- Kogut, M., Pollock, M. R. & Tridgell, E. J. (1956). *Biochem. J.* **62**, 391.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
- Manson, E. E. D., Pollock, M. R. & Tridgell, E. J. (1954). *J. gen. Microbiol.*, **11**, 493.
- Miller, G., Bach, G. & Markus, Z. (1965). *Biotech. Bioengng.* **7**, 517.
- Pollock, M. R. (1952). *Brit. J. exp. Path.* **33**, 587.
- Pollock, M. R. & Torriani, A. M. (1953). *C.R. Acad. Sci., Paris*, **237**, 276.
- Ron-Zenziper, E. & Citri, N. (1963). *Nature, Lond.*, **198**, 887.
- Sabath, L. D. & Abraham, E. P. (1965). *Antimicrobial Agents and Chemotherapy*, p. 392.
- Sabath, L. D. & Abraham, E. P. (1966). *Biochem. J.* **98**, 11c.
- Sabath, L. D., Jago, M. & Abraham, E. P. (1965). *Biochem. J.* **96**, 739.

Biochem. J. (1967) **103**, 30c

Acid Phospholipase A in Lysosomes of the Bovine Adrenal Medulla

By H. BLASCHKO, A. D. SMITH and H. WINKLER

Department of Pharmacology, University of Oxford

H. VAN DEN BOSCH and L. L. M. VAN DEENEN

Department of Biochemistry, Organic Chemistry Laboratory, State University, Utrecht, The Netherlands

(Received 21 March 1967)

In recent studies of the phospholipid content of different particulate fractions from the bovine adrenal medulla it was found that the chromaffin granules, i.e. the particles that contain the hormones, were characterized by a high content of lysolecithin (Blaschko, Firemark, Smith & Winkler, 1966, 1967; Winkler, Strieder & Ziegler, 1966). These observations have led us to investigate the presence and distribution of particulate phospholipase A activity in the bovine adrenal medulla. The large-granule fraction obtained from homogenates of bovine adrenal medulla contains three different types of cell particle, i.e. mitochondria, lysosomes and chromaffin granules. These three types of cell particle can be separated by sucrose-density-gradient centrifugation. The present communication deals with the first outcome of this study which leads us to conclude that lysosomes contain acid-phospholipase A activity.

Methods. Homogenates of bovine adrenal medulla were fractionated as described by Smith & Winkler (1966). By this method a large-granule fraction is sedimented at $2.42 \times 10^5 g\text{-min.}$; this is further resolved into its constituent particles by centrifugation over a sucrose density gradient. The gradient tubes contained 0.5 ml. each of 1.3M-, 1.4M-, 1.5M-, 1.6M-, 1.7M-, 1.8M-, 1.9M- and 2.0M-sucrose, and 0.25 ml. of 2.5M-sucrose. The tubes were centrifuged at $87.4 \times 10^5 g\text{-min.}$ in the Spinco swing-out rotor SW39L and seven fractions were collected. Each fraction was dialysed against 25 mM-glycylglycine-NaOH buffer, pH 6.5. The

dialysed fractions were analysed for fumarase activity (Racker, 1950), acid-deoxyribonuclease activity (Gianetto & de Duve, 1955) and catecholamines (Euler & Hamberg, 1949).

Phospholipase activity was determined by incubating 22 $\mu g.$ of [^{32}P]lecithin or of [^{32}P]phosphatidylethanolamine (prepared as described by van den Bosch & van Deenen, 1965) with up to 0.3 ml. of each fraction in sodium acetate buffer, pH 4.16 (37°) and I 0-0125 (final volume 1.0 ml.). The phospholipids were dispersed in water by brief ultrasonic treatment. After incubation for 1 hr. at 37° the reaction was stopped by the addition of 4 vol. of a mixture of chloroform (1 vol.) and methanol (2 vol.). The solvents were removed by evaporation *in vacuo*, and methanol was added to the residue. The [^{32}P]phospholipids and their breakdown products were separated by thin-layer chromatography according to the method of Skipski, Peterson & Barclay (1964). Phospholipids extracted from rat liver were co-chromatographed with each sample to act as markers. The radioactivity of the spots corresponding to phosphatidylethanolamine, lecithin, their respective lyso compounds, and glycerophosphorylethanolamine and glycerophosphorylcholine were measured with a scintillation counter. It was established, for each fraction, that the breakdown of substrate was linear both with time of incubation and with enzyme concentration under the conditions of assay.

Results and discussion. The pH optimum of the acid phospholipase in the large-granule fraction was

investigated with acetate buffers of pH 3.0–5.5, and was found to be between pH 4.0 and 4.2. More than 80% of the breakdown products formed from lecithin or from phosphatidylethanolamine were

the corresponding lyso derivatives: this indicates that the enzyme is of the phospholipase A type.

The results of the analyses of fractions from the density gradient are shown in Fig. 1. The distribution of fumarase, acid deoxyribonuclease and catecholamines is in agreement with what is known about the localization of these constituents in the different particulate fractions (Smith & Winkler, 1966). Fumarase, a marker for mitochondria, was recovered at the top of the gradient; the catecholamines, typical constituents of the chromaffin granules, were mainly present in the lower part of the gradient; and the maximum of acid-deoxyribonuclease activity, a lysosomal marker, was in an intermediate position in the gradient.

The distribution of phospholipase A in the gradient was similar to that of acid deoxyribonuclease. The region rich in the catecholamines was poor in the activity of these enzymes and most of the enzyme activity was recovered in a layer (fraction 4) intermediate between that of the mitochondria and that of the chromaffin granules.

These results show that chromaffin tissue contains an acid phospholipase A. Phospholipases with a similar pH optimum have been described in rat brain (Gatt, Barenholz & Roitman, 1966) and in rabbit alveolar macrophages (Elsbach, 1966). The present work shows that, at least in the bovine adrenal medulla, the acid phospholipase is a constituent of the lysosomal fraction. The presence of phospholipase activity in lysosomes has not hitherto been established. However, de Duve & Wattiaux (1966) have predicted that this type of cell particle may contain phospholipase activity, and studies by D. Thinès & G. L. Scherphof (personal communication) appear to indicate the presence of phospholipase in highly purified lysosomes from rat liver. The demonstration of such enzymic activity in lysosomes extends our knowledge of the range of substrates that these particles can digest. Further, it is possible to speculate that the action of phospholipase A may be related to changes in the properties of the lysosomal membrane that could be involved in the phenomenon of membrane fusion (see de Duve & Wattiaux, 1966).

Preliminary evidence indicates that the large-granule fraction from bovine adrenal medulla contains, in addition to the acid phospholipase, a phospholipase with a pH optimum close to 6.5; this enzyme is probably present in chromaffin granules (A. D. Smith & H. Winkler, unpublished work). This latter finding may account for the presence of lysolecithin in chromaffin granules.

A. D. S. is a Royal Society Stothert Research Fellow and is grateful to the Royal Society for a travel grant. H. W. holds a British Council Scholarship. This work has been supported by a grant from the Medical Research Council.

Fig. 1. Analysis of fractions from the sucrose density gradient. The columns in each histogram from the left to the right correspond to fractions from the top to the bottom of the density-gradient tube. The abscissa is divided according to the volumes of the fractions; the total volume in each tube was 4.75 ml. The ordinates are arbitrary units/ml. and the actual values of each arbitrary unit are shown in parentheses below. (a) Fumarase (5.0 μ moles/hr./ml.); (b) acid deoxyribonuclease (1.2 μ moles/hr./ml.); (c) acid phospholipase A (0.07 μ mole of lecithin/hr./ml.); (d) catecholamines (0.82 μ mole/ml.). The recoveries of the enzymic activities and catecholamines were between 72% and 85% of that applied to the sucrose gradient.

- Blaschko, H., Firemark, H., Smith, A. D. & Winkler, H. (1966). *Biochem. J.* **98**, 24p.
- Blaschko, H., Firemark, H., Smith, A. D. & Winkler, H. (1967). *Biochem. J.* (in the Press).
- de Duve, C. & Wattiaux, R. (1966). *A. Rev. Physiol.* **28**, 461.
- Elsbach, P. (1966). *Biochim. biophys. Acta*, **125**, 510.
- Euler, U. S. von & Hamberg, U. (1949). *Acta physiol. scand.* **19**, 74.
- Gatt, S., Barenholz, Y. & Roitman, A. (1966). *Biochem. biophys. Res. Commun.* **29**, 169.
- Gianetto, R. & de Duve, C. (1955). *Biochem. J.* **59**, 433.
- Racker, E. (1950). *Biochim. biophys. Acta*, **4**, 211.
- Skipiski, V. P., Peterson, R. F. & Barclay, M. (1964). *Biochem. J.* **90**, 374.
- Smith, A. D. & Winkler, H. (1966). *J. Physiol.* **183**, 179.
- van den Bosch, H. & van Deenen, L. L. M. (1965). *Biochim. biophys. Acta*, **106**, 326.
- Winkler, H., Strieder, N. & Ziegler, E. (1966). *Arch. exp. Path. Pharmacol.* **255**, 94.

Biochem. J. (1967) **103**, 32c

Stimulation of the Phosphoroclastic System of *Desulfovibrio* by Nucleotide Triphosphates

By M. G. YATES

Agricultural Research Council Unit of Nitrogen Fixation, University of Sussex, Brighton, Sussex

(Received 22 March 1967)

Dilworth, Subramanian, Munson & Burris (1965) reported stimulation of the pyruvic phosphoroclastic system of *Clostridium pasteurianum* by ATP, which they proposed might be due to an allosteric or cofactor-binding action. While studying cell-free preparations of normal and nitrogen-fixing strains of *Desulfovibrio* a comparable stimulation of the phosphoroclastic reaction was encountered as is reported below.

Methods. *Desulfovibrio vulgaris* strain Hildenborough (N.C.I.B. 8303) and *Desulfovibrio desulfuricans* Berre S (N.C.I.B. 8388) were grown with fixed nitrogen in batch culture in medium C (Postgate, 1966); nitrogen-fixing Berre S was grown on a modification of that medium in semi-continuous culture. Extracts were prepared by freezing in liquid nitrogen drops of a 1:1 (v/v) suspension of fresh cells in 0.85% NaCl. Phosphoroclastic activity was measured manometrically as described under Table 1. The cofactors added were those needed by the phosphoroclastic system of *Desulfovibrio* (Millet, 1955) plus α -lipoic acid, although this latter was not shown to affect the reaction rate, and acetyl phosphate, which sometimes stimulated the initial reaction rate with these preparations. The concentrations of cofactors and substrates listed in Table 1 were those that gave optimum activity: high concentrations of pyruvate, phosphate, acetyl phosphate or ATP were inhibitory so these, when used, were well below the inhibitory concentration.

Nucleotide phosphates were detected by thin-

layer chromatography on cellulose in butanol-acetone-acetic acid-aq. 5% ammonia-water (7:5:3:3:2, by vol.) (Randerath, 1962). The cellulose was purchased from Machurey und Nagel Co., Düren, Germany, and the nucleotides were from Sigma Chemical Co., St Louis, Mo., U.S.A.

Results. The phosphoroclastic systems from nitrogen-fixing Berre S regularly showed marked stimulation by ATP; those from Hildenborough were intrinsically more active and required aging at 4° under argon to show ATP stimulation (Table 1). With Berre S extracts, ADP, ITP or IDP could substitute for ATP; with Hildenborough extracts ADP was active but ITP showed much lower ability to stimulate the phosphoroclastic reaction than ATP. IDP was not tested with Hildenborough extracts. *S*-Adenosylmethionine was inactive for Hildenborough though it has been implicated in the pyruvate metabolism of *Escherichia coli* (Knappe, Bohnert & Bruemmer, 1965). It was not tested with Berre S extracts. As well as revealing ATP stimulation, anaerobic aging led to a decline in phosphoroclastic activity (Table 1) that was more pronounced with extracts of Berre S. Some preparations showed a lag before hydrogen evolution occurred.

Extracts of both strains showed acetokinase activity measured as the disappearance of acetyl phosphate in the absence of pyruvate, and this reaction was also accelerated by ATP (Table 1, Expt. 5). ITP was also active and, in contrast with its action on the whole phosphoroclastic process, it