n- or isobutanol. Removal of the lipid is accompanied by simultaneous release of the enzyme into true solution. No other organic solvent tested was effective in releasing the bound phosphatase, indicating a unique action of butanol.

I wish to thank Dr M. Dixon, F.R.S., for his encouraging interest and advice during this work. This work was carried out while holding a Travelling Scholarship awarded by the Gowrie Scholarship Trust of Australia, whose assistance is gratefully acknowledged.

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Alkaline Phosphatase of Milk

2. PURIFICATION OF THE ENZYME

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It has been shown in the previous paper (Morton, 1953a) that alkaline phosphatase in milk is intimately associated with insoluble lipoprotein particles. In this respect the enzyme resembles the alkaline phosphatases of animal tissues (Kabat, 1941; Hers, Berthet, Berthet & Duve, 1951), but unlike the tissue enzymes (see Roche & Thoai, 1950) milk phosphatase is only partly released from the particulate material when this is treated with trypsin or lipase (Zittle & Della Monica, 1952).

A far more effective method of obtaining the phosphatase in true solution, and one which avoids undesirable exposure of the enzyme to proteolysis, is the treatment of the whole milk or milk product with butanol (Morton, 1950, 1953a). Using this

procedure to obtain the enzyme in true solution, and by further treatments, the alkaline phosphatase has been highly purified. The final product is considered to be essentially homogeneous protein. The details of the method are described in this paper.

METHODS

The analytical methods have been described in the previous paper (Morton, 1953a).

Preparation of activated charcoal. Animal charcoal (British Drug Houses) (180 g.), was treated successively with 5 l. of 0.1 N-HCl, 2 l. 5% KCl, 200 ml. 0.1 M-Ba(OH)₂, and finally with 2 l. of 5% KCl. After each treatment the charcoal was sucked dry over no. 42 Whatman paper on a large Büchner funnel. After the last treatment it was dried at 100° in an oven, and stored in an air-tight jar. I am indebted to Dr P. D. Mitchell for suggesting this procedure for obtaining an active charcoal, free of inorganic phosphate.

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EXPERIMENTAL AND RESULTS

Preliminary studies

Because of the very low specific activity of whole milk (Morton, 1953*a*) it was clear that very large bulks of material would have to be treated in order to obtain sufficient of the enzyme to enable effective purification.

Preliminary experience in the treatment of a large bulk was obtained using 33 l. of cream (71 % butterfat) obtained by commercial separation of 500 l. of whole milk. After dilution with water to 43 l., it was adjusted to pH 5.5 and treated with *n*-butanol (3.5 l./6 l. cream) at 38° for 5 min. The emulsion was centrifuged (1800 g, 30 min.) in 1 l. glass cups in an International Serum Centrifuge, and the clear, greenish, butanol-saturated aqueous layer (which contained the phosphatase in true solution) was collected by suction. The enzyme was precipitated with acetone, adsorbed from the dialysed solution on to calcium phosphate gel (prepared essentially as described by Keilin & Hartree, 1951), and eluted with 0.1M-NaHCO₃. This gave a brown solution from which the enzyme was obtained by addition of solid (NH₄)₂SO₄, taking the fraction between 65 and 85 % saturation (at pH 7.0). The clear colourless solution so obtained had a specific activity of 3050 units/ml., representing a purification of 985 times as compared with the

initial whole milk, and an overall yield of 2 %. Efforts to obtain further purification by a wide variety of procedures were unrewarding. However, electrophoretic examination in 0.033M phosphate buffer, pH 8.0 showed at least three proteins to be present.

A large number of small-scale trials, using a variety of procedures, were carried out with the partially purified enzyme (specific activity 3050 units/ml.) obtained from cream. The results of these studies provided the basis for the method of purification of the enzyme described in the following section. They also serve to indicate some of the properties of the milk phosphatase, and therefore are briefly summarized here.

Effect of pH. The pH of a sample of the enzyme solution of specific activity 3050 units/ml. was carefully and progressively lowered at room temperature, by slow addition of 0.05N acetic acid. There was no visible precipitation of any protein, although a very faint opalescence appeared at about pH 4.5, and no purification was gained by this procedure. There was no change in appearance by adjustment to alkaline pH values. The results shown in Table 1 indicate the difference in the recovery of the enzyme when in true solution and when bound by particles.

Effect of heating. Heating a sample of the same enzyme solution gave the results shown in Table 2.

Adsorption. A large number of adsorbents were examined, including calcium phosphate gel, magnesium phosphate gel, alumina gel (Cγ), animal charcoal, glass dust, Hyflo Supercel (Johns-Manville and Co.), kieselguhr, kaolin of various grades, magnesium and calcium carbonates, diphenylcarbinol (Neumann, 1949) and salicylic acid. Particularly at pH values about 5.0–5.5, the enzyme can be adsorbed by addition of sufficient of most of these materials. Only calcium phosphate gel, alumina gel, and specially prepared charcoal deserved further examination. Over the pH range 5.0–8.5, the enzyme can be adsorbed and then readily eluted from calcium phosphate and alumina gel, although more difficulty is experienced in eluting the enzyme from charcoal. NaHCO₃ (0.1N), or 5 % (NH₄)₂SO₄ or Na₂SO₄ at pH 8.0 are all equally effective for elution of the enzyme. However, adsorption of inert protein, rather than of enzyme, generally gave more satisfactory results.

Final purification method

Stage 1. Whole milk was commercially separated at 35° to give about 50 l. of cream of 69 % butterfat.

Stage 2. After cooling to 10° and dilution with water to give 50 % butterfat, the cream was churned in two wooden hand-churns, each of 5 gal. capacity. The buttermilk and two wash waters were combined and cooled to about 10°. Further treatment was carried out after transporting to the laboratory.

Stage 3. The buttermilk, at pH 6.6, was treated in batches, adding 2.4 l. (29 %, v/v) of *n*-butanol to 6 l. of buttermilk, previously heated to 32°. The material was vigorously stirred and heated to 35° for approximately 5 min., and then adjusted to pH 4.95 by addition of 750 ml. of 0.25N acetic acid. The casein was thus precipitated, and on centrifuging, the material separated into distinct layers comprising a light white precipitate, a clear yellow

Table 1. *Effect of pH on milk alkaline phosphatase*

(Each preparation treated for 15 min. at 20° at pH indicated and then readjusted to pH 7.5 prior to testing at pH 9.8 (37°) using sodium β-glycerophosphate as substrate. Enzyme preparations: preparation 1 was dialysed, phosphatase-enriched, fat-free separated milk (Morton, 1953*a*). Preparation 2 was a partially purified enzyme preparation of specific activity 3050 units/mg. N, obtained in true solution by treatment of cream with butanol.)

pH (20°)	Relative activity	
	Preparation 1 (particle-bound enzyme)	Preparation 2 (enzyme in true solution)
9.5	96	22
9.0	96	67
7.5	100	100
5.0	60	96
4.7	38	80
4.6	27	68
4.4	5	15
4.3	5	5

Table 2. *Heat stability of milk alkaline phosphatase*

(Enzyme preparation: the partially purified enzyme, of specific activity 3050 units/ml., was used. The preparation, at pH 7.8, was heated for 2 min. at temperature indicated, and then rapidly cooled to 20°.)

Temp. (°)	Relative activity
45	102
50	91
55	76
60	46

aqueous zone overlaid by light particles, and a brown surface layer of excess of *n*-butanol, containing dissolved material.

Stage 4. After removing the excess of butanol, the serum was removed by suction through a narrow glass tube and filtered through a 1 cm. layer of Hyflo Super-cel over no. 1 Whatman paper on a large Büchner funnel. It was then adjusted to pH 8.5 with 0.2N sodium hydroxide and held overnight at 5°, when a bulky white precipitate settled. The supernatant was largely decanted and the remainder centrifuged to remove the precipitate.

Stage 5. The clear yellow-green serum, adjusted to pH 6.3, was treated in batches by adding 10% (v/v) ether to the material at 0°, and precipitating the protein by the addition of a further 45% (v/v) of acetone, at -3 to -5°. The batches were held overnight at -7°, the supernatant carefully decanted, and the pink precipitate collected by centrifuging in a refrigerated centrifuge at -5°.

Stage 6. The precipitate, dissolved in a minimum of distilled water and filtered overnight at 0° through Whatman no. 542 filter paper, gave a cherry-red solution, completely water-clear. After dialysis to remove organic solvents, it was fractionated with ammonium sulphate at pH 7.1. The lower ammonium sulphate fraction (0-63% saturation) containing the red protein had little phosphatase activity and was discarded. The fraction which precipitated between 63 and 85% saturation of ammonium sulphate was pale straw-yellow in colour. After dialysis for 3 days against frequent changes of distilled water at 5°, it gave 127 ml. of protein solution of specific activity 4102 units/mg. nitrogen, a purification of 1520 times compared with the initial whole milk, with a yield of 6.5%.

Stage 7. The dialysed enzyme was adjusted to pH 8.6 with 0.1N sodium bicarbonate, diluted with

water to 180 ml., and then treated by slow addition of 9 g. of treated animal charcoal, added as a slurry in distilled water. After thorough stirring for 30 min., the charcoal was removed by centrifuging and discarded. To the supernatant 12 ml. of alumina gel (Cγ, 22 mg. dry weight/ml.) was added, mixed, and the precipitate discarded.

Stage 8. The resultant supernatant of specific activity 7261 units/mg. nitrogen (purification factor, 2690 times) was dialysed for 2 hr. against distilled water at pH 7.5, adjusted to pH 6.4, and fractionated by addition of ether (10%, v/v) followed by acetone (40-49%, v/v), at -3°. The precipitate was dissolved and dialysed against distilled water to remove organic solvents, and centrifuged at 14000 *g* for 20 min.

Stage 9. The supernatant was readjusted to pH 8.6, 5 g. of treated charcoal was added, and centrifuged at 14000 *g* for 30 min.

Stage 10. The supernatant from centrifuging was immediately fractionated with ammonium sulphate at pH 7.2, largely to eliminate traces of charcoal apparently in stable colloidal suspension. The active fraction between 69 and 82% saturation of ammonium sulphate was dialysed at pH 7.5 to low ionic strength.

Stage 11. After adjusting to pH 6.5 and 0.015M magnesium acetate, and following addition of ether to 10% (v/v) at 0°, the dialysed solution was fractionated with acetone between 42 and 48% (v/v). After dialysis for 4 hr. against 5 l. of distilled water (adjusted to pH 7.5) at 0°, the product was dried in vacuum over sulphuric acid. The fine white powder so obtained had a specific activity of 15314 units/mg. nitrogen (*Q*₁₀, i.e. equivalent μl. P liberated by 1 mg. protein in 1 hr. at 38°, 106000), representing a purification of 5660 times compared with the initial whole milk on a protein nitrogen

Table 3. *Purification of milk alkaline phosphatase*

(Summary of stages: (1) Whole milk. (2) Buttermilk and washings from cream (69% butterfat). (3) Filtered aqueous layer following butanol treatment. (4) Filtrate obtained after treatment at pH 8.5, 12 hr., at 5°. (5) Red solution of precipitate obtained with ether (10%, v/v) and acetone (0.45%, v/v). (6) Yellow solution of precipitate obtained with (NH₄)₂SO₄, 63-85% saturation. (7) Supernatant from charcoal adsorption at pH 8.6. (8) Colourless solution of precipitate obtained with ether (10%, v/v) and acetone (40-49%, v/v). (9) Supernatant from second charcoal adsorption. (10) Fraction obtained with (NH₄)₂SO₄, pH 7.2, 69-82% saturation. (11) Final solution obtained with ether (10%, v/v) and acetone (42-48%, v/v).)

Stage	Vol. (ml.)	Activity (units/ml.)	Total (units × 10 ³)	Protein N (mg./ml.)	Specific activity (units/mg. N)	Yield (%)	Purification
(1)	1.3 × 10 ⁶	13.9	18 070	5.10	2.7	100.0	1
(2)	32 500	80	2 600	2.80	28.6	14.4	11
(3)	27 120	66	1 790	0.21	314.3	9.9	116
(4)	27 000	65	1 755	0.20	325.0	9.7	121
(5)	1 480	1 092	1 616	1.20	910.0	8.9	337
(6)	127	9 230	1 172	2.25	4 102	6.5	1 520
(7)	280	3 340	935	0.46	7 261	5.2	2 690
(8)	75	9 800	735	1.04	9 423	4.1	3 490
(9)	89	5 730	510	0.49	11 690	2.8	4 330
(10)	12	29 000	348	2.04	14 200	1.9	5 270
(11)	10	26 800	268	1.75	15 314	1.5	5 660

basis. This corresponds to a purification of about 11 000 times on a non-dialysable dry-weight basis, or about 24 000 times on a total dry-weight basis. The final yield of purified enzyme was 1.5%. All attempts at further purification beyond this stage caused a decline in the specific activity of the preparation, which was considered to be essentially pure milk phosphatase.

A summary of the purification is given in Table 3.

Electrophoretic studies

The preparations obtained during purification of the enzyme were examined at several stages, using a Tiselius electrophoresis apparatus. The material of specific activity 4102 units/mg. nitrogen (Table 3, stage 6) showed the presence of three protein components which separated in 0.033M phosphate buffer, pH 8.0 (Fig. 1, upper photograph). The leading peak separated sufficiently after 1 hr. to enable removal of a sample without contamination with either of the other two proteins. The specific activity of this fast-moving peak was 192 units/mg. nitrogen, while that of the sample, which contained some of each of the other two remaining proteins, was 9600 units/mg. nitrogen.

Fig. 1. Electrophoretic analyses during purification of milk phosphatase. The upper photograph shows material of specific activity 4102 units/mg. N (Table 3, stage 6) after 1 hr.; the lower photograph, material of specific activity 9423 units/mg. N (Table 3, stage 8) after 1 hr. 45 min. On the left are the ascending (anodic), and on the right the descending (cathodic) boundaries obtained with phosphate buffer (pH 8.0, 0.033M) at 5.0 v/cm.

The material of specific activity 9423 units/mg. nitrogen (Table 3, stage 8) was examined under the same conditions. Only two proteins separated (Fig. 1, lower photograph). A sample of the leading protein gave a specific activity of 100 units/mg. nitrogen, while that of the slower-moving protein gave 12 000 units/mg. nitrogen. Since this protein

showed no tendency to separate further, the activity of the sample should approximate to that of the pure enzyme.

The material of specific activity 9423 units/mg. was also examined in 0.125M acetate buffer, pH 5.5. However, there was very little separation of the proteins, even after 2 hr.

Insufficient material remained after final purification (Table 3, stage 11) to permit of electrophoretic examination since the apparatus available required about 12 ml. of a 1% protein solution.

DISCUSSION

When the alkaline phosphatase of milk has been released into true solution by disruption of the lipoprotein particles which bind the enzyme, it is capable of considerable purification by procedures of a more or less standard type. The essential step in the purification of this enzyme is the complete removal of the phospholipid fraction with which the enzyme is so intimately associated. This can be achieved only by treatment of the milk, or milk product, with *n*- or isobutanol, the butanol being unique in this reaction (Morton, 1950; Zittle & Della Monica, 1952).

While some disruption of the particulate component can be achieved by prolonged treatment of rennin-whey with trypsin, difficulties are experienced in later stages of purification. One of the most pronounced changes of milk phosphatase following butanol treatment is in the behaviour on adsorption and elution. Before butanol treatment, the enzyme is fairly readily adsorbed, but a small portion only may be regained by elution. After removal of the lipoprotein complex, the enzyme can readily be quantitatively recovered by proper elution. Zittle & Della Monica (1951, 1952) have also drawn attention to this change in behaviour following butanol treatment. In using the valuable adsorption method for enzyme purification, it is essential that the enzyme to be purified is in true solution and free of any associated lipid. Where difficulty is experienced in using the adsorption method, the presence of contaminant phospholipid should be suspected.

Although animal charcoal has been widely used for adsorption of non-protein materials, it has not commonly been employed in enzyme purification. Dixon & Kodama (1926) used charcoal for purification of xanthine oxidase, while Abul-Fadl & King (1949) used it for decolorization of a preparation of alkaline phosphatase from dog faeces. In the purification of milk phosphatase animal charcoal, treated as described to remove inorganic phosphate, has been found to be superior to other adsorbents for selective removal of inert protein. It also removed traces of compounds absorbing at 260 m μ ., presumably nucleic acids or derivatives of them. It

may be found useful in the purification of enzymes other than alkaline phosphatase.

At stage 6 of the purification procedure (p. 797), the lower ammonium sulphate fraction contained a red protein, the identity of which has not been established. The colour was completely reduced on addition of sodium dithionite. The fraction showed some xanthine dehydrogenase activity, and the absorption spectrum was like that of xanthine dehydrogenase (see Morell, 1952). However, it is considered that the very strong red colour of this fraction could not arise from xanthine dehydrogenase alone, and it is probable that another red protein was present. It is noteworthy that Sørensen & Sørensen (1938-41) and Theorell & Åkeson (1943) have both described red proteins found in separated milk.

Although insufficient protein was recovered to enable electrophoretic examination of the final product, it had a specific activity (15314 units/mg. nitrogen) greater than that of the most active material separated by electrophoresis (specific activity, 12 000 units/mg. nitrogen). Moreover, the final product was found to be a specific phosphomonoesterase, free of any diesterase or pyrophosphatase activities (see Morton, 1953*b*). Hence it is considered that the purified enzyme is essentially pure.

Since this work was carried out and briefly reported (Morton, 1950), Zittle & Della Monica (1952) have obtained a 1000-fold purification of milk phosphatase by using butanol treatment of rennin- whey from separated milk, followed by careful acetone fractionation and adsorption of non-active protein on 'Filter-cel'. This relative purification is based on a comparison with separated milk, which has a specific activity about 0.6 times that of whole milk (Morton, 1953*a*). On this basis, the purification factor for the product obtained in this study would be about 9500 times. It is not surprising, therefore, that Zittle & Della Monica (1952) found their final product showed several components when examined electrophoretically, and that the preparation had diesterase activity.

Caputto & Marsal (1941) obtained a partial purification of the alkaline phosphatase of cow mammary gland using prolonged autolysis followed by ethanol fractionation, a procedure introduced by Albers & Albers (1935) for purification of kidney phosphatase. The final product obtained by Caputto & Marsal (1941) had a specific activity equivalent to 3850 units/mg. N (Q_p 26800). Their preparation may be directly compared with the milk phosphatase of specific activity 15300 units/mg. nitrogen (Q_p 106 000) obtained in the present study, since the milk phosphatase is derived from the mammary gland (Folley & Kay, 1936-7; Folley & White, 1936). Moreover, the milk and mammary gland alkaline phosphatases are identical (Morton, 1953*b*).

While milk itself has a very low specific activity (Morton, 1953*a*), it should be noted that buttermilk from high-fat cream is quite as active as some animal tissues (e.g. kidney and liver). Where available as an industrial by-product, raw-cream buttermilk therefore represents an inexpensive and convenient source from which purified alkaline phosphatase may be obtained with a yield of about 10% (see Table 3). From whole milk, of course, the yield is very much lower (1.5%, as in Table 3).

Although the preparation of milk alkaline phosphatase as described in this paper has not been repeated, there should be no great difficulty in reproducing these results. It is suggested that a useful modification would be to sediment the enzymically active lipoprotein particles from buttermilk by centrifuging at high speed (50 000 *g* or greater), wash the particles free of soluble proteins as in the preparation of intestinal phosphatase (see Morton, 1950) and then to apply the procedures herein described. Such a modification is possible since equipment for high-speed centrifuging of relatively large quantities is now available, whereas it was still lacking in the period 1949-50 when the work described above was completed.

SUMMARY

1. The alkaline phosphatase of milk was purified 5600 times compared to the initial whole milk on a protein nitrogen basis. The yield was 1.5%. The details of the method are described.

2. An essential step in the purification procedure depends on the unique ability of butanol to disrupt the lipoprotein complex with which the phosphatase is normally associated.

3. The final product had a specific activity of 15300 units/mg. nitrogen (Q_p 106 000), estimated from hydrolysis of sodium β -glycerophosphate at 37°. Comparison of the activity with a protein fraction separated electrophoretically indicated that the purified enzyme was probably homogeneous. Homogeneity was also indicated by the lack of diesterase or pyrophosphatase activity in the preparation.

4. A red protein fraction was separated from the buttermilk during the purification. The identity of this protein was not established.

I wish to thank Dr M. Dixon, F.R.S., for his encouraging interest and advice during this work, and particularly for carrying out the electrophoretic examinations of the milk proteins. I am grateful to the Manager of the Milk Marketing Board Creamery, Norwich, for his co-operation in supplying raw materials for this work. I also wish to record my thanks to Mr R. Brown for some technical assistance. This work was carried out while holding a Travelling Scholarship awarded by the Gowrie Scholarship Trust of Australia, whose assistance is gratefully acknowledged.

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Oxidative Pathways in a Fluorescent *Pseudomonas*

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During an investigation of the oxidative metabolism of a strain of a fluorescent *Pseudomonas* (KB 1) it was noted that when succinate was oxidized by washed suspensions there was a sharp break in the oxygen-uptake curve corresponding to approximately 1.5 moles oxygen/mole succinate added. Examination of the suspending fluid for possible end products showed the presence of keto acids which were tentatively identified as pyruvate and α -ketoglutarate by partition chromatography. The production of α -ketoglutarate by micro-organisms has been reported by Lockwood & Stodola (1946) using a strain of *Ps. fluorescens*; by Dagley, Fewster & Happold (1952) using *Vibrio*, strain 01; by Hockenhull, Wilkin & Winder (1951) using *Pencillium chrysogenum*; by Walker, Hapton & Hall (1951) using *Aspergillus niger*; and by Hunter (1952) using *Mycobacterium butyricum*. Lockwood & Stodola (1946) showed that when their strain of *Ps. fluorescens* was grown upon a medium containing glucose and with a high carbon/nitrogen ratio, with carefully controlled aeration, up to 18 moles α -ketoglutarate/100 moles glucose consumed accumulated in the growth medium. Dagley *et al.* found that this keto acid was produced when *Vibrio* 01 oxidized either benzoate, phenylacetate, citrate, acetate or succinate, though the amounts formed were not determined.

The formation of α -ketoglutarate from succinate by *Ps. fluorescens* suggested that some, at least, of the reactions of the tricarboxylic acid cycle played a part in the oxidative metabolism of this organism.

A detailed study was therefore made of the oxidation of succinate and other tricarboxylic-acid-cycle intermediates by this organism using whole cells and cell-free extracts. Since the oxidation of some of the substrates appeared to be adaptive, it was also possible to examine the application of the simultaneous adaptation technique (Stanier, 1947) to problems of this type. A preliminary account of this work has already been given (Kogut, 1952a, b).

MATERIALS AND METHODS

Organism. The organism was isolated from soil and had the following properties: Gram-negative motile rods $1.0-2.0 \mu \times 0.4-1.0 \mu$; with a single polar flagellum; a green, fluorescent pigment was produced which diffused into the growth medium; opt. temp. 30° , growth occurred at 37° ; gelatin not liquefied; nitrites not produced from nitrates; catalase positive; indole not produced; litmus milk, no change. These properties are compatible with either *Ps. conveza* or *Ps. ovalis*. These two species are differentiated from one another by Bergey (1948) according to their temperature range. Thus *Ps. ovalis* has an optimum temperature of 25° with poor or no growth at 37° , whereas *Ps. conveza* is said to grow readily at 37° and to have an optimum temperature of 30° . For convenience we propose to use the term *Ps. fluorescens* species group.

Cultures were maintained in the dry state (Stamp, 1947), and when in use, were subcultured monthly on 10% (v/v) yeast autolysate (Barker & Beck, 1942) agar slopes. Unless otherwise stated, the organisms were grown on the following medium: K_2HPO_4 , 0.1%; NH_4Cl , 0.1%; $Mg_2SO_4 \cdot 7H_2O$, 0.05%; all (w/v), in distilled water and supplemented with the following trace elements: 100 $\mu g./l.$ Fe^{2+} as $FeSO_4$, - $(NH_4)_2SO_4$, 6 H_2O ; 200 $\mu g./l.$ B as H_3BO_3 ; 100 $\mu g./l.$ Co^{2+} as