PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The 484th Meeting of the Society was held at the University of Cambridge on Tuesday and Wednesday, 2 and 3 July 1968, when the following papers were presented:

COLLOQUIUM ON 'STORAGE OF BIOLOGICALLY ACTIVE SUBSTANCES IN CELLS'

The Storage of Hormones

By A. D. SMITH (*Department of Pharmacology, University of Oxford*)

Three types of endocrine tissue can be distinguished according to the mode of storage of the hormones. In the first type, the gland does not store significant amounts of hormone but may store a precursor; in the second, the hormones are stored extracellularly; and, in the third, the hormones are stored intracellularly.

The steroid-secreting glands are an example of the first type of endocrine tissue, and contain only small amounts of hormones relative to the amounts they secrete (Vogt, 1943). The adrenal cortex, the ovary and the testis contain only as much corticosteroid (Holzbauer, 1957), progesterone (Short, McDonald & Rowson, 1963) and testosterone (Lindner, 1961) as they secrete in 3, 10 and 18 min. respectively. However, in several species these glands store large quantities of cholesterol: as much as 7% of the wet wt. of the ovary (Claesson, 1954) or of the adrenal cortex (Cook, 1958) is cholesterol ester. Analysis of the adrenal cortex (Long, 1946) and ovary (Claesson, Hillarp & Högberg, 1953) indicated an inverse relationship between the amount of cholesterol stored and the secretory activity of the gland. A similar relationship has been found by electron microscopists between the number of membrane-limited lipid granules and secretory activity in the adrenal zona glomerulosa (Giacomelli, Wiener & Spiro, 1965) and zona fasciculata (Luse, 1967) and in the lutein cells of the ovary (Blanchette, 1966).

Direct evidence for the storage of esterified cholesterol in lipid granules of the ovary was provided by Claesson (1954), who centrifuged tissue homogenates and obtained (by flotation) a fraction containing 95% of the total cell cholesterol esters. Freshly isolated granules, about 1μ in diameter, had a marked birefringence approaching that of crystalline cholesterol. It is likely that the lipid granules of steroid-secreting cells represent a reserve store of precursor, and that the rate-limiting step for the synthesis and secretion of steroids, at least in the adrenal cortex, is the

cleavage of the side chain of cholesterol (Holzbauer, 1968).

The second type of endocrine tissue, in which the hormones are stored outside the cell, is exemplified by the thyroid gland. The lumina of thyroid follicles contain a colloid composed mostly of thyroglobulin, a glycoprotein of high molecular weight to which the hormones are bound in peptide linkage (Edeloch & Hall, 1964). The follicular cell (Lupulescu & Petrovici, 1968) has a dual function: first, it synthesizes thyroglobulin and releases it into the lumen (exocrine function) and, secondly, the cell takes up thyroglobulin from the store in the lumen, transports it across the cell while hydrolysing the peptide bond and then releases the hormones (endocrine function). The transport of thyroglobulin from apical to basal pole of the cell takes place in membrane-limited colloid droplets ($1-2\mu$ in diameter), which can be sedimented from thyroid homogenates (Balasubramaniam, Deiss, Tan & Powell, 1965). During transport, the colloid droplets acquire new enzymic activities (acid hydrolases), which result from fusion with lysosomes (Wetzel, Spicer & Wollman, 1965; Seljelid, 1967). Accordingly, there has been much speculation that a lysosomal protease is involved in the release of the hormones (Peake, Balasubramaniam & Deiss, 1967). Undegraded thyroglobulin is also released from the gland and is recovered in the lymph (Daniel, Pratt, Roitt & Torrigiani, 1967).

In most endocrine tissues the hormones are stored intracellularly in membrane-limited granules, which range in diameter from 100 to $400m\mu$. The capacity of this type of tissue to store hormones can be very high. Insulin comprises 14.5% of the dry weight of pancreatic islet tissue of rat (Dixit, Lowe & Lazarow, 1962) and rabbit (Lacy & Williamson, 1962). The adrenal medullae of the rat store as much adrenaline as is normally secreted in 1 week (Leduc, 1961).

Unequivocal evidence for the storage of a hormone in a subcellular particle requires a combination of biochemical and morphological studies. Centrifugation and filter techniques should show that the bulk of the hormonal activity in homogenates is present in particles, and that the hormone-containing

particles are distinct from other types of cell particle. Electron-microscopic analysis should show that the endocrine cells contain secretory granules that undergo characteristic changes when the gland is stimulated, and these granules should be identified in the hormone-containing particles isolated from homogenates. It is not always possible to satisfy all these criteria, especially when the tissue contains more than one type of hormone.

Many hormones have been shown to be localized in particulate fractions of homogenates of endocrine glands. One of the first such studies was that of McShan & Meyer (1949) on the gonadotrophins, and later work showed that somatotrophin, mammothrophin, thyrotrophin and corticotrophin were also localized in granules (Hymer & McShan, 1963; Perdue & McShan, 1966). Homogenates of the neurohypophysis include neurosecretory granules and some pinched-off nerve endings (neurosecretosomes), which contain the polypeptide hormones. The results of density-gradient centrifugation led to suggestions that oxytocin and vasopressin are stored in different nerve endings (Bindler, Labella & Sanwal, 1967) as well as in different granules (see review by Hope & Dean, 1968). The islet tissue of fish is readily obtained and this enabled Maske (1957) to demonstrate that insulin is particle-bound and Lazarow, Bauer & Lindall (1964) to distinguish the insulin granules from mitochondria. Insulin is also present in particulate fractions of mammalian pancreas (Hjertén, Höglund & Hellman, 1964; Randall & Shaw, 1964).

Other protein or polypeptide hormones that are present in particulate fractions are thyrocalcitonin (Bauer & Teitelbaum, 1966; Cooper & Tashjian, 1966), renin (Dengler & Reichal, 1960; Cook & Pickering, 1962) and, possibly, parathyroid hormone (L'Heureux & Melius, 1956). Insect neurosecretory material of the corpus cardiacum is sedimentable (Evans, 1962). Hormones of low molecular weight, such as the catecholamines of the adrenal medulla (see review by Smith, 1968), are also present in granules.

The isolation of purified storage granules from the adrenal medulla and from the neurohypophysis has enabled the composition of these unique particles to be studied. Hillarp (1959) found that the dry weight of adrenal chromaffin granules was made up of protein (35%), lipid (22%), catecholamine (21%) and ATP (15%). A knowledge of the composition of chromaffin granules led to the finding that secretion of the catecholamines takes place directly from the chromaffin granules (exocytosis) and is accompanied by the secretion of ATP and soluble proteins (see Schneider, Smith & Winkler, 1967). It has long been known that a hormone-binding protein (neurophysin) is present

in the neurohypophysis, and recent work has shown that the neurosecretory granules contain two hormone-binding proteins, neurophysin-I and -II (Dean & Hope, 1968); this finding also led to a new method for the isolation of the native neurophysins of the gland. Crystalline complexes of the two native neurophysins with vasopressin have been obtained (Hollenberg & Hope, 1968); the complex with neurophysin-I contained 3 molecules of vasopressin/molecule of protein.

It is likely that the biochemical analysis of purified storage granules from other endocrine tissues will lead to valuable information on the mechanisms of storage and secretion of the hormones.

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The Uptake of Biologically Active Substances by Lysosomes

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Living cells exposed to acridine orange show metachromatic fluorescent staining of subcellular structures (Allison & Young, 1964). Recent observations have shown that uptake of the dye also occurs readily in isolated lysosomes and other organelles (Dingle & Barrett, 1967). Further investigations into the mechanism of this phenomenon have led incidentally to a rapid method for the examination of fractions obtained during the separation of subcellular particles. In this technique 50 μ l. of each fraction is mixed with an acridine orange solution in suspension medium to give a concentration of 50 μ g. of dye/ml. After allowing 5 min. for uptake of the acridine orange, slides are examined by fluorescence microscopy with blue-violet dark-ground illumination and a yellow secondary filter with a magnification of 220 $\times$. Under these conditions nuclei (pale green), mitochondria (green), lysosomes (bright orange) and

membrane fragments (green) can be readily identified and counted. Among the advantages of this method are its speed and convenience, so that the purity of large numbers of fractions can be estimated and the organelle morphology can be examined immediately. Thus it has been found that counts of lysosomes in gradient-centrifugation fractions agree well with measurements of specific enzyme activity, and damage to organelles resulting in mitochondrial swelling or lysosomal abnormalities can be easily recognized. Further, it has been found that fractions apparently pure by conventional enzymological methods may, in fact, be seriously contaminated.

Measurement of the uptake of tritium-labelled acridine orange by isolated organelles has shown that, at the concentration used for staining, lysosomes take up several times as much of the dye as mitochondria. This factor may be sufficient to explain the orange fluorescence of the lysosomes, since solutions of the dye show a shift from green to orange emission with increasing concentration, but a specific molecular 'stacking' effect at lower concentration cannot yet be precluded. The possibility exists that the acridine orange-binding substance extracted from lysosomes and shown to bind dye after gel electrophoresis (Barrett & Dingle, 1967) may be present as a component of the lysosomal membrane. The bright orange fluorescence observed in the fine filamentous processes of lysosomes subjected to mechanical stress is compatible with a high concentration of dye within the membrane, as is the peripheral orange fluorescence of small vesicles sometimes observed within large lysosomes of rat kidney.

Experiments *in vivo* still in progress demonstrate that acridine orange injected intraperitoneally into rats persists in the kidney lysosomes, but not the mitochondria, for at least 1 week. These findings are similar to those obtained with labelled dimethyl-benzanthracene (Dingle & Barrett, 1967), and with [14 C]cetyltrimethylammonium bromide, which remains in the lysosomes for more than 4 weeks. The binding of acridine orange is thought to be a useful model system in which to study the processes by which lysosomes bind biologically active substances.

Results so far obtained support the hypothesis (Barrett & Dingle, 1967) that kidney lysosomes contain a strongly acidic lipid capable of binding lipophilic and cationic molecules. This substance may confer upon lysosomes a role in the scavenging and metabolism of drugs, carcinogens and other toxic materials from the cytoplasm.

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