5. The causes for the observed results are discussed.

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The Phospholipids of Pigeon and Ox Skeletal Muscle

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As a preliminary to the ultimate object of determining the phospholipid composition of the sub-cellular particles of skeletal muscle, an investigation was undertaken of the total phospholipid of pigeon breast muscle. The phospholipids of skeletal muscle have not been studied to the same extent as the phospholipids of tissues such as liver and brain. Two recent papers report the detailed phospholipid composition of sheep skeletal muscle (Dawson, 1960) and of rabbit, pigeon and trout skeletal muscle (Gray & Macfarlane, 1961). In these investigations modern methods of phospholipid analysis were used. The results of the present investigation, which also includes a determination of the phospholipids of ox longissimus dorsi muscle, are in sub-

stantial agreement with the results of these two papers, except that the major ninhydrin-positive component in both pigeon breast muscle and ox longissimus dorsi muscle is not phosphatidylethanolamine, as was assumed, but another compound. The pigeon-breast-muscle lipid extracts contained considerable amounts of proteolipid which dissociated on silicic acid. Evidence is also presented for the possible occurrence of phenolic components in the polyglycerolphospholipid fraction.

EXPERIMENTAL

Extraction of lipid. Sample A. Three pigeons were beheaded and the breast muscles blended with chloroform-methanol (2:1, v/v) by using 20 ml. of solvent/g. of muscle. The blend was boiled for 10 min. and then kept at room temperature overnight. It was filtered through sintered glass, 0.9% sodium chloride (1 ml./5 ml. of filtrate) was

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added and the mixture was shaken thoroughly. After the two phases had separated, the upper aqueous-methanolic phase was rejected, and the lower chloroform phase was filtered through three thicknesses of filter paper and evaporated, and the residue weighed. It was redissolved in chloroform-methanol and stored in solid carbon dioxide. The wet wt. of muscle was 170 g.; the lipid content was 5.91%, and 41% of this was phospholipid of N:P ratio 1.49.

Sample B. Breast muscle from another pigeon was extracted as above except that the whole extraction was carried out at 0°. The wet wt. of muscle was 79 g.; the lipid content was 5.17%, and 28% of this was phospholipid of N:P ratio 1.77.

Sample C. Ox longissimus dorsi muscle was extracted as described for sample B. The wet wt. of muscle was 200 g.; the lipid content was 1.6%, and 24% of this was phospholipid of N:P ratio 1.14.

Silicic acid chromatography. A preliminary separation of the lipid was made on silicic acid (Mallinckrodt silicic acid, from which the fines had been removed by washing with methanol and which had been reactivated at 110°). The procedure was essentially that of Hirsch & Ahrens (1958). Three fractions were collected by elution successively with 10% (v/v) diethyl ether in light petroleum (b.p. 60–80°), diethyl ether and methanol. The three fractions were evaporated to constant weight in a rotary vacuum evaporator at 37°.

Resolution of the phospholipid fraction (methanol eluate) was achieved by chromatography on the silicic acid, by using stepwise elution with increasing concentrations of methanol in chloroform.

All solvents were distilled before use. The methanol was distilled from oxalic acid to remove traces of basic nitrogenous contaminants.

Hydrolytic determination of the phospholipids. The individual phospholipids in the total mixtures and in the silicic acid fractions were determined by successive chemical hydrolyses (Dawson, 1960), and in one case by a later modification of the method (Dawson, Hemington & Davenport, 1962).

Phosphorus was determined after perchloric acid digestion, by the method of Allen (1940). Nitrogen and phosphorus were both determined on a Kjeldahl digest (Davenport & Dawson, 1962). Egg-yolk phospholipid, treated by passage through a column of cellulose powder (Lea & Rhodes, 1953), and carefully analysed for phosphorus by the Allen (1940) method and for nitrogen by Kjeldahl digestion and titration, was used as a standard for this determination (N:P ratio 1.12). The percentage content of phospholipid was obtained by multiplying the percentage content of lipid phosphorus by 25. For analysis for proteolipid the protein analysis of Daughaday, Lowry, Rosebrough & Fields (1952) was adapted to lipid samples. A suitable amount of a lipid solution was evaporated to dryness in a 10 ml. graduated stoppered test tube. Alkaline copper reagent (10 ml.) was added and, after the mixture had been thoroughly shaken and left for 15 min., 1.0 ml. of the Folin-Ciocalteu reagent was added rapidly, the tube being immediately shaken. After maximum colour had developed, 2.0 ml. of water-saturated chloroform was added, and the mixture was shaken thoroughly and then centrifuged. The chloroform extraction technique was adequate to give a clear coloured supernatant layer. It had

no effect on the colour derived from a bovine serum albumin standard. The reagents gave no colour with egg-yolk phospholipid. Thus, although conditions for complete reaction of the Lowry reagents with the protein moiety of proteolipids were not studied, the results are believed to indicate the approximate proportion of lipid nitrogen present as polypeptide (Chou & Goldstein, 1960). Ester groups were determined by the method of Rapport & Alonzo (1955).

Ion-exchange chromatography of the phosphate esters. Samples of lipid containing about 5 mg. of phosphorus were subjected to the mild alkaline hydrolysis procedure of Dawson (1960), but with ten times the quantities of reagents. The resulting aqueous solution of water-soluble phosphate compounds was passed through a column of Amberlite IRC-50 (H⁺ form) to remove cations; the phosphorus compounds retained on the column were then eluted with 80% (v/v) ethanol. The combined eluates were evaporated to dryness, dissolved in 0.1 N-formic acid and poured on to a column (1 cm. × 15 cm.) of Dowex 2 (Cl⁻ form) that had first been washed with 200 ml. of 0.1 N-formic acid. The column was developed with a convex gradient of increasing concentration of 0.05 M-sodium formate buffer, pH 4.0, in 0.1 N-formic acid. This was formed by two vessels in series. The first contained initially 300 ml. of the buffer, which fed by gravity into the second vessel containing initially 300 ml. of 0.1 N-formic acid. The second vessel was stirred magnetically, and fed directly into the column.

RESULTS

The preliminary separation of the three samples of muscle lipid on silicic acid is summarized in Table 1. Both samples of pigeon breast muscle had high N:P ratios on first extraction. Only about 50% of the nitrogen was recovered after passage through silicic acid, although the phosphorus recovery was high in both cases. Thus the N:P ratio of the methanol eluate was much less than that of the original lipid. Also, there was a weight loss: this was 13% for sample A and 9% for sample B. The N:P ratio of the methanol eluate of the ox-muscle phospholipid was, however, much the same as the original N:P ratio, and the weight recovery was 100%.

Folch & Lees (1951) first reported the occurrence of proteolipids in many tissues, including skeletal muscle. It seemed likely, therefore, that the loss of nitrogen on silicic acid chromatography was due to dissociation of proteolipids on the adsorbent, with subsequent elution of the lipid and retention of the protein. Hence the modified Lowry determination was applied to the original lipid extract and to the methanol eluate of sample B. The weight of lipid taken for chromatography was 586 mg. and there was a loss of 2.45 mg. of nitrogen. If we assume that the proteolipid protein contains 12% of nitrogen (Folch & Lees, 1951) then this would represent 20.4 mg. of protein. The Lowry determination gave 28 mg. of protein in the original

sample and 15 mg. in the methanol eluate. This loss of 13 mg. of protein thus accounts, in part at least, for the loss of nitrogen recorded. The Lowry determination was used in preference to less equivocal methods of protein determination (e.g. biuret) because of its greater sensitivity. Other non-peptide nitrogenous contaminants not removed by washing with 0.9% sodium chloride may also be

retained on silicic acid. Also, this loss of protein accounts for only part of the weight loss on silicic acid chromatography. Folch & Lees (1951) reported 0.4 mg. of proteolipid protein/g. of skeletal muscle. The corresponding value in the pigeon breast muscle is 1.1–1.8. However, a good deal of species variation is indicated since the lipid extracted from ox longissimus dorsi muscle at 0° contained almost no proteolipid.

Table 2 gives the results of the hydrolytic determination of the individual phospholipids in samples A, B and C, and in sample A after passage through silicic acid. The lower plasmalogen content of A after passage through silicic acid is consonant with decomposition of the plasmalogens on the acidic adsorbent, and a consequent increase in material determined as phosphatidylcholine and phosphatidylethanolamine in the Dawson procedure.

The substance listed in Table 2 as 'unknown component' yielded, on mild alkaline hydrolysis, a compound that had the same R_f in phenol–acetic acid–ethanol–water as glycerylphosphorylethanolamine, but a slightly lower R_f in methanol–formic acid–water. It was ninhydrin-positive and gave a phosphate colour with a Hanes & Isherwood (1949) spray. In an attempt to identify this lipid further, sample B was subjected to silicic acid chromatography, the results of which are presented in Fig. 1 and Table 3. The first peak (fractions 18–22), eluted with 10% (v/v) methanol in chloroform, was principally polyglycerolphospholipid. Fractions 18–22 were combined and evaporated to dryness, leaving a greenish-brown solid. This was separated into an ethanol-soluble fraction and an ethanol-insoluble fraction. The latter was a white solid and the former a greenish-brown oil. On moving through the column the band corresponding to fractions 18–22 darkened

Table 1. *Properties of the muscle-lipid fractions and their behaviour on silicic acid*

Experimental details are given in the text.

Extraction conditions ...	Sample A (pigeon breast) Boiling chloroform–methanol	Sample B (pigeon breast) Chloroform–methanol at 0°	Sample C (ox longissimus dorsi) Chloroform–methanol at 0°
Lipid (% of wet wt.)	5.91	5.17	1.6
Phospholipid (% of total lipid)	41	28	24
N:P ratio	1.49	1.77	1.14
Wt. eluted by 10% diethyl ether in light petroleum (%)	34	53	77
Wt. eluted by diethyl ether (%)	4	2	1
Wt. eluted by methanol (%)	48	36	22
Recovery of phosphorus from silicic acid (%)	93	97	—
Recovery of nitrogen from silicic acid (%)	51	53	—
N:P ratio of methanol eluate	0.82	0.99	1.08

Table 2. *Composition of phospholipids of the various samples as determined by the hydrolytic procedure*

Samples A and B were analysed by the procedure of Dawson (1960) and sample C by a later modification of the method (Dawson *et al.* 1962). The water-soluble phosphate esters resulting from the mild alkaline hydrolysis were separated by two-dimensional paper chromatography. Experimental details are given in the text.

Extraction conditions ...	Percentage composition			
	Sample A (pigeon breast) Boiling chloroform–methanol	Sample A (methanol eluate)	Sample B (pigeon breast) Chloroform–methanol at 0°	Sample C (ox longissimus dorsi) Chloroform–methanol at 0°
Phosphatidylcholine	24.0	28.6	32.2	35.2
Phosphatidylethanolamine	2.3	4.6	3.4	5.4
Unknown component	22.8	21.6	24.1	19.9
Phosphatidylserine	1.1	1.4	1.2	—
Phosphatidylinositol	4.5	4.5	4.8	2.7
Polyglycerolphospholipid	7.4	8.1	6.7	5.8
Choline plasmalogen	11.6	7.9	7.5	13.6
Ethanolamine plasmalogen	9.7	4.3	4.1	13.3
Sphingomyelin	1.6	1.2	1.2	3.0
Recovery (%)	85.0	82.2	85.2	98.9

appreciably, and it gave a strong colour with the Lowry reagent. This was obviously not due to peptide because the nitrogen content of the fraction was too low to account for the intensity of the colour. However, the strong Lowry colour could be due to phenolic substances in this fraction reacting with the Folin-Ciocalteu reagent. Siegel (1960) has reported the oxidation of *o*-diphenols by silica gel to brown catechol polymers. The presence of phenolic substances in the phospholipid fraction could also explain the Lowry reaction of the phospholipids which was observed after the preliminary separation on silicic acid (see above).

Fractions 23-40 were subjected to mild alkaline hydrolysis and the water-soluble products were examined by ion-exchange chromatography (Fig. 2). The major part was not held on the column and, in contrast with glycerylphosphorylethanolamine, behaved very similarly to glycerylphosphoryl-

choline from egg-yolk phospholipids. Further evidence that it was not glycerylphosphorylethanolamine was obtained by paper chromatography of the material separated by ion exchange. It had R_F 0.68, very similar to that of glycerylphosphorylethanolamine (R_F 0.71), in the methanol-formic acid-water solvent system of Dawson (1960) when they were chromatographed separately. When, however, a mixture of the two was chromatographed in the same solvent system, two distinct spots were obtained: the glycerylphosphorylethanolamine (R_F 0.68) and the unknown component (R_F 0.52). Further, the unknown component gave a mauve colour with a ninhydrin-acetone spray whereas the glycerylphosphorylethanolamine gave a pink colour. Different amounts of the two substances were co-chromatographed and the separation was confirmed by phosphorus analysis of the spots. The two substances did not separate with the phenol-acetic acid-ethanol-water solvent system, nor on paper electrophoresis at pH 3.2 (Dawson, 1960). Other small ninhydrin-positive, but phosphorus-negative, spots appeared on the papers and probably account for the N:P ratios greater than 1 in the Dowex 2 fractions (Fig. 2).

To ascertain whether the unknown phospholipid was characteristic of avian species or whether it occurred in the muscle of a non-avian species, a sample of ox-longissimus-dorsi-muscle lipid was prepared as described in the Experimental section. The results of the hydrolytic determination of the whole lipid sample are listed in Table 2. The recovery in this case was almost 100%, as the modified hydrolytic procedure of Dawson *et al.* (1962) had been used. A sample of the mild alkaline hydrolysate was then mixed with a sample of a mild alkaline hydrolysate of egg-yolk lipid and subjected to two-dimensional paper chromatography with the solvent systems of Dawson (1960). The same procedure was also carried out on the muscle-phospholipid hydrolysate alone. Both the glycerylphosphorylcholine and glycerylphosphorylethanol-

Fig. 1. The separation of the pigeon-breast-muscle phospholipids (sample B: 2.77 g. of lipid; 32.25 mg. of phosphorus) on 100 g. of silicic acid. The % (v/v) of methanol in chloroform used for elution is given at the top of the Figure. The phosphorus was determined after digestion with perchloric acid. The volume of each fraction was 10 ml. The neutral lipids were eluted first with 200 ml. of chloroform. The amounts (μ equiv.) of ester/ μ g. atom of phosphorus for various fractions were: fraction 28, 2.04; fraction 30, 2.16; fraction 34, 2.04; fraction 83, 1.92; fraction 86, 1.89; fraction 90, 1.93; fraction 94, 2.11.

Table 3. Characteristics of the lipid fractions separated by silicic acid chromatography (Fig. 1)

Experimental details are given in the text.

Fraction nos.	Percentage of total lipid P applied to column	N:P ratio	Products of mild alkaline hydrolysis (% of lipid P in fraction)
18-22 (ethanol-soluble)	2.5	0.4	Polyglycerophosphate (60) Unknown component (20)
18-22 (ethanol-insoluble)	3.0	< 0.05	Polyglycerophosphate (80) Unknown component (13)
23-40	33.6	1.58	Glycerylphosphorylethanolamine (5) Glycerylphosphorylserine (9) Unknown component (68)
41-75	10.8	1.2	—
76-100	42.5	1.15	Glycerylphosphorylcholine (67)

amine spots were increased by amounts of phosphorus equivalent to the amounts added by the hydrolysate of egg-yolk phospholipid above that of the muscle-lipid hydrolysate alone. The unknown ninhydrin-positive spot, which migrated to the same position relative to glycerylphosphorylethanolamine as did the unknown component from the hydrolysate of pigeon-breast-muscle phospholipid, contained the same amount of phosphorus with and without the hydrolysate of egg-yolk phospholipid. Thus a ninhydrin-positive phosphorus-containing substance distinct from glycerylphosphorylethanolamine was also present in the hydrolysate of ox-muscle lipid. No direct comparison was made of the hydrolysis products from ox-muscle and pigeon-breast-muscle phospholipids. Also, no attempt was made to determine whether the ninhydrin-positive component released

on mild acid hydrolysis was definitely glycerylphosphorylethanolamine, and in Table 2 the parent lipid is listed as ethanolamine plasmalogen.

DISCUSSION

From the outset in this work, when it was realized that proteolipids were dissociating on silicic acid and two ninhydrin-positive spots were appearing on two-dimensional paper chromatograms in the region of glycerylphosphorylethanolamine, it was deemed essential to show that the unknown ninhydrin-positive spot was a single compound containing both nitrogen and phosphorus. The possibility existed that the second spot was a complex of a phosphorus-containing hydrolysis product of one of the other phospholipids and a water-soluble ninhydrin-positive contaminant possibly arising from the dissociation of the proteolipids. However, the nitrogen (ninhydrin reaction) and the phosphorus stayed together during the successive procedures of silicic acid chromatography of the intact lipid, partition after mild alkaline hydrolysis, chromatography on an ion-exchange resin and paper chromatography with a methanol-formic acid-water solvent system. It is therefore very likely that this is a single compound. It has also been clearly distinguished from glycerylphosphorylethanolamine, both by ion-exchange chromatography and by paper chromatography. The final proof that it is indeed the hydrolysis product of a new phospholipid must await the isolation of the intact parent substance in a pure form and its complete characterization by the classical methods of organic chemistry.

The ester values of some of the ninhydrin-positive fractions from silicic acid chromatography (Fig. 1) are consistent with the unknown phospholipid having a glycerophosphatide structure, although no analyses for glycerol were carried out. Also, the N:P values of the Dowex 2 fractions (Fig. 2) suggest that the hydrolysis product contains equimolar amounts of nitrogen and phosphorus.

There have been a number of reports of the occurrence of amino substances, other than ethanolamine and serine, released on hydrolysis of naturally occurring phospholipids. Barbier & Lederer (1952) reported the release of hydroxylysine on acid hydrolysis of the phospholipid fraction of *Mycobacterium phlei*. Also, hydroxylysine phosphate has been detected in minced-muscle dialysates (Gordon, 1949). A threonine-containing phosphatide is present in the muscle of the tunny fish (Igarashi, Zama & Katada, 1958). Kittredge, Roberts & Simonsen (1962) identified 2-aminoethylphosphonic acid in the alkaline hydrolysate of the phospholipids of a sea anemone. The N:P ratio of the un-

Fig. 2. Chromatography of the water-soluble product of mild alkaline hydrolysis of fractions 23-40 (Fig. 1) on Dowex 2 with a gradient of formic acid-sodium formate. The right-hand ordinate gives the pH of the eluent. The volume of each fraction was 10 ml. ○---○, μg. atoms of nitrogen; ●—●, μg. atoms of phosphorus; ·····, pH of eluent. A sample of the water-soluble products resulting from the mild alkaline hydrolysis of egg-yolk phospholipids was chromatographed under exactly the same conditions. All the egg-yolk glycerylphosphorylcholine emerged from the column in fractions 1 and 2, and the egg-yolk glycerylphosphorylethanolamine was retained until fractions 22-28, the pH of the eluent being 2.5-2.6.

known hydrolysis product reported in the present paper suggests that it is not a simple phosphate ester of hydrolylysine or indeed a peptide monophosphate. Neither 2-aminoethylphosphonate nor its derivatives would be likely to react with the acid molybdate spray of Hanes & Isherwood (1949) as does this present substance. Also, a phosphate ester of threonine should be adsorbed even more strongly on Dowex 2 than glycerylphosphorylethanolamine, which suggests that the unknown component is neither glycerylphosphorylthreonine nor phosphorylthreonine.

The detailed phospholipid composition of the two muscles reported in the present paper are in good agreement with the composition of pigeon and rabbit skeletal muscle reported by Gray & Macfarlane (1961), and of sheep skeletal muscle reported by Dawson (1960). The muscle phospholipids are characterized by a high content of polyglycerophospholipid (though not as high as that of heart muscle) and by a high content of plasmalogens in comparison with other tissues.

Gray & Macfarlane (1961) used silicic acid chromatography and paper-chromatographic separation of the phosphate esters liberated by mild alkaline hydrolysis (Dawson, 1954) for the identification of the individual phospholipids. It is now clear that this will not necessarily distinguish phosphatidylethanolamine from the unknown phospholipid, and it is very likely that the values for the percentage content of phosphatidylethanolamine quoted by these authors refer principally to the new phospholipid. R. M. C. Dawson (personal communication) in his examination of the sheep-muscle lipids used a combination of paper chromatography in phenol-acetic acid-ethanol-water in one direction and paper electrophoresis at pH 3.2 in the other direction to separate the water-soluble phosphate esters. The results in the present paper show that this again would not distinguish the two substances.

The methods currently in use for the determination of phospholipids in complex mixtures (for a review see Marinetti, 1962) rely on column and paper chromatography of the intact lipids or paper chromatography of their hydrolytic products. The phospholipids are fairly large molecules among which there may be only slight differences in physical properties. Hence their separation by chromatographic methods depending on one or other of these physical properties may be difficult. By working with the much smaller hydrolytic products, as in the Dawson procedure, slight differences between the parent phospholipids may be emphasized.

Thus, although the unknown phospholipid did not separate from phosphatidylethanolamine on silicic acid, the product of mild alkaline hydrolysis did separate from glycerylphosphorylethanolamine

provided that two-dimensional paper chromatography was used. Finally, resort to another method of separation, and direct comparison with glycerylphosphorylethanolamine derived from egg-yolk phosphatidylethanolamine, was required to establish the difference with any degree of certainty. Frequently the assumption is made that ninhydrin-positive phospholipid is phosphatidylethanolamine, with only chromatographic behaviour to support the identification.

SUMMARY

1. The phospholipids of pigeon breast muscle and ox longissimus dorsi muscle have been investigated.

2. Considerable amounts of proteolipid were present in the lipids extracted from pigeon breast muscle by chloroform-methanol both at the boiling point of the solvent and at 0°. The lipids extracted from ox longissimus dorsi muscle by chloroform-methanol at 0° contained very little proteolipid.

3. The major ninhydrin-positive component of the phospholipids of both muscles, which was eluted from silicic acid by a concentration of methanol in chloroform similar to that which elutes phosphatidylethanolamine, was different from this compound. Mild alkaline hydrolysis liberated from it a water-soluble compound containing phosphorus and reacting with ninhydrin, which behaved differently from glycerylphosphorylethanolamine on an ion-exchange column and separated from glycerylphosphorylethanolamine on paper chromatography in one solvent system.

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Effects of Iron Deficiency and of Zinc Deficiency on the Activities of some Enzymes in *Mycobacterium smegmatis*

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When the growth medium of *Mycobacterium smegmatis* is depleted of iron or zinc, limitation of growth of the culture is finally brought about by exhaustion of the supply of the depleted metal. When this occurs certain characteristic changes occur in the composition of the bacteria that give some indications of the synthetic pathways which are affected earliest by deficiencies of these metals (Winder & O'Hara, 1962; Ratledge & Winder, 1962). Further information on the effects of these deficiencies has been sought by following the activities of some enzymes in these depleted cultures and these results are reported in the present paper. A preliminary report on some of this work has been published (Winder, O'Hara & Ratledge, 1961).

EXPERIMENTAL

Organism. *Mycobacterium smegmatis* is a strain of unknown origin used for previous studies (Winder & O'Hara, 1962).

Growth and harvesting. The preparation of metal-depleted medium, treatment of glassware and growth of the bacteria were described by Winder & O'Hara (1962) and Ratledge & Winder (1962). Metals were added to the depleted medium to give the following concentrations (per ml.): in normal medium, 2.16 μg . of Fe^{3+} and 0.42 μg . of Zn^{2+} ions; in iron-deficient medium, 0.27 μg . of Fe^{3+} and 0.42 μg . of Zn^{2+} ions; in zinc-deficient medium, 2.16 μg . of Fe^{3+} and 0.035 μg . of Zn^{2+} ions. Surface cultures only were used. After 2, 3 and 4 days one or more flasks of each type of culture were taken, the cells were collected in a Buchner funnel on

Whatman no. 1 paper and washed with water under gentle suction.

Preparation of extracts. The cells were transferred to Mickle disintegrator tubes (about 50–100 mg. of cell protein/tube) and about 5 ml. of the buffer appropriate to the enzyme determination (usually 0.05 M-potassium phosphate, pH 7.5) was added together with a volume of Ballotini beads about equal to the volume of the cells. The tubes were placed in a refrigerator at -10° until just frozen and were then shaken in a Mickle disintegrator at maximum amplitude for three 10 min. periods, refreezing at -10° between periods. The extracts were centrifuged at 2000g for 15 min. at 4° and the supernatant was removed for assay of enzyme activity and for protein determination by the biuret method (Layne, 1957). Preliminary studies were carried out to determine for each enzyme the optimum disintegration time. The above procedure led to extraction of all the enzymes studied except aconitate hydratase, which was inactivated to an undesirable extent. Aconitate hydratase was extracted by grinding the cells with an equal volume of Ballotini beads in a chilled mortar for about 5 min. with gradual addition of chilled buffer. The extracts were centrifuged as before and the supernatants were used as enzyme. This method gave minimal inactivation of the enzyme.

The enzymes were assayed also in the residues after centrifuging but in all the enzymes the activity was much greater in the supernatant and it is this latter value which is given throughout this paper. All enzyme assays except that of the aminotransferase were done at $18-20^\circ$.

Assay procedures

Glycerol dehydrogenase (glycerol-NAD oxidoreductase, EC 1.1.1.6). This enzyme was determined according to the method of Burton (1955). The reaction mixture contained, in a final volume 3.0 ml.: 500 μmoles of glycerol, 0.5 μmole of NAD, 100 μmoles of sodium pyrophosphate buffer

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