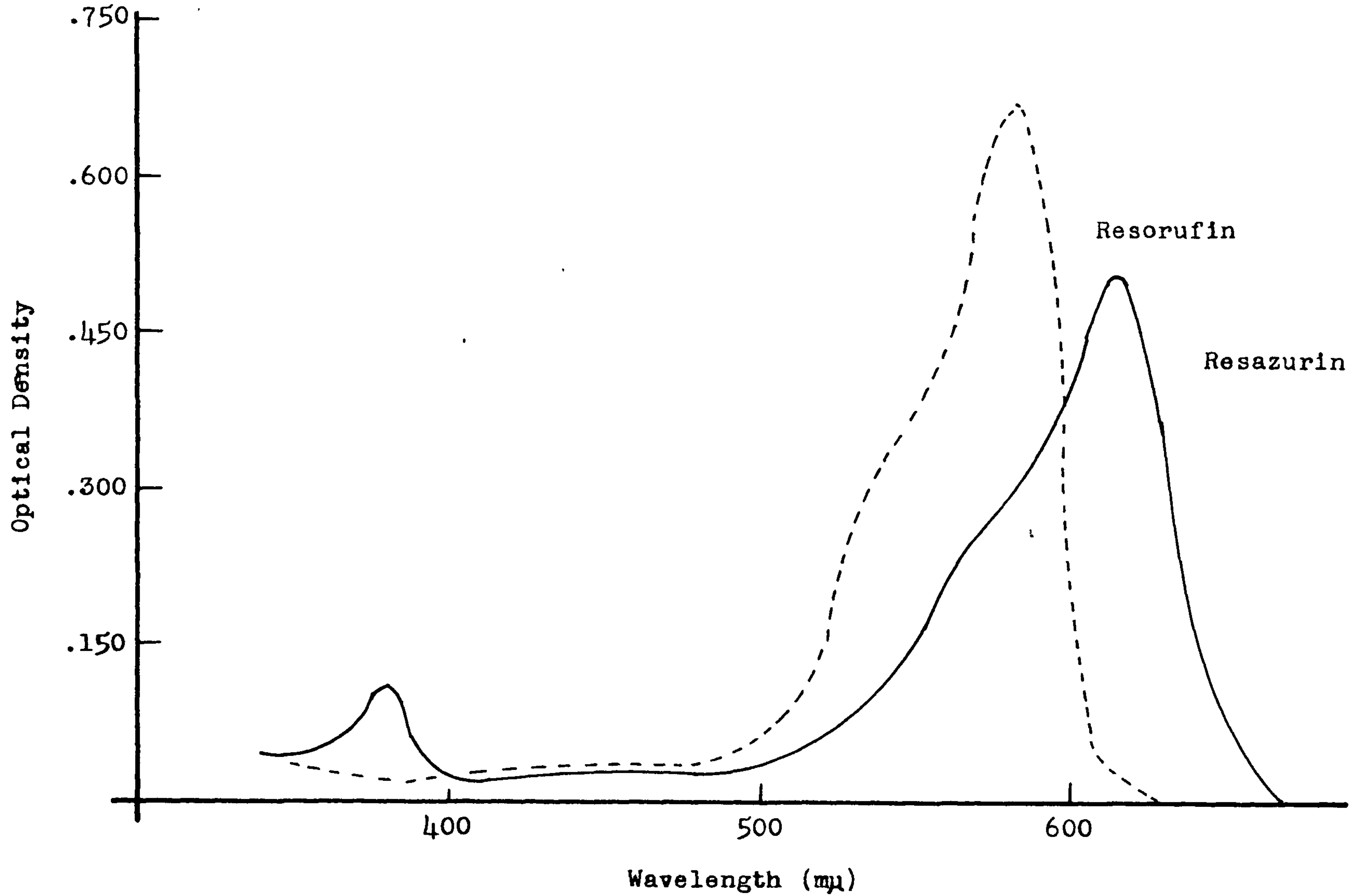


Figure 1

Spectra of Resazurin and Resorufin in butanol saturated with sodium bicarbonate

butanol and shaking in the presence of excess sodium bicarbonate. The butanol layer was cleared by filtration and read against butanol which had been given similar treatment, except that distilled water was substituted for dye solution. In order to obtain the curve for resorufin, the resazurin solution was reduced with sodium isoascorbate prior to addition of butanol and treated as above. The E max for resazurin and resorufin in butanol, saturated with sodium bicarbonate, were 615 and 582 mμ respectively. The blanks, butanol shaken with distilled water and sodium bicarbonate, with and without added sodium isoascorbate, showed no absorption at either of these wavelengths when read against distilled water. The addition of sodium bicarbonate did not influence the absorption spectra of resazurin and resorufin extracted from milk at pH 6.6 or from phosphate buffer at pH 6.4, but did markedly increase the optical density at the points of maximum absorbance.

Since resazurin changes color from violet to orange in the pH range 6.5 to 3.8 (22), its absorbance at more alkaline reactions was also expected to vary with pH. The variation in absorbance over the range pH 6.2 to 7.0 is shown in column A of table 1. The readings in column B were the result of saturating the extracts from column A with sodium bicarbonate, and show that the variations in the values of column A were due to absorbance rather than to the actual amount of dye present. The data as a whole illustrate that extraction of dye from phosphate buffer is constant throughout the physiological pH

range of milk and that absorbance increases with rising pH. It would appear logical to calibrate the method for a number of pH values and then measure reduction and dye recovery at the pH of the sample being examined. While the optical density measurements of samples from phosphate buffer were sufficiently reproducable to provide the calibration data, replicate extracts from the same sample of milk tended to differ considerably in absorbance, making readings unreliable. Since these replicate extracts from milk produced a common optical density on saturating with sodium bicarbonate, the variation was probably the result of slight differences in pH. Milk containing partially reduced resazurin probably also contains some colorless dihydroresorufin. It is remotely possible that some of the colorless compound remains, even after shaking in air. Shaking in sodium bicarbonate should ensure that the equilibrium resorufin $\rightleftharpoons$ dihydroresorufin is shifted far to the left, the E_o' values being: (18)

$$E_o' = \begin{array}{l} -0.021 \text{ v at pH } 6.583 \\ -0.163 \text{ v at pH } 8.338 \\ -0.231 \text{ v at pH } 9.186 \end{array}$$

Saturating with sodium bicarbonate, besides eliminating effects of pH and traces of dihydroresorufin on the assay, also removes some water and salts from the butanol extract, leaving these at a more constant concentration and so adding to the precision of the method.

TABLE 1

OPTICAL DENSITY OF RESAZURIN AND RESORUFIN EXTRACTED FROM PHOSPHATE BUFFER OF VARYING pH

	A				B			
	* First Extract				# First Extract Saturated with Sodium Bicarbonate			
pH of Buffer	Resazurin		Resorufin		Resazurin		Resorufin	
	582mμ	615mμ	582mμ	615mμ	582mμ	615mμ	582mμ	615mμ
6.2	.193	.273	.232	.009	.295	.520	.690	.025
6.3	.215	.330	.250	.010	.295	.520	.690	.024
6.4	.221	.350	.291	.011	.295	.520	.690	.024
6.5	.235	.377	.348	.013	.295	.520	.690	.024
6.6	.245	.404	.390	.015	.295	.520	.690	.024
6.8	.290	.470	.520	.015	.295	.520	.690	.025
7.0	.298	.482	.550	.020	.295	.520	.690	.024
H_2O	.197	.288	.367	.015	.295	.520	.690	.025

* 0.5 ml resazurin solution or 0.5 ml resorufin (resazurin solution reduced with sodium isoascorbate) added to 5.0 ml 1% phosphate buffer at above pH values in test-tubes: held at 37°C for 30 minutes: added to 18 ml n-butanol in 9 gm Babcock cream test bottles: shaken 10 minutes at room temperature: centrifuged, and optical density of butanol layer measured at 582 and 615 mμ.

Butanol layer from column A added to excess sodium bicarbonate: shaken 5 minutes at room temperature: filtered through Whatman #1 paper and optical density measured at 582 and 615 mμ.

The effect of varying the shaking time of a butanol-dye-milk mixture is shown in table 2. The data also serve to illustrate the precision of the method. Although a shaking time of 5 minutes was found to be adequate for the extraction of the dye, a 10 minute shaking interval was chosen for subsequent determinations.

TABLE 2

INFLUENCE OF SHAKING TIME ON AMOUNT OF DYE EXTRACTED FROM MILK

Time on Shaker (Minutes)	Optical Density 582 mμ	615mμ
5	.407	.320
10	.407	.321
15	.406	.320
20	.407	.321
25	.406	.320
30	.407	.320

0.5 ml dye solution plus 5.0 ml milk held at 37°C for 60 minutes in test tubes and then added to 9.0 ml n-butanol in 9 gm Babcock cream test bottles: shaken on mechanical shaker at room temperature for times indicated: centrifuged 10 minutes at room temperature: butanol layer decanted to clean flasks containing excess sodium bicarbonate: shaken 5 minutes at room temperature: filtered through Whatman #1 paper and optical density read at 582 and 615 mμ.

Calibration curves for resazurin and resorufin in butanol saturated with sodium bicarbonate were obtained on a freshly prepared dye solution (figure 2). Increments of dye and water were added to 9 gm Babcock cream test bottles to a total volume

of 0.6 ml. Sodium isoascorbate was added to one series of flasks to reduce the resazurin to resorufin. The remaining procedure was as outlined for table 2, except that the single phase resulting from the first shaking was not centrifuged; sodium bicarbonate being added directly to the original flasks after shaking 10 minutes. The stock solution from which these data were obtained was the arbitrary standard described, and subsequent solutions of dye were corrected to it. Since it was more convenient for subsequent work to calibrate the method directly in terms of quantity of dye added to milk, extinction coeffecients for the dye in butanol were not obtained.

The constants derived from the slopes of these calibration curves were used to determine the relative quantities of resazurin and resorufin in an unknown mixture (23). The calculations were carried out, and the final results expressed, in terms of µM resazurin or resorufin in the sample. It should be noted that the calibration was carried out on 0.6 ml samples rather than on the 5.5 ml samples which would usually be employed in measuring resazurin reduction in milk. The smaller volume was completely miscible with butanol and was used for standardization to eliminate any partition effects due to the presence of two phases.

Calculation of the relative concentrations of resazurin and resorufin in unknown mixtures was as follows:

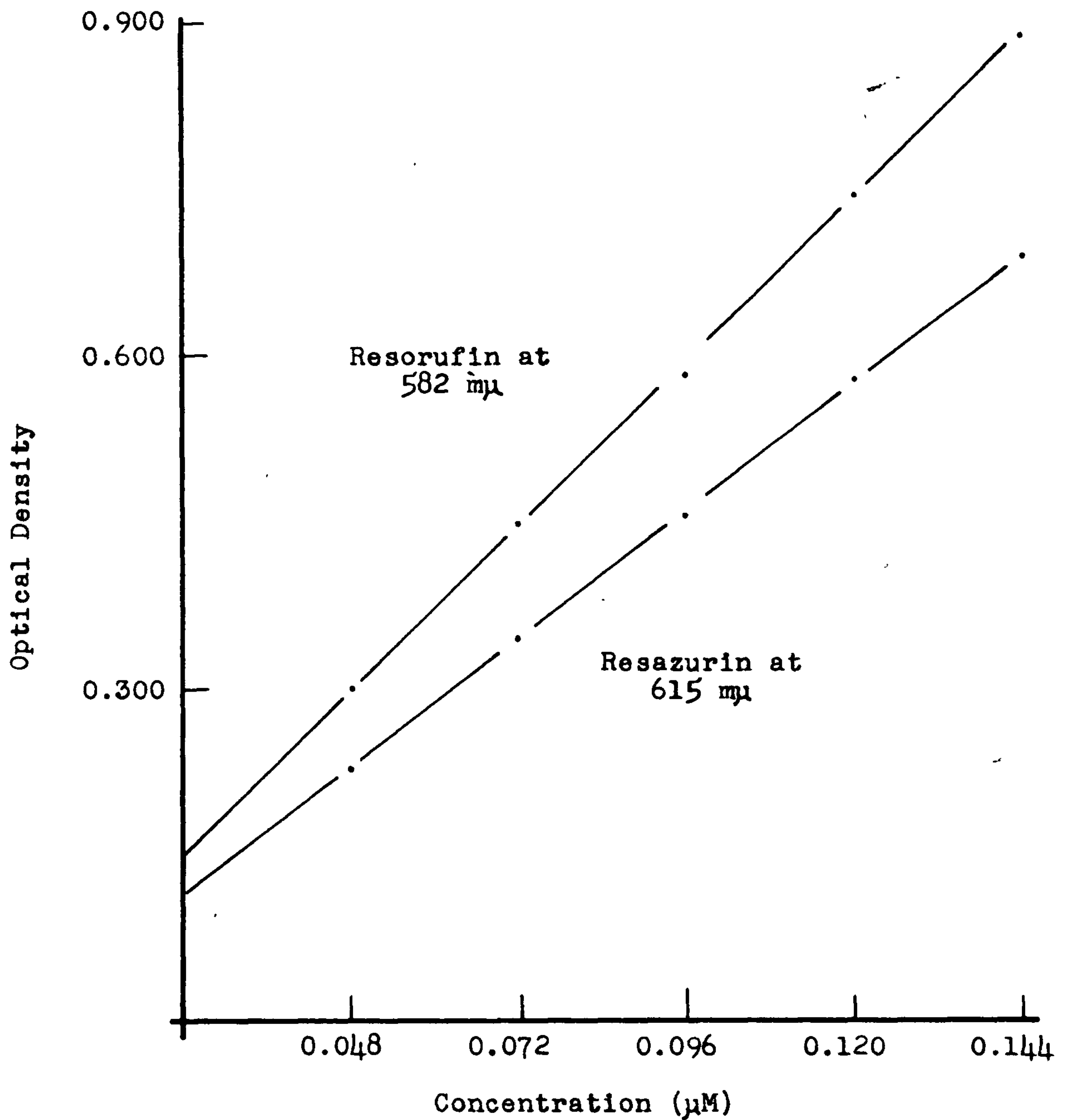


Figure 2

Calibration curves for the method. Dye at each concentration was in 9.0 ml butanol saturated with sodium bicarbonate.

D_1 = optical density at 615 mμ due to resazurin.

D_2 = optical density at 615 mμ due to resorufin.

D_3 = optical density at 582 mμ due to resazurin.

D_4 = optical density at 582 mμ due to resorufin.

R = concentration resazurin (μM per sample).

R' = concentration resorufin (μM per sample).

$$D_1 = K_1R \qquad D_3 = K_3R$$

$$D_2 = K_2R' \qquad D_4 = K_4R'$$

$K_1 = D_1/R = 4.82$

$K_2 = D_2/R' = .02$

$K_3 = D_3/R = 2.57$

$K_4 = D_4/R' = 6.14$

Each value was calculated as an average of 6 readings <u>i.e.</u> at 6 different concentrations

$E_{615} = D_1 + D_2 = K_1R + K_2R'$ = observed OD at 615 mμ

$E_{582} = D_3 + D_4 = K_3R + K_4R'$ = observed OD at 582 mμ

$$\therefore R = \frac{K_4E_{615} - K_2E_{582}}{K_1K_4 - K_2K_3}$$

$$= 0.208\ E_{615} - 0.007\ E_{582}$$ = μM resazurin in sample

$$R' = \frac{K_1E_{582} - K_3E_{615}}{K_1K_4 - K_2K_3}$$

$$= 0.164\ E_{582} - 0.087\ E_{615}$$ = μM resorufin in sample

TABLE 3

EFFECT OF INCUBATION TIME ON DYE RECOVERY

Milk or Buffer System	Time to Reduce to 7/4 std. by visual assay	Incubation time at 37°C — 30 Minutes			90 Minutes			150 Minutes		
		Resazurin (µM)	Resorufin (µM)	Total (µM)	Resazurin (µM)	Resorufin (µM)	Total (µM)	Resazurin (µM)	Resorufin (µM)	Total (µM)
Cow #1	60 min.	.061	.040	.101	.034	.069	.103	.023	.079	.102
2	70 min.	.066	.033	.099	.040	.062	.102	.031	.075	.106
3	90 min.	.076	.025	.101	.057	.047	.104	.043	.057	.100
4	130 min.	.076	.030	.106	.050	.048	.098	.042	.062	.104
5	175 min.	.078	.022	.100	.062	.042	.104	.052	.053	.105
.4% Phosphate Buffer at pH 6.4 with added Sodium isoascorbate		<.001	.107	.107	<.001	.106	.106	<.001	.106	.106
.4% Phosphate Buffer at pH 6.4		.107	<.001	.107	.108	<.001	.108	.108	<.001	.108

Conditions were as outlined in table 2: 0.5 ml resazurin solution containing 0.12 µM dye was added to 5.0 ml milk. The recovery range was 82% to 90%.

The recovery of dye from milk and phosphate buffer after incubation is shown in table 3. Visual observations of dye color are included as well. Milk from both Ayrshires and Holsteins is represented. Dye recoveries from both milk and buffer were low, but that recovered after 30 minutes incubation was comparable with that after 150 minutes. This indicated that loss of dye resulted from a partition effect of some sort rather than destruction. Since recoveries from milk were comparable with those from buffer, adsorption of dye to protein or interference from milk fat did not significantly influence the butanol extraction. Butanol and water are mutually soluble to some extent (24), and part of the apparent dye loss could be due to a net gain in volume of the butanol layer. If this gain in volume were to account for all of the apparent dye loss, it would have to amount to 10% in the case of extracts from buffer, and up to 20% for extracts from milk.

An estimate of dilution of butanol extracts from various volumes of buffer, milk, and gravity cream was obtained (table 4). The difference between 10 ml and the volume of butanol retreivable from a 10 ml portion of extract was taken to represent the dilution. The net gain in volume of the butanol after extraction of dye from 10.0 ml buffer and saturating the extract with sodium bicarbonate was 9.2%. When a solution of dye in butanol, such as that used for standardization, was diluted to the same extent, the apparent dye loss was 8.0%. This accounted for the low dye recovery calculated for extracts from buffer. The dilution of butanol by whole milk, 11.8%, only accounted for a

10% loss of dye, while apparent losses up to 18% were encountered for whole milk in table 3. This difference could not be due solely to dilution by milk fat because losses up to 40% were found with milk fat in table 5, while gravity cream could only account for a 12% loss on the basis of dilution in table 4. The milk fat used in table 5 was, however, much more concentrated than the gravity cream in table 4, making the actual difference less than the difference between these values indicates.

It is evident from the foregoing that the original calibration could not be used directly to calculate dye recoveries unless dilution of the extract was considered as well and some additional allowance made for milk fat. Since milks from different sources differ in fat content, it would be difficult to obtain reproducible dilution values for direct calculation.

The apparent dye recovery from whole milk, skim, and milk fat is shown in table 5. Equal quantities (0.06 μM) resazurin and resorufin were added to each sample and the dye extracted immediately. The extraction was carried out before any significant resazurin reduction occurred to change the ratio of the two forms of the dye. The decrease in recovery shown for milk fat was more rapid than it would have been for gravity cream, but it illustrated that, even at recoveries as low as 60%, the ratio, resazurin/resorufin, remained constant regardless of source of extract.

TABLE 4

DILUTION OF BUTANOL EXTRACT FROM DIFFERENT SOURCES

Source of Extract	Vol. BuOH (ml) per Extract After Saturation with $NaHCO_3$	Percent Dilution (100 minus Vol. BuOH per 100 ml extract)
* Buffer (ml)		
1.0	9.73	2.7
2.0	9.24	7.6
4.0	9.08	9.2
10.0	9.08	9.2
Whole Milk (ml)		
1.0	9.72	2.8
2.0	9.42	5.8
4.0	9.13	8.7
10.0	8.82	11.8
Skim Milk (ml)		
1.0	9.66	3.4
2.0	9.26	7.4
4.0	9.03	9.7
10.0	9.10	9.0
Gravity Cream (ml)		
1.0	9.96	0.4
2.0	9.50	5.0
4.0	8.78	12.2
10.0	8.63	13.7

*.04% phosphate, pH 6.4

Materials shown in first column added to 18 ml butanol in Babcock flasks and shaken 10 minutes: centrifuged and butanol layer decanted: excess $NaHCO_3$ added to butanol extract and shaken 5 minutes: filtered: 10 ml extract added to 50 ml H_2O and distilled at 92.6°C: volume butanol calculated from weight of 25 ml distillate.

TABLE 5

APPARENT DYE RECOVERY FROM DIFFERENT SOURCES

Whole Milk (ml)	OD 582mμ	OD 615mμ	Resazurin (μM)	Resorufin (μM)	Total (μM)	Resazurin/ Resorufin
0	.428	.260	.052	.048	.100	1.1
1	.435	.255	.051	.049	.100	1.0
2	.430	.248	.049	.049	.098	1.0
3	.430	.242	.048	.050	.098	1.0
4	.430	.240	.048	.050	.098	1.0
5	.430	.238	.048	.050	.098	1.0
Skim Milk (ml)						
0	.420	.260	.052	.048	.100	1.1
1	.440	.260	.052	.052	.104	1.0
2	.435	.255	.051	.052	.103	1.0
3	.440	.250	.050	.051	.101	1.0
4	.430	.240	.048	.050	.098	1.0
5	.440	.240	.048	.052	.100	0.9
*Milk Fat (ml)						
0	.428	.260	.052	.048	.100	1.1
1	.400	.235	.047	.045	.092	1.0
2	.380	.220	.044	.043	.087	1.0
3	.360	.210	.042	.041	.083	1.0
4	.340	.200	.040	.038	.078	1.0
5	.312	.175	.035	.036	.071	1.0

*obtained by centrifuging whole milk and therefore containing more fat than gravity cream.

Milk, skim, or fat diluted to a total volume of 5.0 ml with water prior to adding .12 μM dye (1:1 resazurin:resorufin mixture): assay procedure was the same as that for table 3 except that samples were not warmed to 37°C before extraction.

Generally, in whole milk, more than 80% of added dye was recovered. Of the 20% loss, half could be accounted for by gain in volume of the butanol extract, leaving a net 10% loss that could not be accounted for. On the basis of the data in table 5, it was considered valid to assume that the ratio, resazurin/resorufin was the same in this 10% as in the extract, and that recovered dye could be extrapolated to 100% using this ratio to arrive at a quantitative estimate of extent of reduction.

This assay does not correlate exactly with the visual method. The absorbance of resazurin and resorufin varies over the normal pH range of milk and it is possible that the shades of color observed in the visual method vary in a similar manner. Calculation of apparent resazurin and resorufin concentrations for the data in table 1 presents the following picture:

pH	Resazurin (μM)	Resorufin (μM)	Resazurin/ Resorufin
6.3	.069	.041	1.68
6.4	.073	.047	1.55
6.5	.078	.057	1.37
6.6	.084	.064	1.31
6.8	.098	.085	1.15
Sat. with bicarbonate	.108	.112	0.97

Since resazurin and resorufin were at the same concentration, the ratio should be 1.0. While it is true that the constants used for the above calculation are valid only for extracts

saturated with sodium bicarbonate, the illustration shows that the apparent concentration of resorufin decreased more rapidly with pH than did that of resazurin. If the same reasoning were applied to the visual assay, a milk sample at pH 6.8 could appear more reduced than one at pH 6.5, though both contained the same concentrations of dye with the same ratios of resazurin/resorufin. For purposes of comparison with the visual assay, a resazurin/resorufin ratio of 1.0, which was frequently obtained from samples which appeared to be reduced to the Munsell 7/4 color standard, was accepted as being similar to the 7/4 standard.

II. MEASUREMENT OF REDUCING CAPACITY OF MILK

It was found necessary to have an estimate of reducing capacity of milk in the presence of added resazurin. Potentiometric measurements of Eh, since they give no indication of poise, or capacity of the system to resist change, are of no use for this purpose. This is analogous to pH giving no indication of buffer capacity. The Eh of fresh milk, in the absence of extensive bacterial activity, ranges from +0.2 to +0.3 volt (25,26). It is possible to assess degree of poising of the system by adding an excess of an oxidant having E_0' in this range and measuring extent of reduction of added oxidant. The oxidant selected in our experiments was the reversible Eh indicator, 2,6-dichlorophenolindophenol, which has $E_0' = +0.247$ v at pH 6.6 (27).

It was found from preliminary experiments that indophenol was decolorized on addition to milk and that the blue sodium salt of the dye could be extracted from milk with n-butanol. The problems involved in butanol extraction of dye from milk which were encountered in measuring resazurin reduction, were also expected to occur with indophenol. The method of extraction and treatment of extracts were therefore exactly the same as those described for resazurin. The absorption spectrum of a butanol extract of indophenol from phosphate buffer (0.04%, pH 6.6) is shown in figure 3. Maximum absorbance occurred at 660 mμ. Spectra of extracts obtained under the same conditions from milk or cream were superimposable on that shown, except for concentration differences. Neither blank extracts from milk or

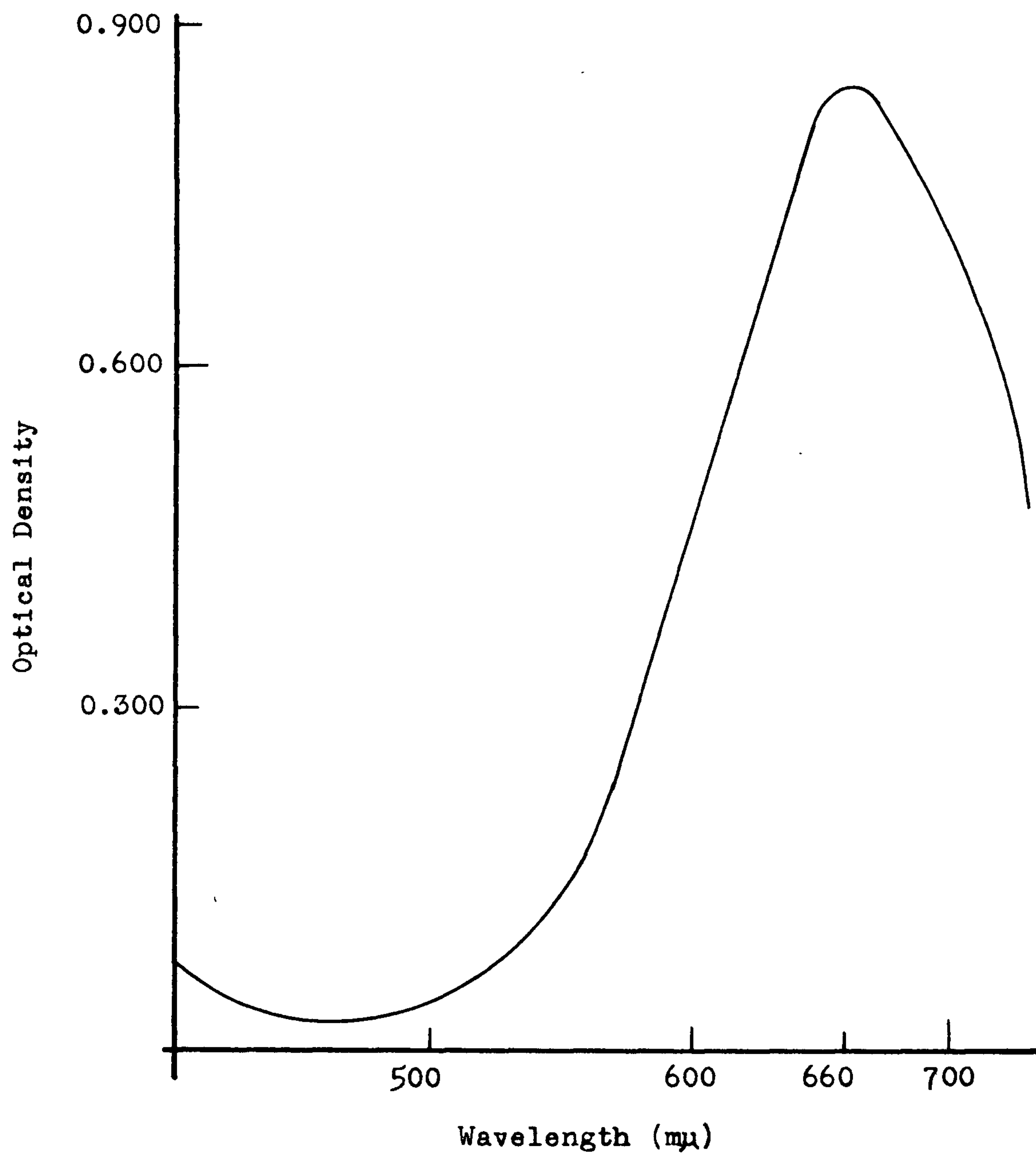


Figure 3

Absorbance spectrum of 2,6-dichlorophenolindophenol in butanol saturated with bicarbonate.

cream, nor extracts of resazurin or resorufin (0.12 µM) had significant optical density at this wavelength.

Since the poising of normal fresh milk is believed due to its ascorbic acid content (26,28), indophenol solutions were standardized with ascorbic acid immediately prior to use. An example of such a standardization is shown by figure 4. The indophenol solution (0.4 mg/ml) was prepared dy dissolving the dye, along with an equal weight of sodium bicarbonate, in water. The bicarbonate was added to ensure maximum solubility of dye and was not present in sufficient concentration to affect the pH of milk or buffer solution. The dye solution was filtered and 1.0 ml added to a series of test tubes containing 5.5 ml M/50 phosphate buffer (pH 6.6) and graded increments of ascorbic acid. The tube contents were mixed by inversion and, after 3 minutes, added to 10 ml butanol in Babcock flasks. The extraction and treatment of extracts were as described for resazurin. The filtered extracts from solutions of low ascorbic acid concentration were diluted with butanol to an optical density in the range 0.100 to 0.600. The optical densities tabulated in figure 4 were obtained by direct multiplication of observed OD by the appropriate dilution factor.

The apparent dye losses encountered in butanol extraction of resazurin from buffer and milk (tables 4 and 5) probably also occurred in butanol extraction of indophenol. The losses with the method described for indophenol were, however, of much less significance. The apparent losses, (10%), due to dilution of butanol by water were eliminated entirely by use of the same

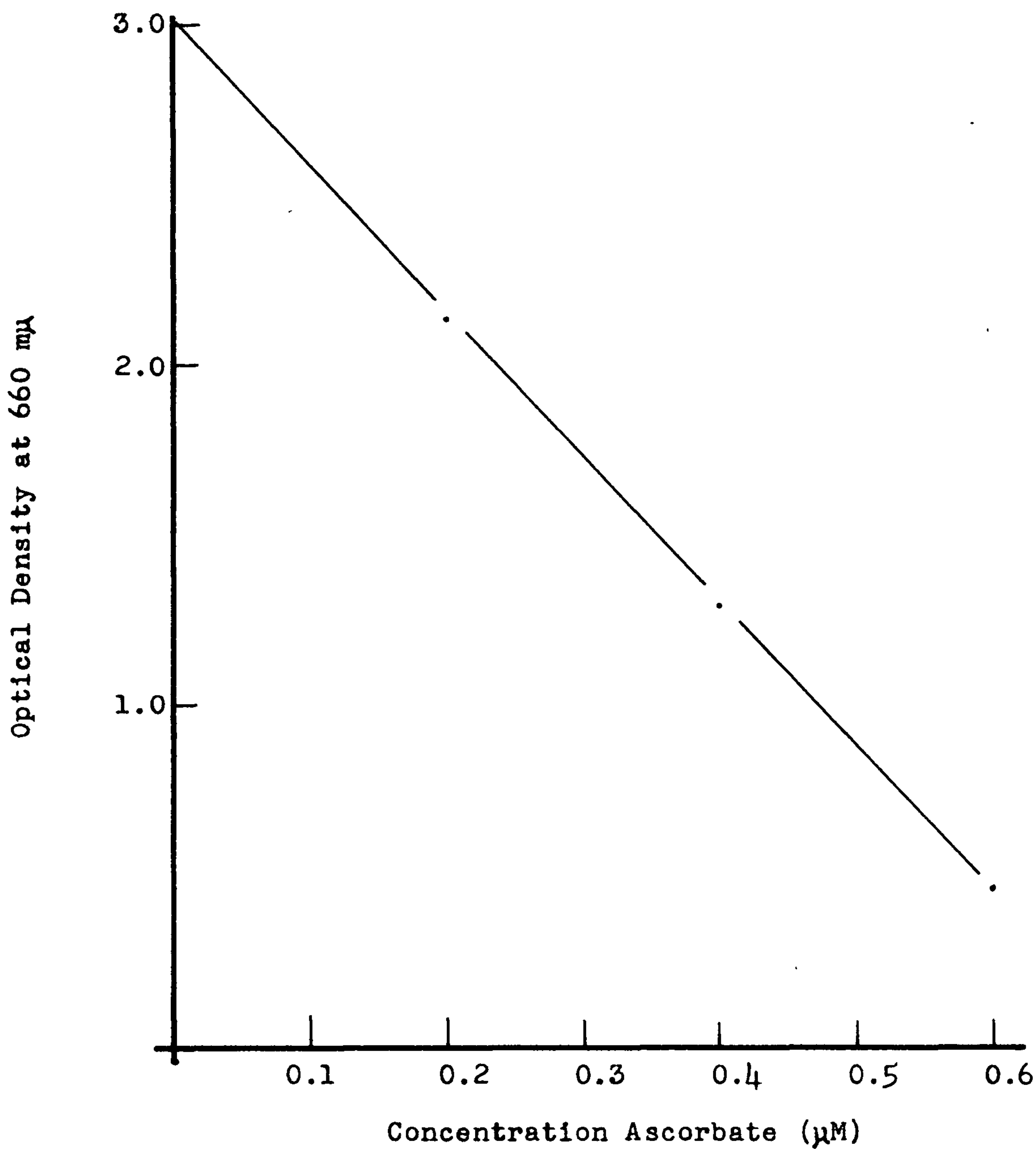


Figure 4

Standardization of indophenol solution with ascorbate: extracts read in butanol saturated with sodium bicarbonate.

conditions for calibration as were used for testing unknown samples. The maximum losses of optical density found in table 5 were more severe than those normally encountered, being a decrease of 25 percent in the observed values. With indophenol extracts, 25 percent dilution of the original extract would cause a loss of only 5 percent in the observed values. The difference, in sensitivity to errors of extraction, between the two methods results from the difference in optical density ranges for which they were calibrated: resazurin, OD range = 0 to 0.7; indophenol, OD range = 0 to 3.0.

The reducing capacity of a sample of fresh milk is shown by table 6. The extracts were prepared as described for figure 4, except that 5.0 ml milk with 0.5 ml added water or resazurin (resorufin) solution replaced buffer. Aliquots of the same sample of milk were used for all the readings shown. It is evident from these data that the method was reproducible and that neither resazurin nor resorufin interfered with reduction of added indophenol.

That indophenol reduction was quantitative, and not merely a function of the Eh of the system, is illustrated by the data in table 7. A sample of the milk used in table 6 was diluted with water as shown, and the observed reducing capacities compared with values calculated from that observed for the undiluted sample. Though there was no direct evidence that ascorbic acid was responsible for reduction of indophenol added to milk, the reducing capacities were expressed in terms of

ascorbic acid. This was mainly to provide a scale by which milk samples could be compared.

TABLE 6

REDUCING CAPACITY OF MILK
(expressed as ascorbate)

Milk (ml)	H_2O (ml)	OD at 660 mμ	Equiv. Ascorbate (μM)
5.0	0.5	0.580	0.57
5.0	0.5	0.540	0.58
	Resazurin (μM/0.5 ml)		
5.0	0.12	0.560	0.58
5.0	0.12	0.540	0.58
	Resorufin (μM/0.5 ml)		
5.0	0.12	0.540	0.58
5.0	0.12	0.560	0.58

TABLE 7

INFLUENCE OF DILUTION ON REDUCING CAPACITY

Milk (ml)	H_2O (ml)	OD at 660 mμ*	Equiv. Ascorbate (μM) Found	Calculated
1.0	4.0	2.580	0.10	0.11
2.0	3.0	1.900	0.26	0.23
3.0	2.0	1.480	0.36	0.34
4.0	1.0	0.960	0.48	0.46
5.0	0	0.560	0.57	---

*Extracts were diluted with butanol to an OD in the range 0.100 to 0.600 before reading. The value tabulated was extrapolated back to original concentration.

On application of this method to a wide variety of milk samples, it was found that the milk reduced a portion of the added indophenol in every case, the quantity depending on the previous history of the sample. If the milk was aged, held at 37°C for several hours, or treated with a mild oxidizing agent, it lost some of its ability to reduce the dye. This argues in favor of dye reduction rather than adsorption or destruction. The following discussion is based on this wider application of the method as well as on the specific results reported in tables 6 and 7. The discussion is presented in an effort to explain the apparently anomalous behavior of indophenol under the conditions previously described and to show that the reduction of the dye under these conditions is a measure of the reducing capacity, or poise, of milk.

Since indophenol, having $E_0' = +0.247$ volt at pH 6.6, is quantitatively reduced by milk with Eh in the same range, its reduction must be determined by factors other than the potentiometrically determined Eh of the milk. Tha fact that resazurin and resorufin have no measurable effect on the indophenol-milk system helps substantiate this conclusion. The rapid, quantitative reduction of indophenol indicates that milk must be strongly poised at an Eh lower than that determined by potentiometric measurement. The Eh of fresh milk appears to be a function of its oxygen content, since removal of oxygen by flushing with nitrogen results in an immediate decrease of over 0.4 volt in the measured potential (26). The potential in the presence of oxygen, over a 0.075 volt range, depends, however, on reduced

ascorbic acid content (28). These observations indicate that Eh must be the result of a contribution from both dissolved oxygen and the reducing system, though not necessarily the result of an interaction between them. Both oxygen and the reducing system maintain their identity and appear to function independently of each other, as evidenced by the behavior of the system on removal of oxygen or addition of indophenol. This type of behavior toward oxygen is typical of many biological reducing systems in situations which approximate their natural environment, for example, in tissue preparations or cell-free extracts. Oxidation, under these conditions, is extremely slow, the reducing system and oxygen behaving as if they were physically separated so that their respective potentials are superimposed to produce a net Eh for the system as a whole, without affecting the actual potentials of the components. This means that the true potential of the reducing system can be measured only after removing the contribution to Eh due to oxygen, that is by removing oxygen. Reducing systems of this type are usually fully active toward many oxidants other than oxygen and react with them rapidly and quantitatively in the presence or absence of oxygen. This appears to be true for the reduction of indophenol added to milk. On the basis of the preceding discussion, the actual Eh of the reducing system of milk would be approximately -0.1 volt, assuming that a decrease of 0.4 volt would result on removal of the potential due to oxygen. Since the presence of oxygen, and hence the measurable Eh of milk, has no influence on the reaction of indophenol with the

reducing system, the quantity of dye needed to raise the Eh of the reducing system from -0.1 volt to +0.2 volt is a measure of the poise, or reducing capacity of the milk. In the method proposed for estimating reducing capacity, the quantity of indophenol decolorized by a sample is measured to assign a relative value to the poise of the reducing system.

EXPERIMENTAL

I GENERAL OBSERVATIONS

A. Decrease in Rate of Resazurin Reduction on Aging of Milk

In early experiments, using visual estimates of resazurin reduction, it was observed that the time taken to reduce the dye increased with age of the milk sample, provided extensive bacterial growth had not occurred. In order to obtain a more quantitative estimate of the aging effect, fresh milk and skim were stored at 0°, 4°, and 12.8°C for 50 hours. The skim was obtained by centrifuging warm whole milk within 30 minutes of milking. Resazurin/resorufin ratios were obtained after 5, 10, 20 and 50 hours on all samples. Representative results are shown by figure 5 (a and b). Resazurin/resorufin ratios were determined after samples had been held with 0.12 µM dye for 60 minutes at 37°C.

Both of the milks in figures 5 show a rapid loss of the ability to reduce resazurin, even when storage was at 0°C. Differences in temperature were reflected to a much greater extent in skim than in whole milk. The break in the curves between 5 and 10 hours was probably a real effect rather than an experimental artifact. It was largely absent from the whole samples and much less pronounced in the skim from sample 1 (figure 5a) than in that from sample 2 (figure 5b). Since all of the samples, for any given time, were assayed simultaneously, it is unlikely that related samples should have differed due to random errors. The effect was also found in other samples in the same experiment, its magnitude varying as in the examples

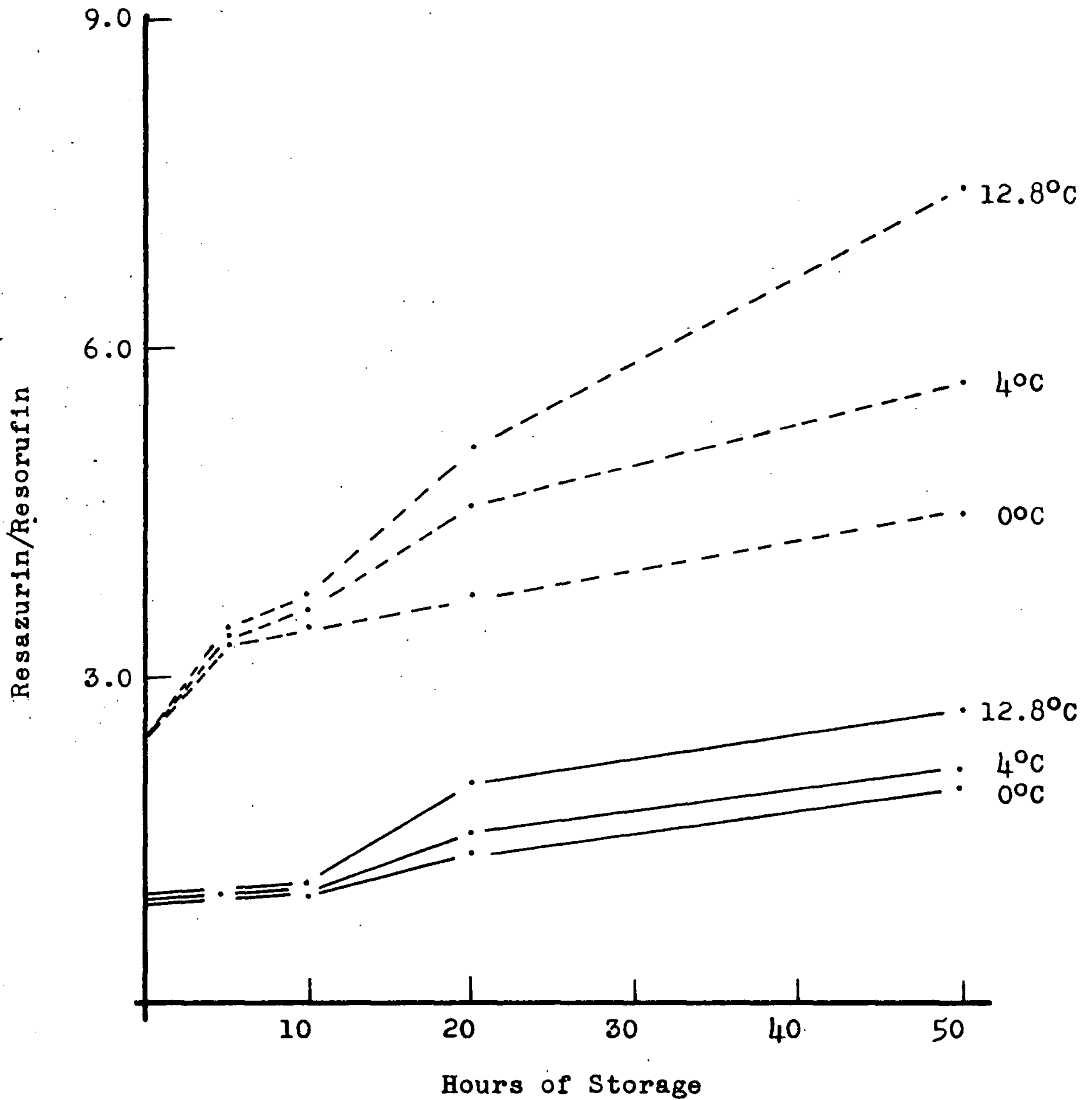


Figure 5a (Milk 1)

Influence of storage at various temperatures on ability to reduce resazurin. Broken lines represent skim and solid lines whole milk. Resazurin/resorufin determined after incubation with dye at 37°C for 60 minutes.

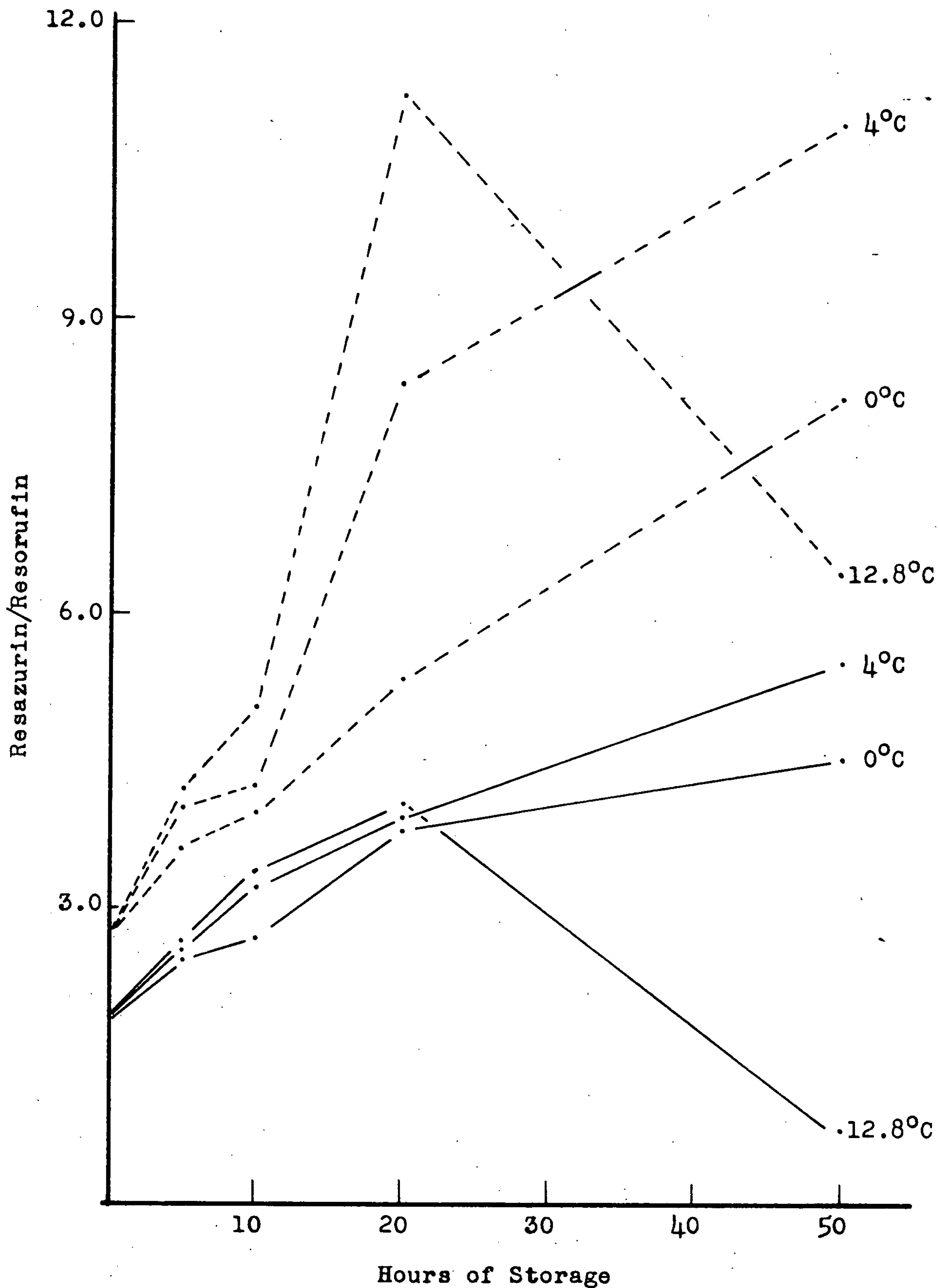


Figure 5b (Milk 2)

shown, and always occurring at the same point in the curves.

It should be noted that, although milk 1, when fresh, reduced resazurin more rapidly than did milk 2, it retained more of its ability to reduce dye on storage and was less sensitive to temperature of storage. This is contrary to what would be expected if rapid resazurin reduction depended on a more mobile and hence, a more labile, reducing system. In fact, these data indicate that the reverse may have been true, rapid reduction depending on a more stable reducing system.

The major point illustrated by these figures is that the resazurin reduction observed with fresh milks, or those refrigerated for short intervals, was due to the normal reducing systems of milk rather than to bacterial activity. Reduction due to bacterial action would be expected to increase with storage time. The rapid reversal of slope shown at 20 hours by the lines representing milk 2 stored at 12.8°C (5b) resulted from bacterial activity. This illustrates that, though milk 2 contained a more active bacterial population, it was a less active resazurin reducer initially, and also lost its reducing ability more rapidly than milk 1.

B. <u>Reduction Rate in Milk Fractions</u>

The differences in reducing rate and stability on storage between whole milk and skim, shown in figure 5, led to interest in the dye reducing ability of the various fractions of normal milk. The results of a typical experiment are pictured in figure 6. Fresh milk was permitted to cream for 20 hours in a cylinder at 4°C. The cream was removed and a portion warmed

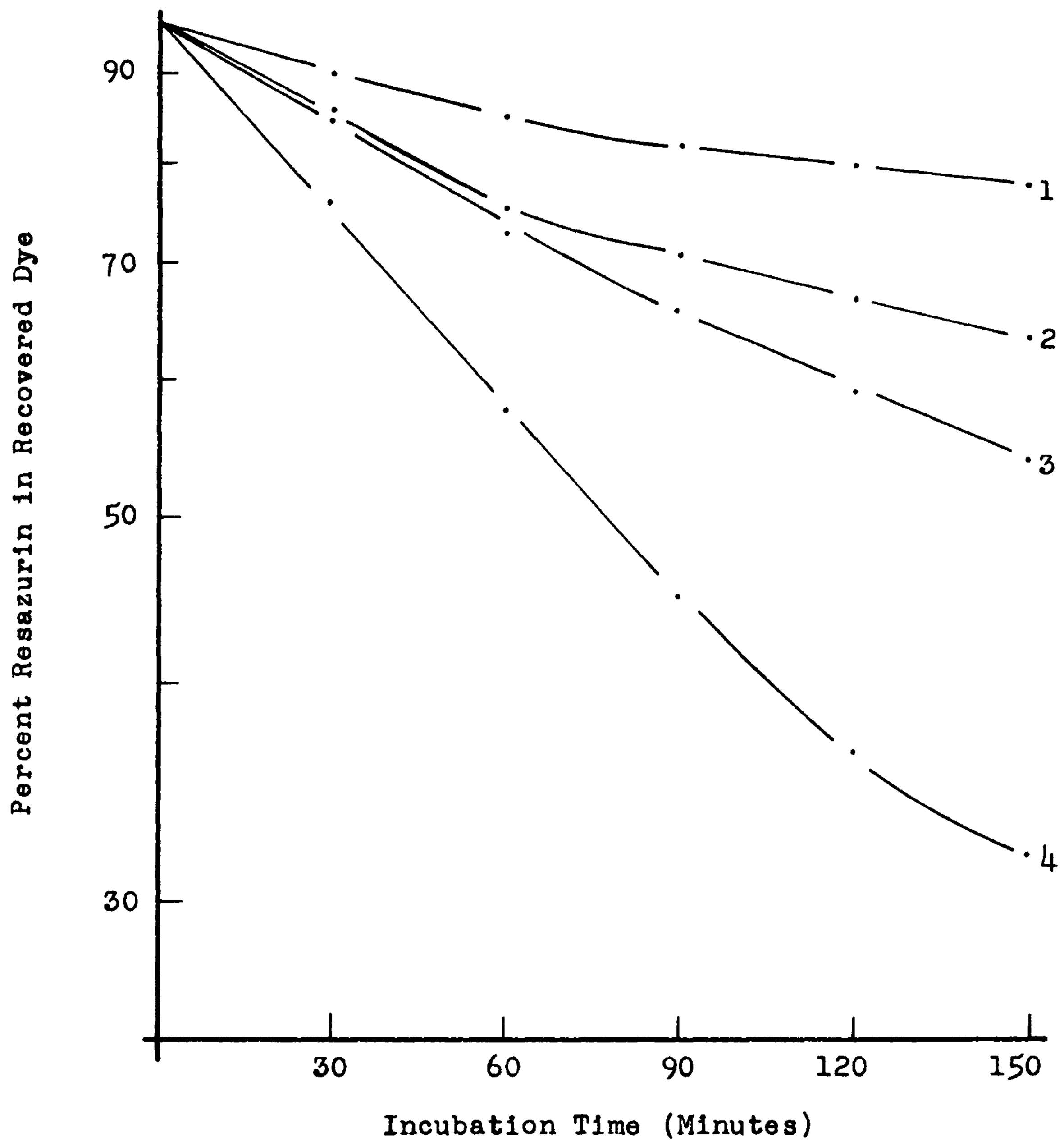


Figure 6

Reduction Rate in Milk Fractions

1 - Gravity skim
2 - Whole milk
3 - Plasma (aqueous phase from centri-
fuging warm cream)
4 - Gravity cream

to room temperature, centrifuged, and the aqueous phase used to obtain the curve labelled "plasma". The remaining curves in the figure represented whole milk which had been held at 4°C in a separate container, and the gravity cream and skim from the cylinder referred to above. Each point in the figure was derived from a 5.0 ml sample, with 0.5 ml added resazurin solution (0.12 µM dye), held at 37°C for the time intervals shown on the arithmetic scale. The spectrophotometric assay for resazurin and resorufin was applied to obtain the values plotted on the logarithmic scale. These values are tabulated as percent resazurin in the recovered dye and were interpreted to represent percent of added dye remaining as resazurin in the sample. The validity of this extrapolation was discussed in section 1 of "Preliminary Experiments".

As predicted by the results on aging, skim reduced dye more slowly than did whole milk, the cream layer apparently accounting for the difference. No effort was made to relate the differences in reducing activity between milk and skim to the fat content of the original whole milk. The position of the curve for plasma, in relation to those for the other fractions, was of considerable interest. Since this fraction was obtained by centrifuging warm, gravity cream, it contained a much greater concentration of the material which had been loosely adsorbed to the fat globule surfaces, i.e. the readily removable, outer layer of the fat globule membrane (26), than did the skim or whole milk. The fat globule membrane has been shown to contain the bulk of the enzyme activity as well as

most of the copper and iron of milk (26). The plasma fraction, as obtained in this experiment, would also have contained the majority of the bacteria and leucocytes present. In view of the membrane constituents as well as the bacteria and leucocytes, it is to be expected that this fraction would have enhanced resazurin reducing activity.

The reducing rates plotted in figure 6 provide some information concerning the kinetics of resazurin reduction. The plots were linear over the initial portion, which would have been typical of spontaneous decay or of a first order enzyme reaction. The rate, however, decreased before half the added dye had been reduced. If reduction were spontaneous decay dependent on Eh, or if the dye were involved in electron transport linked to some enzyme system, the increasing concentrations of the reversible, resorufin $\rightleftharpoons$ dihydroresorufin, forms of the dye could possibly change the poise of an Eh system or mediate electron transport, so that rate of further resazurin reduction would be decreased. Other factors, for example, depletion of a substrate, inactivation of an enzyme, or removal of a reductant through reaction with oxygen, could be presented as equally probable explanations.

C. Poising by Dye

The following experiment, illustrated by figure 7, was designed to determine the influence of resorufin on rate of resazurin reduction. Resorufin was prepared by reducing resazurin solution with ascorbic acid, extracting the resorufin with ether, and readjusting to volume after evaporation of ether. When

checked spectrophotometrically, this solution contained 0.24 µM resorufin per ml and no resazurin. Resazurin and resorufin solutions were mixed to produce dye solutions having the relative concentrations shown in figure 7. Dye solution (0.5 ml) was added to tubes containing 5.0 ml milk and held at 37°C. Resazurin and resorufin were measured, and results expressed as described for figure 6.

The decreasing rates of resazurin reduction with time of incubation (figure 6) had suggested that the increasing concentrations of the reversible, resorufin $\rightleftharpoons$ dihydroresorufin, forms of the dye could have inhibited resazurin reduction through poising of the Eh system or by mediating electron transport. That this was unlikely, is shown by the curves in figure 7. These are roughly parallel, indicating that poising was not of particular significance in decreasing the rate of reduction. The dotted curve (above curve 1) was calculated from curve 4 by eliminating the contribution of added resorufin to the recovered dye. If it is considered that the actual quantity of resorufin present initially in this set of samples was 20 percent greater than that finally generated in the samples represented by curve 1, and that the rate of change of these two curves was comparable over the first hour of incubation, the influence of added resorufin was negligible. Concentration differences must, however, be remembered in comparing these two curves. The total resazurin reduced during the first hour by the system represented by the dotted curve was 9×10^{-4} µM,

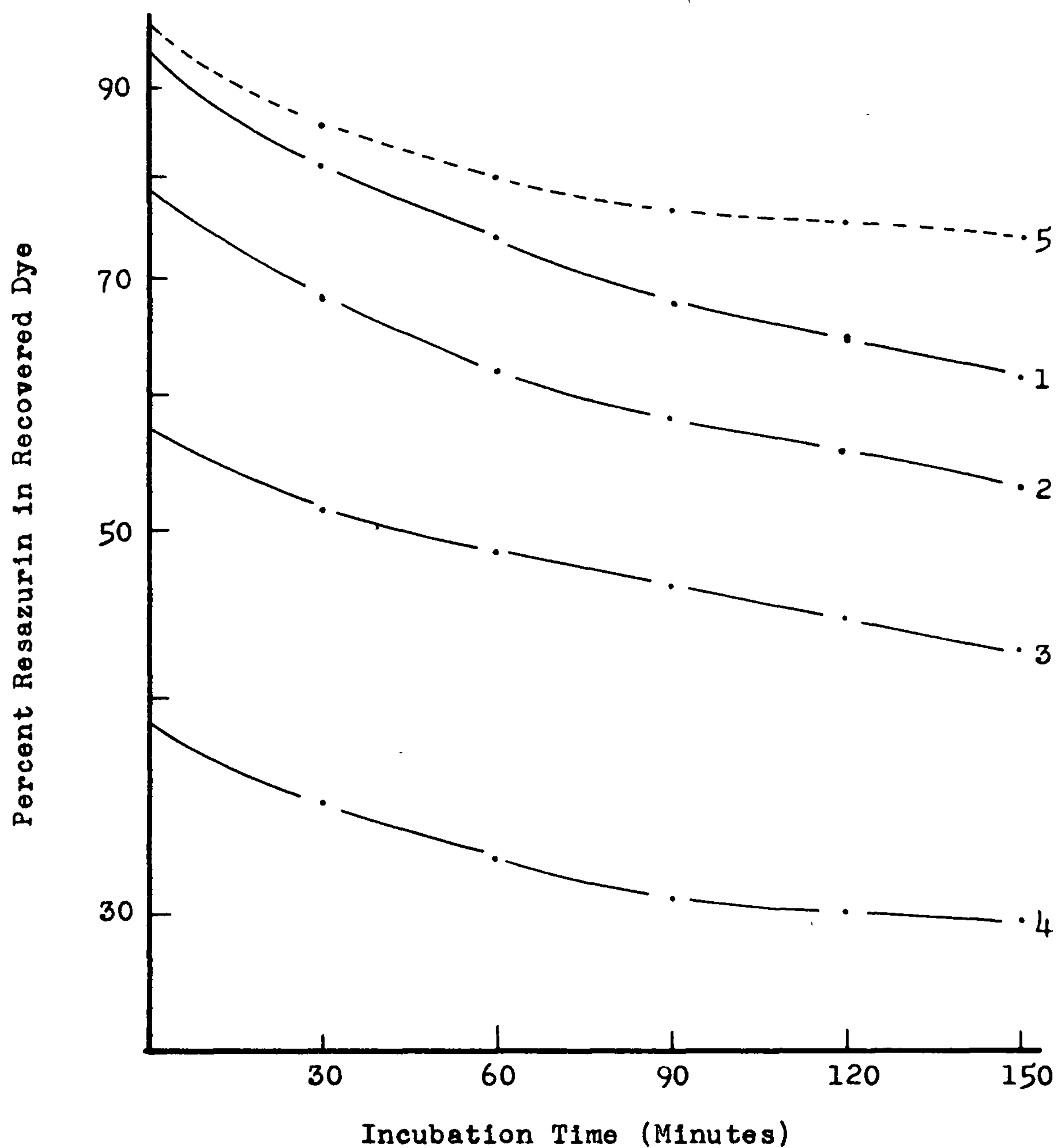


Figure 7

Poising by Dye Added to 5 ml Milk

1 - 0.12 µM Resazurin
2 - 0.096 µM Resazurin and 0.024 µM Resorufin
3 - 0.072 µM Resazurin and 0.048 µM Resorufin
4 - 0.048 µM Resazurin and 0.072 µM Resorufin
5 - Percent resazurin remaining in samples of curve 4 if resorufin added initially was disregarded.

while that by the system represented by curve 1 was 22.8 X 10^{-4} µM, or 2.5 times as much. The greater decrease in reduction rate shown by the dotted curve on incubation beyond one hour could be due to the low concentration of resazurin or, possibly, to competition from the resorufin/dihydroresorufin system, whose concentration at one hour incubation was 1.55 times that of the resazurin present.

D. Influence of Dye Concentration on Reduction Rates

It was considered possible that the decrease in reduction rate shown by the dotted curve in figure 7 on incubation beyond one hour could have been due to the low concentration of resazurin. The data in figure 8 illustrate that the fraction of added resazurin reduced changed more rapidly with incubation time if dye was present at lower concentrations than that normally employed for milk testing. The curves were obtained using the conditions and techniques described for figure 6, except that volumes of resazurin solution containing 0.024, 0.048, and 0.120 µM dye were diluted to 0.5 ml and added to 5.0 ml milk samples. Two samples of milk are represented in figure 8. The upper set of curves (milk 1) represent a slow dye reducer and the lower set (milk 2), a rapid reducer.

In both samples, the higher dye concentrations showed greater deviations from typical first order plots than did the lower concentrations. Examination of these curves and calculation of the actual quantities of resazurin reduced could imply that the deviation from linearity was due to poising by the resorufin $\rightleftharpoons$ dihydroresorufin system. However, it was

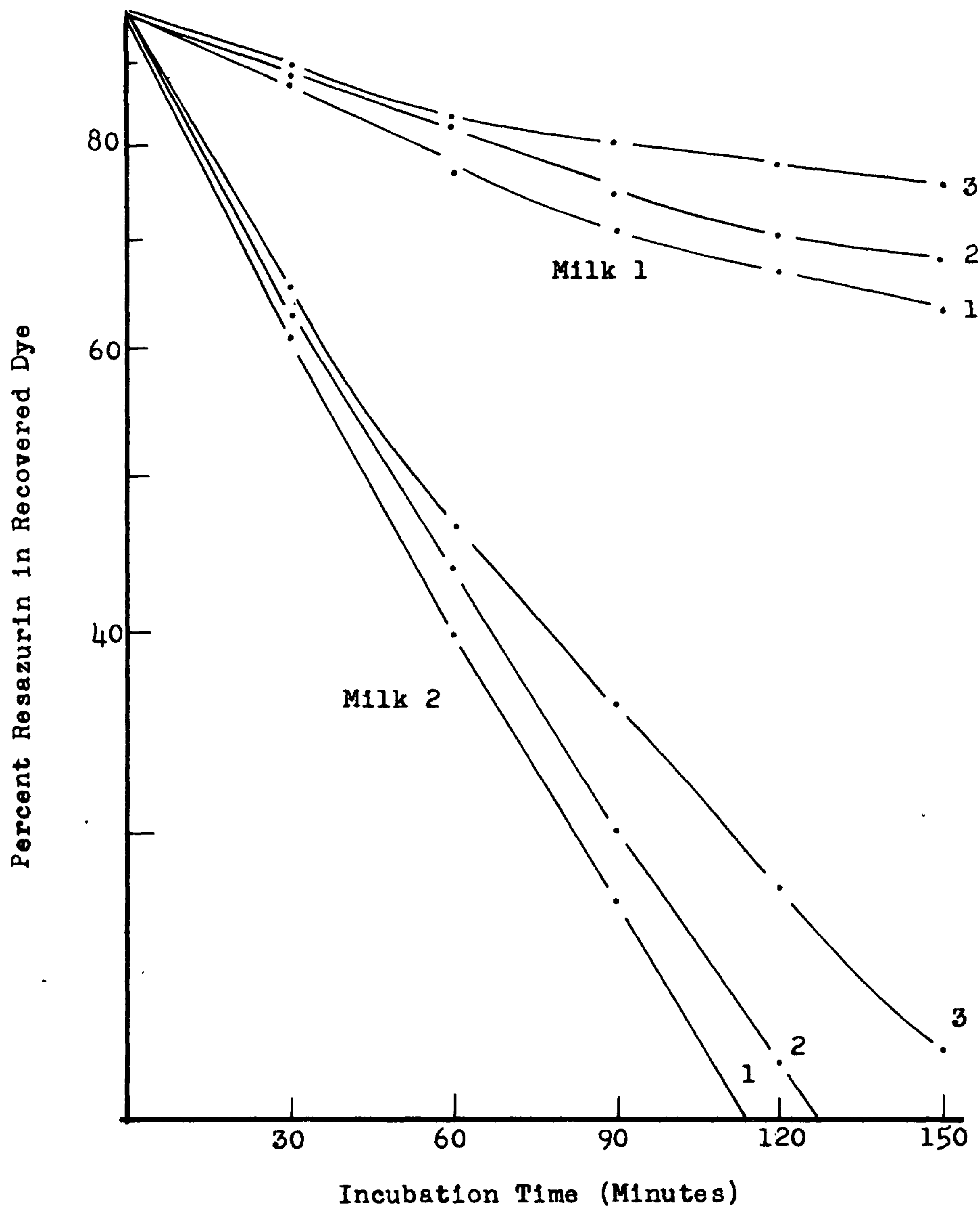


Figure 8

Influence of Dye Concentration on Reduction Rates
Added to 5 ml milk:

1 - 0.024 µM Resazurin
2 - 0.048 µM Resazurin
3 - 0.120 µM Resazurin

demonstrated in figure 7 that the interference from this system was negligible in the initial stages of reduction and had only a small influence in the later stages. It is unlikely that the resorufin/dihydroresorufin system required a 60 minute induction period before becoming functional, since the equilibrium is supposedly freely reversible. A more plausible explanation is that both resazurin and resorufin were capable of interfering with some reducing process in the milk without being reduced themselves, *i.e.* acting catalytically to deplete the system of a reducing substance. The explanation that the higher concentration of resazurin depleted the milk of some reducing substance by quantitative interaction, cannot be applied to this system. Though all samples showed a decelerated reduction rate with time of incubation, in any given time interval, the actual quantity of dye reduced in the more concentrated solutions was 4 to 5 times as great as that in the most dilute.

E. Influence of pH and Bacteria on Rate of Reduction

Before attempting to locate and study the actual systems responsible for resazurin reduction in milk, it was considered advisable to determine the influence of added bacterial populations and small changes in pH on rate and pattern of reduction. Typical results of experiments in this regard are summarized in figures 9 (a and b). Bacteria were grown on yeast litmus milk at 37°C for 12 to 18 hours and harvested immediately before use. Cells were washed once in M/50 phosphate buffer at pH 6.8, resuspended in a minimum volume of buffer and added to the milk at the same time as dye. The pH of the milk

samples was dropped to 6.4 with N-HCl immediately prior to addition of bacteria and dye. The remaining procedure was as described for figure 6. In order to illustrate a maximum effect, the curves shown are for normal milk and milk at pH 6.4, which is below the physiological range of milk. Curves for rates within the physiological range and below pH 6.6, occurred between those shown, while those for pH above 6.6, occurred above the upper curve and showed a greater decrease in reduction rate with time of incubation. Curves for bacterial activity were only obtained at the pH values shown but variations with pH would probably have been as predicted by the behavior of milk with no added bacteria.

The constant (or accelerating) rate of reduction with time of incubation of the curves representing added bacteria, as compared with the decelerating rate of the controls, further substantiates the earlier observations that bacterial activity was normally of little significance in resazurin reduction by fresh milk. The curves shown represented an intermediate concentration of bacteria. Higher concentrations of these same organisms caused proportionately faster reduction, while lower concentrations produced curves which were barely distinguishable from the controls. Some effort was made to correlate the drop in Eh of milk, in the presence of added bacteria, with resazurin reduction due to bacteria. The results were not conclusive, and more elaborate experiments would have been necessary to demonstrate a relationship.

Oxidation-reduction systems generally, show a strong

positive increase in E_o' with decreasing pH. According to the data of Twigg (18), dropping the pH from 6.6 to 6.1 produced an increase in the E_o' value of the resorufin/dihydroresorufin system of +0.03 volt. A change of this magnitude was, however, found to be compensated for by a rise of +0.03 volt in the Eh of milk, on dropping the pH from 6.7 to 6.2. The relative potentials of dye and milk should therefore have remained unchanged, so that the effect of pH could not be accounted for on this basis.

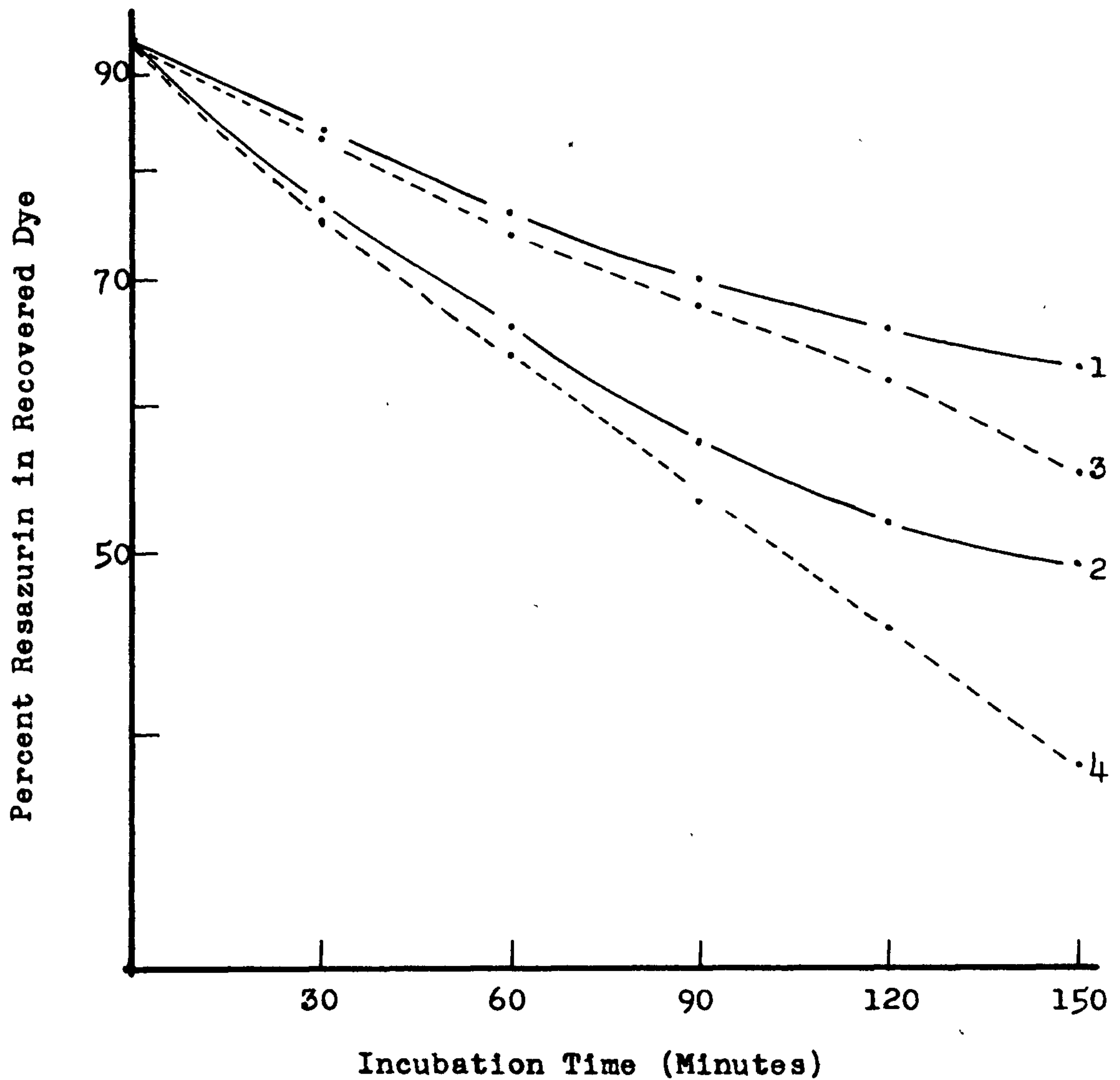


Figure 9a (Milk 1)

Influence of pH and Bacteria on Rate of Reduction

1 - Normal milk at pH 6.7

2 - pH of milk dropped to 6.4 with N HCl

3 - Milk at pH 6.7: 2×10^6/ml unidentified Streptococcus isolated from milk added

4 - pH dropped to 6.4: 2×10^6/ml unidentified Streptococcus isolated from milk added

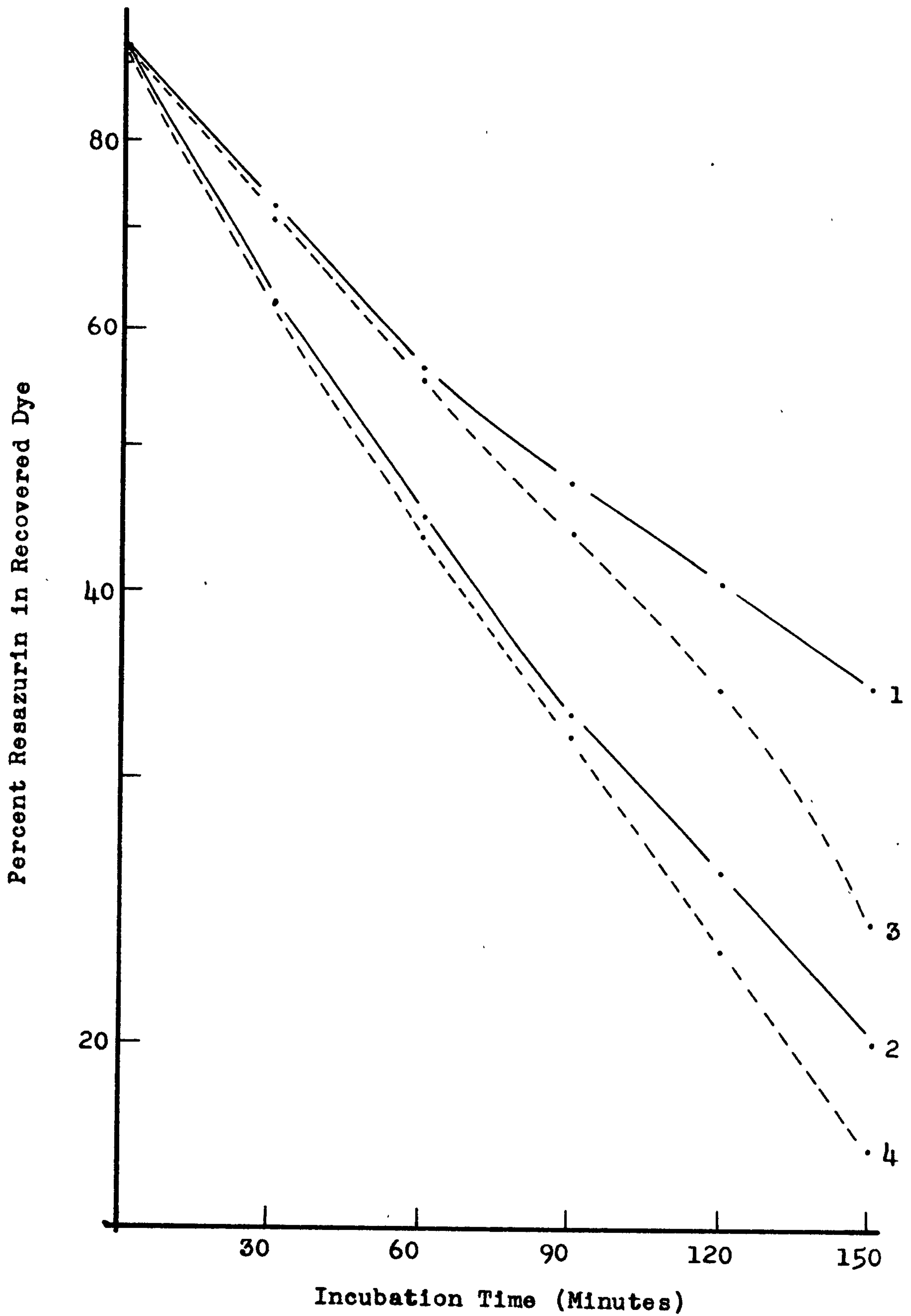


Figure 9b (Milk 2)

Bacteria added; 750,000/ml S. <u>faecalis</u>

II REDUCING SYSTEM OF MILK

A. Behavior of Resazurin at Low pH

The pH dependence of resazurin reduction in milk suggested that useful information concerning the mechanism of reduction could be obtained by dropping the pH to lower levels. The results of such an experiment are shown in figure 10. Samples of whole milk were adjusted to the pH values shown using 6 N HCl, and held at 37°C for 15 minutes in the presence of dye before butanol extraction. The extraction was carried out at the pH of the samples and resazurin remaining estimated by the spectrophotometric method. The values plotted in figure 10 are percent resazurin in the recovered dye. Since dye recoveries in the acid pH range were as high as those normally encountered (approximately 80% for all samples), the values plotted were interpreted as percent of added dye remaining as resazurin in the samples.

Curves very similar to that pictured in figure 10 were obtained using buffered ascorbic acid solutions (10 mg/litre) in place of milk, and it is possible that the curve obtained from milk was nothing more than reaction of ascorbic acid with resazurin. The situation depicted is, however, the reverse of that explained by Ball (29) for the system, ascorbic acid/ methylene blue. i.e. Methylene blue can be used for assay of ascorbic acid at any pH except 5.0, where the E_0'/pH curves of the two systems intersect. At higher pH, the dye has E_0' below that of ascorbic acid, but is reduced by the ascorbic acid system because of the low Eh generated as a result of the

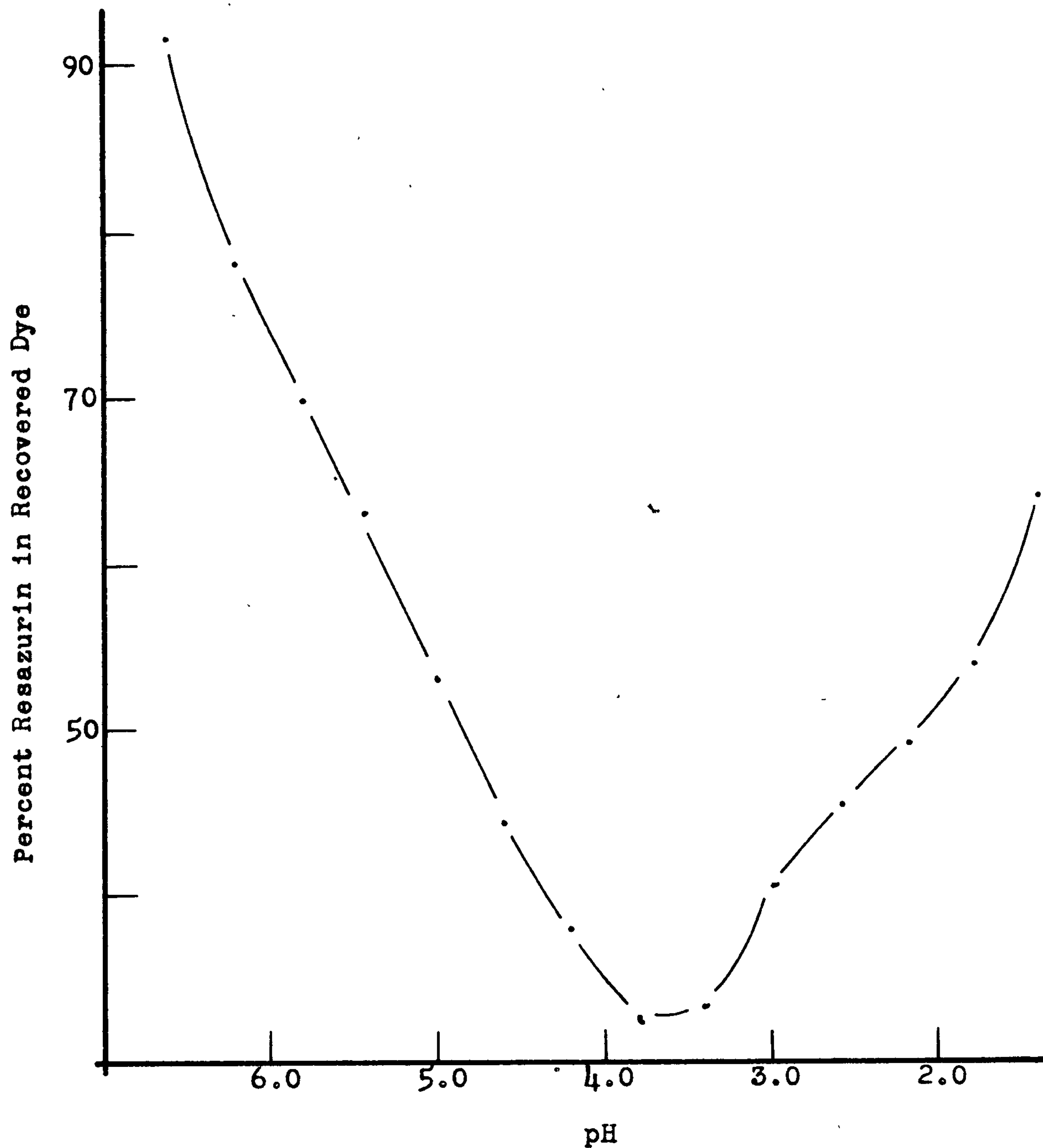


Figure 10

Behavior of Resazurin at low pH

pH adjusted using 6 N HCl: held at 37°C for 15 minutes in the presence of dye.

instability of dehydroascorbic acid. At pH below 5.0, the ascorbic acid system has E'_0 below that of methylene blue and reduction of dye is an obvious consequence of mixing the two systems. That a mixture of ascorbic acid and resazurin behaved in the reverse manner indicates that the interaction was probably not a simple function of Eh and pH. Whether or not resazurin showed this odd behavior in the presence of reducing agents other than ascorbic acid was not determined. The regular increase in rate of reduction from pH 6.6 to 3.8 indicates that the reducing system charted in figure 10 was the normal reducing system in milk. Since it is common knowledge that levels of reduced ascorbic acid, as such, do not correlate with rapid resazurin reduction, and since ascorbic acid was present in sufficient concentration in the milk to produce the curve shown in figure 10, no advantage could be seen in investigating this particular effect any further. The observation is included merely as an illustration that the interaction of the reducing system of milk with resazurin probably involves more than a simple oxidation-reduction process.

B. Influence of Resazurin

The deceleration in rate of resazurin reduction with time of incubation noted in earlier experiments suggested that resazurin had some inactivating influence on the reducing capacity of milk. The magnitude of this influence is illustrated in figure 11. Reducing capacity was measured as described in section II of "Preliminary Experiments" and was expressed as µM ascorbate per sample. The values plotted to produce the three upper curves

on the logarithmic scale represent µM ascorbate remaining at the times shown. Concentrations of resazurin (µM/sample) are shown by the two lower curves. The volume of all milk samples was 5.0 ml with 0.5 ml added water or resazurin solution. Resazurin solutions contained either 0.12 µM or 0.024 µM dye per 0.5 ml.

The data in figure 11 illustrate that added dye had a catalytic effect on destruction of the reducing system of the milk. Calculation of half life periods for the stability of the reducing system under the three conditions measured produces values of 576 minutes for the control system with no added dye, 287 minutes in the presence of 0.024 µM dye, and 155 minutes in the presence of 0.12 µM. The reaction with added dye was not quantitative, 0.024 µM causing a loss equivalent to 0.17 µM ascorbate. This was 7.1 times the quantity of dye added. The higher dye concentration was not so efficient, 0.12 µM dye causing a loss of only 0.34 µM, or 2.8 times the amount added. The quantities of dye reduced during this interval were 61.5 percent of that added, or 0.015 µM at the low concentration, and 50 percent, or 0.06 µM at the high concentration.

It is evident from inspection of figure 11 that rate of destruction of the reducing system was constant for a given dye concentration, over a time interval in which significant concentrations of resazurin were reduced in the same system. On this basis, oxidized resazurin could not have been the sole catalytic agent involved. The effect observed could have been

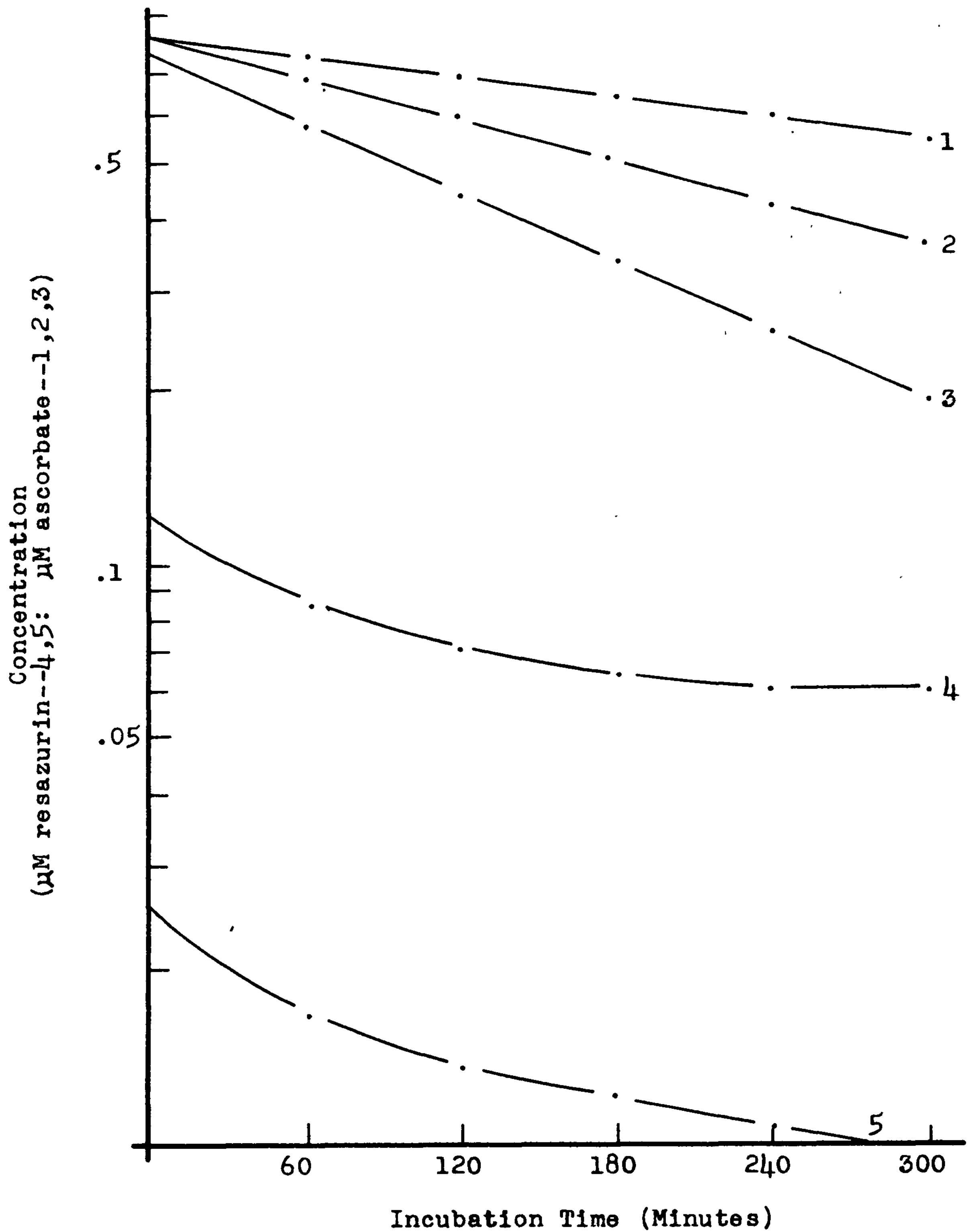


Figure 11

Influence of Resazurin on Reducing Capacity

Reducing Capacity (µM ascorbate)
1 - No added dye
2 - 0.024 µM resazurin
3 - 0.12 µM resazurin

Resazurin Remaining (µM)
4 - 0.12 µM added
5 - 0.024 µM added

due to the dye molecule as such, since concentrations of total dye remained constant while those of the oxidized and reduced species varied. An additional possibility is that catalysis resulted from an artifact, such as a metal ion introduced with the dye and therefore varying with dye concentration.

C. Stability of Ascorbate

Milk usually contains approximately 20 mg/litre or 0.57 µM/5 ml reduced ascorbic acid. The reducing capacity, as measured by ability to decolorize neutral indophenol, was usually 0.7 µM/5 ml. Since any ascorbic acid present would react with the indophenol, titratable ascorbate may be said to constitute an average of 80 percent of the reducing capacity of milk by this measurement. It was therefore probable that the effect of resazurin on the reducing system involved oxidation of ascorbate. Several unsuccessful attempts were made to measure this effect in buffered solutions of ascorbic acid but the ascorbate was too unstable at pH 6.6 and 37°C, even when precautions were taken to minimize contamination by metal ions. Some success was attained by making up all solutions in ethylenediamine tetraacetate (EDTA), though it was known that EDTA at neutral pH was a pro-oxidant for ascorbate (30). Results of an experiment employing buffered solutions of ascorbate in two concentrations of EDTA are plotted in figure 12. Samples contained M/50 phosphate buffer at pH 6.6 and either 0.02% or 0.2% EDTA. Ascorbate (0.76 µM) was added to 5.0 ml buffer followed by 0.12 µM resazurin solution. The final volume was 5.5 ml per sample. Ascorbate was measured using the method described for reducing capacity using the

general procedure outline for figure 11.

Comparison of figures 12 and 11, neglecting differences in arithmetic scales, shows that added resazurin had a similar affect on both ascorbate solutions and milk. In ascorbate solutions, however, no significant resazurin reduction accompanied loss of half the reducing system present. While it is true that ascorbate was much less stable in the buffered solution than in milk, some resazurin reduction was expected in the 90 minute interval measured. In the milk, 0.05 μM resazurin was reduced in the first 90 minutes at the high dye concentration though the milk contained only 0.58 μM titratable ascorbate per sample. The relative effects of different concentrations of resazurin on ascorbate in the buffered solutions was similar to that obtained in milk and it would appear that added resazurin probably stimulated the oxidation of ascorbate in the milk. The fact that resazurin was not reduced in the presence of 0.76 μM ascorbate in buffered solutions (figure 12) leads to the conclusion that the 0.58 μM titratable ascorbate in milk would not have caused resazurin reduction. Unless the greater stability of ascorbate in milk caused it to reduce added dye, reduction must have been due to the 20 percent reducing capacity that could not be accounted for as titratable ascorbate.

The decreased stability of ascorbate in higher concentrations of EDTA shown in figure 12 has been studied in other systems (30). It is possible that if still lower concentrations of EDTA had been used, an ascorbate solution as stable at pH 6.6 as the ascorbate in milk could have been obtained. It was found

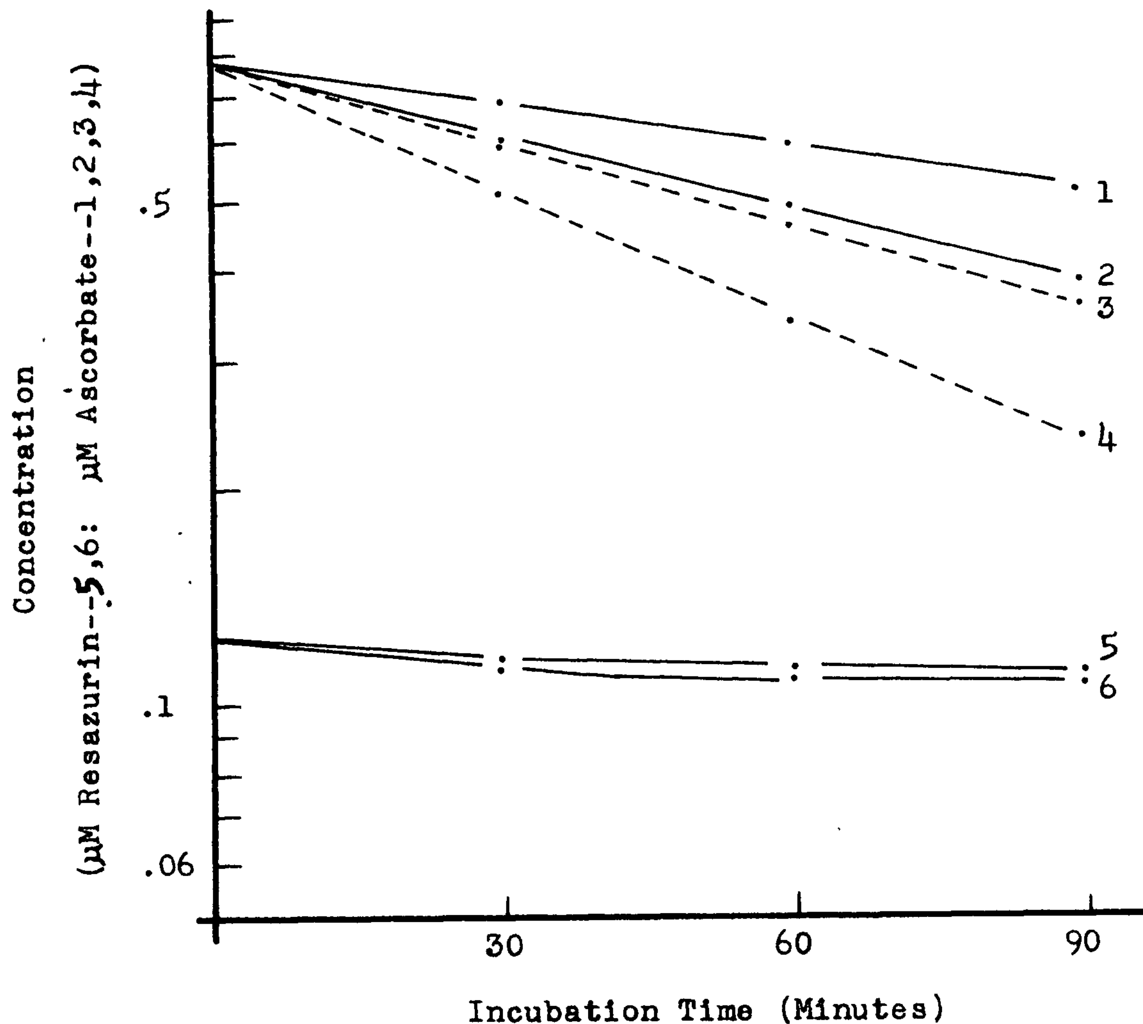


Figure 12

Influence of Resazurin on Ascorbate in EDTA

Ascorbate Remaining (µM)

1 - 0.02% EDTA
2 - 0.02% EDTA, 0.12 µM resazurin
3 - 0.2% EDTA
4 - 0.2% EDTA, 0.12 µM resazurin

Resazurin Remaining (µM)

5 - 0.02% EDTA, ascorbate
6 - 0.2% EDTA, ascorbate

that ascorbate added to powdered skim milk was as stable as that occurring in most normal milk. Addition of resazurin to these stabilized solutions resulted in plots similar to those illustrated in figure 12 i.e. accelerated ascorbate disappearance in the presence of added dye but no significant reduction of resazurin by samples containing 0.7 µM ascorbate per 5.0 ml.

D. Influence of Reduced Dye on Reducing System of Milk

If the dye molecule was involved in catalysing the inactivation of ascorbate in milk, the oxidized and reduced forms must have been equally efficient, since rate of inactivation was constant for a given dye concentration, though the relative concentrations of resazurin and resorufin varied. This result would also have been obtained if catalysis had been due to artifacts in the dye preparation.

In preparing resorufin solutions for use in constructing figure 7, ascorbic acid was employed to reduce resazurin solutions, and the resulting resorufin extracted with ether. This eliminated both residual ascorbic acid and its degradation products from the resorufin solution. Ether extraction could, however, not be used in the present instance. Because catalysis could have been due to an artifact, it was essential to test the entire dye solution rather than just the ether soluble portions. The data in table 8 illustrate that ascorbate degradation products, at a concentration greater than that occurring in the resorufin solution, had no influence on the reducing system of milk nor on the measurements made on this system. For preparation of resorufin, ascorbate was added to resazurin

solution at 5 times the dye concentration. The system was evacuated and held at 37°C for 20 minutes, after which, residual ascorbate was destroyed by aeration. Spectrophotometric examination showed this solution to contain 0.12 µM resorufin and no resazurin. Since no fractionation was applied, the solution should still have contained any impurities present in the original dye.

TABLE 8

INFLUENCE OF REDUCED DYE ON THE REDUCING SYSTEM

Sample 5.0 ml milk, 0.5 ml H_2O	Reducing Capacity (µM ascorbate)		Resazurin (µM)	
	0 Time	120 Mins.	0 Time	120 Mins.
Control	.79	.76	---	---
+.12 µM resazurin	.78	.53	.12	.061
+.12 µM resorufin	.79	.77	---	---
+.7 µM dehydroascorbate	.79	.75	---	---
+.7 µM dehydroascorbate +.12 µM resazurin	.78	.52	.12	.059

Reducing capacity and resazurin were measured as described previously.

As shown by the data in table 8, reduced dye had no ability to decrease the reducing capacity of milk. This indicated that the ascorbic acid treatment must have reduced something in addition to resazurin since resazurin alone could not have been responsible for the catalytic influence on the reducing system (figure 11). The dye preparation must, therefore, have contained some artifact that inactivated the reducing capacity of milk

but was itself inactivated by ascorbic acid treatment.

E. Silicic Acid Treatment of Resazurin

A silicic acid column was employed in an effort to purify resazurin. One commercial resazurin tablet was ground in a mortar with repeated 10 ml volumes of ethyl acetate and sufficient 0.01 N HCl to maintain a pH below 5.0. The low pH was necessary for extraction of resazurin by ethyl acetate. The extraction was stopped when approximately 100 ml extract had been obtained, though only a portion of the resazurin had been dissolved. The extract was concentrated to approximately 2 ml and layered on top of a small silicic acid column. The column had been packed in petroleum ether and washed with approximately 2 bed volumes of ethyl acetate. Development was with ethyl acetate, which separated the extract into four bands. Three of these were eluted with ethyl acetate and the fourth with a 50/50 mixture of ethyl acetate/n-butanol.

The fractions were numbered in the sequence of their elution from the column. Water was added to each before evaporation of solvent and the aqueous solutions concentrated to 5.0 ml. Both fractions 1 and 2 were insoluble in aqueous media or in dilute sodium bicarbonate. They appeared as oils heavier than water and were present at approximately 0.2 ml each. Fraction 1 was a muddy grey color and fraction 2, a bright fluorescent red, reminiscent of a very concentrated solution of resorufin. Fractions 3 and 4 were completely soluble in water and both appeared blue, fraction 3 having much less color than 4. Spectra of all fractions were obtained in butanol saturated

with sodium bicarbonate under the conditions employed for spectrophotometric measurement of resazurin. Volumes of the solutions described added to 9.0 ml butanol were: fraction 1, 5.0 ml; fraction 2, 1.0 ml; fraction 3, 3.0 ml; fraction 4, 1.0 ml. The spectra of fractions 1, 2 and 4 are shown by figure 13. The optical densities of fraction 3 were too low to plot on this scale. It had one maximum coinciding with that at 580 mμ shown for fraction 2.

It is obvious, on comparison of these spectra with those previously obtained for resazurin and resorufin (figure 1), that fraction 4 was resazurin. Calculation of the quanity of resazurin present showed that 1.0 ml contained 0.12 μM, making fraction 4 half as concentrated as the resazurin solution usually employed. Though fraction 2 had a fluorescent red color, it was not resorufin. A resorufin solution having optical density of 0.150 at 475 mμ would have had a very strong absorbance at 582 mμ.

All fractions were tested for their ability to catalyse the decomposition of the reducing capacity of milk. Results are shown in table 9. It is evident from these data that fraction 4 was much more active than any of the other fractions. Though it was present at only half the concentration of unpurified resazurin, its effect was almost as great. This fraction represented quite an extensive purification of resazurin and most artifacts should have been removed from it. It would appear, therefore, that the dye itself affected the reducing system. This is at variance with the results obtained using

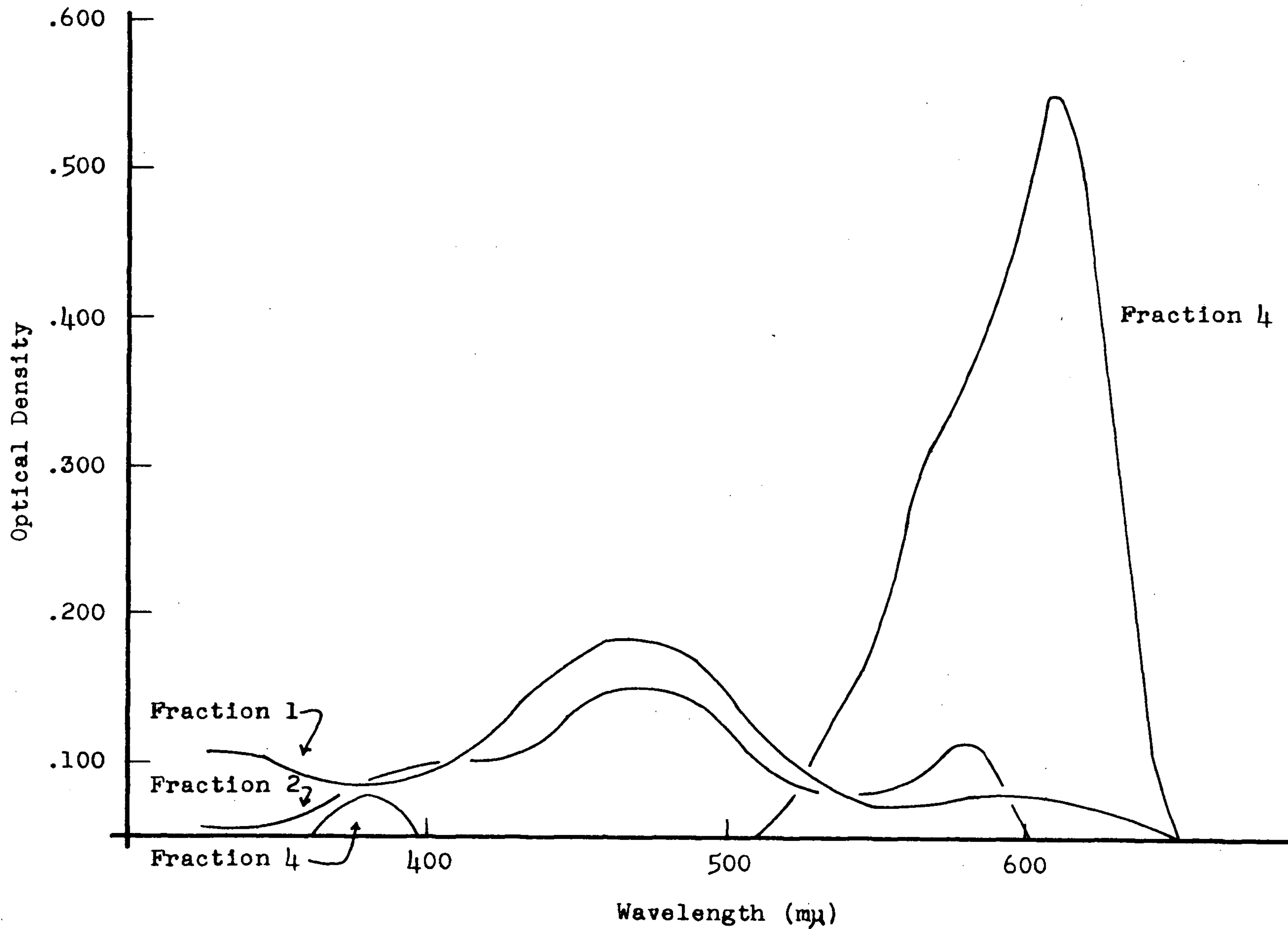


Figure 13

Spectra of Resazurin fractions in butanol saturated with sodium bicarbonate.

reduced dye. However, it is possible that an active form of the dye, possibly a metal chelate, was inactivated, even under the extremely mild conditions employed for reduction in the experiment with reduced dye.

TABLE 9

INFLUENCE OF RESAZURIN FRACTIONS ON REDUCING CAPACITY

Sample (5.0 ml milk)	Reducing Capacity (µM ascorbate) 0 Time	120 Mins.	% Remaining after 120 Mins.
Control (0.5 ml H_2O)	.65	.58	93
Fraction 1 (0.3 ml)	.62	.49	79
Fraction 2 (0.3 ml)	.62	.47	75
Fraction 3 (0.3 ml)	.61	.53	85
Fraction 4 (0.036 µM)	.63	.39	62
Resazurin (0.72 µM)	.63	.36	57

F. Reducing Capacity of Milk Fractions

In experiments on rates of resazurin reduction, it was found that fractions of normal milk differed in their ability to reduce dye. The present experiment was designed to determine whether or not this trend persisted in mastitic samples and how it was related to total reducing capacity. Results are presented in table 10. Whole milk (80 ml) was centrifuged and the fat, combined with sediment, made back to 30 ml with whole milk. This produced a fraction enriched in fat and any solid residues in the milk, but having a lower fat content than gravity cream. Resazurin (0.12 µM/0.5 ml) or water (0.5 ml) was added to 5.0 ml of each fraction before incubation at 37°C.

TABLE 10

REDUCING CAPACITY OF MILK FRACTIONS

Sample	Mastitic Milk				Normal Milk			
	Reducing Capacity (μM Ascorbate)		Resazurin * (μM)		Reducing Capacity (μM Ascorbate)		Resazurin (μM)	
	Time (Minutes)		Time (Minutes)		Time (Minutes)		Time (Minutes)	
	0	60	0	60	0	60	0	60
whole + H_2O	.76	.77	---	---	.61	.54	---	---
whole + resazurin	.75	.79	.12	0	.61	.35	.12	.079
skim + H_2O	.51	.44	---	---	.66	.53	---	---
skim + resazurin	.51	.32	.12	.089	.65	.38	.12	.091
fat + pellet + H_2O	.80	.81	---	---	.53	.47	---	---
fat + pellet + resazurin	.79	.80	.12	0	.53	.34	.12	.072

*Resazurin reduction was very rapid, the whole and fat + pellet samples reaching the 7/4 standard in 10 to 15 minutes: at 30 minutes, the fat + pellet samples were white, and whole samples were approximately 50% white.

Resazurin and reducing capacity were determined as described previously.

In this experiment, reducing capacity of the mastitic milk was abnormally high, and that of the normal milk low. Despite this, the data in table 10 illustrate some significant points. The resazurin reducing ability of the mastitic sample was almost completely in the combined fat and pellet fraction and was largely insensitive to added resazurin, only that of the skim being inactivated by added dye. This substantiates, to some extent, the earlier observation that the free ascorbate of milk was probably the component of the reducing system inactivated by resazurin. The reducing capacity of all the fractions of normal milk was affected by added resazurin and all fractions reduced the dye to some extent, as predicted from earlier experiments.

G. Ascorbic Acid Oxidase

In early experiments employing visual extimates of resazurin reduction, it was observed that treatment of milk with ascorbic acid oxidase increased the time needed for samples of normal milk to become reduced to the 7/4 color standard but had no effect on rapidly reducing milks. It was found at that time that enzyme treatment oxidized all the titratable ascorbate in both normal and abnormal milks. The effect of ascorbic acid oxidase treatment on the reducing capacity and titratable ascorbic acid content of fractions of mastitic milk is shown in table 11. The fat and pellet obtained from centrifuging 40 ml whole milk were each made to 5.0 ml with skim or powdered skim

milk. Ascorbic acid was measured by indophenol titration in metaphosphoric acid-acetic acid, and reducing capacity by the method employed in previous experiments. The volume of all samples was 5.0 ml. Enzyme treatment was for 15 minutes at 37°C.

TABLE 11

INFLUENCE OF ASCORBIC ACID OXIDASE ON REDUCING CAPACITY

Sample	Reducing Capacity (μM Ascorbate)		Titratable Ascorbate (μM)	
	Initial Values	Enzyme Treated	Initial Values	Enzyme Treated
whole	.44	.27	.22	.05
skim	.30	.07	.26	.05
fat + skim	.64	.57	.18	.05
fat + powder*	.59	.53	.07	.05
pellet + skim	.66	.62	.20	.05
pellet + powder*	.62	.62	.15	.05

*Blank values for powdered skim milk subtracted.

Inspection of the initial values in table 11 shows that the concentration of titratable ascorbic acid was much lower than the reducing capacity in all fractions, except skim. In this fraction, the two measurements agreed quite closely. In addition, the reducing capacity of skim was largely eliminated on treatment with enzyme, indicating that ascorbate was the major component of the reducing capacity of this fraction. In the remaining fractions, enzyme treatment removed almost half

the reducing capacity of whole milk but had little effect on samples derived from fat or pellet. In contrast to this, the enzyme removed essentially all the titratable ascorbic acid from all fractions. A number of unsuccessful attempts were made to identify ascorbic acid in these fractions by paper chromatography. Since ascorbate must certainly have occurred in the skim, the negative results meant merely that the techniques used were inadequate. The possibility that the portion of the reducing capacity that could not be accounted for as ascorbate was due to free sulfhydryl groups was investigated. All fractions of the mastitic milk produced a slight pink color with the nitroprusside reagent, while similar fractions from normal milk remained white. Since mastitic skim produced as much color with the reagent as did the fat and pellet fractions, it was concluded that the differences between fractions noted in figure 11 could not have resulted from presence and absence of free sulfhydryl groups.

GENERAL DISCUSSION

I INTERACTION OF RESAZURIN WITH REDUCING SYSTEM

The reducing capacity of milk was inactivated by the amounts of resazurin normally employed in milk testing. The half life of the relative reducing capacities of milk and milk plus resazurin were 576 and 155 minutes respectively (figure 11). Resazurin was also found to decrease the stability of ascorbic acid in buffered solutions containing EDTA (figure 12). This influence of resazurin on the reducing capacity of milk was also noted by Johns (6) who found a rise in Eh of approximately 0.06 volt over a 5 hour interval if resazurin was present in low leucocyte milk. Campbell, Phelps and Keur (28) showed that a loss of 12 mg/litre reduced ascorbic acid resulted in a rise of approximately 0.07 volt in the aerobic potential of milk. On the basis of the similarity of the reducing capacity curves in figures 11 and 12 and of the supporting observations cited, it was concluded that the component of the measured reducing capacity that was unstable to the presence of resazurin was reduced ascorbate.

Comparison of resazurin reduction rates with those for reducing capacity in figure 11 shows an obvious correlation between decrease in rate of resazurin reduction and inactivation of reducing capacity. However, this was probably not a cause and effect relationship, for the amount of ascorbate present in the milk was only 0.58 μM and it was found that in buffered solutions of ascorbate even 0.7 μM would not bring about reduction of resazurin (figures 11 and 12). Employing visual estimates of resazurin reduction, it was found that treatment of milk

with ascorbic acid oxidase, which removed all of the titratable ascorbate, had little influence on the resazurin reduction time of normal milk and no influence on the reduction time of milk that could reduce the dye in less than 2 hours. It is therefore apparent that the concentrations of ascorbate present in milk do not influence the rate of resazurin reduction under the normal aerobic conditions employed in milk testing, in spite of the fact that ascorbate is oxidized during this period and that this oxidation is stimulated by resazurin.

It is doubtful that the 0.06 volt rise in Eh which probably accompanied the loss of ascorbate in figure 11 was responsible for the decreased rate of resazurin reduction. Johns (6) found the same rate of reduction in aerated samples as in unaerated controls, concluding that the aerobic potential had no influence on resazurin reduction in milk. This had also been suggested by the observations of Ramsdell et al. (3), that a single shade of resazurin color could exist over a wide range of Eh values in milk.

The foregoing discussion should not be construed as meaning that resazurin reduction is independent of electrode potential. In milk, the measured aerobic potential has no influence on reduction but resazurin becomes reduced to resorufin immediately on exposure to the anaerobic potential which is approximately 0.4 volt lower, as determined after flushing milk with nitrogen (26). A similar effect is noted on removal of oxygen from solutions containing 5 to 10 mg/litre ascorbate, which is not sufficient to reduce resazurin aerobically but will reduce

it immediately anaerobically. It is therefore obvious that reduction of resazurin is dependent on Eh. In this investigation, no effort was made to measure rates of reduction at fixed Eh values, it being assumed that an irreversible, Eh-dependent reaction would plot as spontaneous decay with rate dependent on applied potential.

Since the change from resazurin to resorufin is irreversible under physiological conditions, reduction can occur in additive increments to produce a progressive rate of color change, in an ambiant Eh at which the dye should be completely stable. If zones of low Eh occurred at the surfaces of particles in the milk, such as bacteria, leucocytes, or fat globules; any dye diffusing into these zones would become reduced and, on diffusion back into the high Eh of the milk plasma, would remain reduced. It is apparent that continuation of this process would result in a measurable reduction of total dye depending on number and Eh of zones and the rate of diffusion of dye through them.

II STABILITY OF REDUCING SYSTEM

The experiments on aging, demonstrate that the resazurin reduction observed with fresh milk resulted from the normal reducing systems present rather than from bacterial activity (figure 5). In addition some questions with regard to the location and stability of the reductants are raised. Milk 1 produced a resazurin/resorufin ratio of 0.9 after 1 hour incubation with dye at 37°C. According to accepted views, this milk would be classified as a rapid resazurin reducer and hence, the product

of an unhealthy udder. Unfortunately, leucocytes were not counted in these samples but, on the basis of the rapid resazurin reduction, they should have been quite numerous (7). If reduction had been due to leucocytes, there should have been a more rapid decrease in rate of reduction on storage, particularly at the higher temperature, the current view being that preincubation at 12.8°C for 18 hours largely dissipates any reducing activity due to leucocytes (1). Contrary to this, there was little change in the rapid reducer on storage and very little influence of temperature of storage. Milk 2, on the other hand, was nearer what would be termed a normal sample but lost reducing ability more rapidly on storage and was more sensitive to temperature of storage. This trend was reflected to a greater degree in the skims from these two milks, which had most of the leucocytes centrifuged off. This type of behavior was not unique in the two milks presented in figure 5, but occurred in all of the samples tested, which included some mixed milks. However, the number of samples tested was not sufficient to state that the behavior was general for milk.

The reducing system in these milks was obviously not bacterial and, from the behavior of the skims, probably not leucocytes alone. From the difference in reduction rates between whole and skim samples, it would appear that the reducing system was associated with the fat or sediment and was stabilized by this association. If, as suggested earlier, resazurin reduction occurred in association with some structure in the milk, it is logical that skim would have reduced dye more slowly

than whole milk, and normal milk more slowly than high leucocyte milk.

It had been found in early experiments that dilution of milk with buffer or water greatly slowed the rate of resazurin reduction and that washed cream lost all its ability to reduce dye. Leucocytes have been reported to lose a large percentage of their reducing capacity on washing in saline or water (2,4,6), and even on washing in milk (8). The tendency of milk to lose reducing activity on dilution leads to the suggestion that the reducing capacity is loosely adsorbed in the form of diffusable chemical reducing agents whose concentration determines the Eh of the surfaces of the structures they occur on. Considered in the light of the earlier discussion on the possible mechanism of resazurin reduction in milk, this suggestion appears more plausible than the removal of cofactors of enzyme reactions, which has been advanced as an explanation for the loss of activity of washed leucocytes (31). If it is assumed that resazurin is reduced by chemical agents adsorbed to particles in the milk, the decreased stability of the skim samples of figure 5 merely reflects an increased rate of diffusion of reducing agent from the surfaces of any remaining particles. The fat globules and leucocytes remaining in the skim would not be protected by the mutual aggregation which occurs in whole milk at lower temperatures (32) and would, therefore, probably tend to lose adsorbed constituents more readily.

III NATURE OF REDUCING SYSTEM

The quantity of indophenol which would be decolorized by

various dilutions of a sample of milk could be calculated from the value obtained for the intact sample (table 7). The reaction of milk with indophenol was extremely rapid, appearing complete on mixing, and produced the same values for reducing capacity at 0°C as at 37°C. Because of the quantitative nature and speed of this reaction, it was concluded that the dye gained electrons from some readily available source in the milk rather than through mediation of some enzyme reaction. It was shown by comparison with ascorbic acid titration that reduced ascorbate comprised approximately 80 percent of this electron source. The remaining 20 percent remained unaccounted for, and it is probable that this was a chemical reducing substance with a behavior toward indophenol and oxygen analogous, in many respects, to that of reduced ascorbate.

It has been shown that human leucocytes and blood platelets contain 290 to 430 mg/Kg bound ascorbic acid in a weakly dissociable complex (33). Extrapolation of this interrelationship to bovine blood produces a hypothetical particle with all the attributes inferred, in the earlier discussion, to be characteristic of the resazurin reducing system of milk. In addition to posession of the characteristics postulated for the reducing system, these particles have an incidence of occurrence and location in milk which correlates well with rate of resazurin reduction. The observation of Campbell and Phelps (8), that leucocytes isolated from milk regained their reducing ability on suspension in bovine plasma, also fits this picture since leucocytes depleted of ascorbate are known to take it up

rapidly from blood plasma (34).

With the exception of their behavior in the presence of ascorbic acid oxidase, these hypothetical particles, considered from the point of view of the proposed mechanism of resazurin reduction, can be used to explain all of the observations made in this investigation and most of those recorded in the literature concerning the peculiarities of resazurin reduction. The fact that it was not possible to account for this part of the reducing capacity as ascorbate by titration in metaphosphoric acid is in favor of the existence of the particles. Buller and Cushman (33) found they could not titrate the ascorbate in their preparations if they precipitated with metaphosphoric acid without first saturating the system with carbon monoxide. They attributed the loss of ascorbate to immediate oxidation by hemoglobin from laking of the few red blood cells present. It has recently been shown that the blood in vat samples of milk ranged from 1 to 15 mg/litre (35). Since the quantity of ascorbate affected was only approximately 4 mg/litre in normal milk, it is probable that there was sufficient hemoglobin present to cause its rapid oxidation on treatment with metaphosphoric acid.

The resistance of the residual reducing capacity to ascorbic acid oxidase is more difficult to rationalize. Buller and Cushman (33) reported complete destruction of the ascorbic acid in platelets and leucocytes in the presence of enzyme, but gave no experimental details of this phase of their work. On more careful examination of the data in table 11, it is evident that the loss in titratable ascorbate was parallelled by a comparable

loss in reducing capacity in all but the pellet fractions. It is possible that the constituents of this fraction stabilized some acid labile intermediate generated by the enzyme. Though this possibility is admittedly remote, the ascorbic acid oxidase data was the only evidence found which was contrary to the proposed structures postulated as instrumental in resazurin reduction.

SUMMARY AND CONCLUSIONS

Spectrophotometric methods were developed to quantitatively measure resazurin reduction and the reducing capacity of milk. Reducing capacity was assessed as the quantity of indophenol reduced by milk at its normal pH. The method could be used in the presence of resazurin. These methods were employed singly and in combination to study interaction of resazurin with the reducing system of milk.

Resazurin was shown to have a destabilizing influence on reducing capacity. This influence was catalytic and dependent on total concentration of dye; rate of inactivation being constant for a given dye concentration. Evidence was presented to show that the component of the reducing capacity that was inactivated was ascorbic acid.

It was concluded from these investigations that the reducing system of fresh milk existed as a measurable entity at any given time rather than as a continuous evolution of electrons from some enzymatic reaction. This system consisted of the measurable ascorbic acid of the milk, which occurred in the plasma, and some reducing agent bound to structural components of the cream and sediment. The measurable ascorbic acid accounted for approximately 80 percent of the reducing capacity but it was concluded to have little influence on resazurin reduction. The bound reducing agent apparently depended on structural elements in the milk for its ability to reduce resazurin, and that it lost this ability on dissociation from the particulate structure. It was postulated that this reducing agent was ascorbate

and that it occurred bound to leucocytes and other cellular debris in the milk in situations analogous to its reported occurrence in blood. Attempts to identify this reducing agent as ascorbate were unsuccessful in this investigation, but the techniques employed were probably inadequate.

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