ISOLATION TECHNIQUES FOR PHARMACOLOGICALLY ACTIVE SUBSTANCES (ANIMAL)[1,2]

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This particular subject has not been reviewed previously, although many reviews on related subjects have covered aspects of it (1–8), and a collection of articles on the separation of biological materials (9) is recommended. The field is extensive, so it has been necessary to be selective in the definition of "pharmacologically active substances." This review deals with methods of isolation which have been used for hormones, neurohumors, and autacoids, a collective name (10) for various autopharmacological agents. Methods for the isolation of substances primarily of biochemical or physiological interest have not been included, although no fine lines divide these disciplines from pharmacology. Some pharmacologically active extracts containing unidentified active substances are included. A summary of the apparently most useful method of isolation is included for each substance where possible.

Isolation Methods, General

The extraction of substances from solid animal tissues may commence with homogenization of the fresh or frozen tissue with water, aqueous solutions, or other solvents, followed by centrifugation or filtration; this technique is suitable for hydrophilic or easily water-soluble substances since the resulting extract will be more or less aqueous. Alternatively, a tissue may be lyophilized or otherwise dried and then extracted with nonaqueous solvents; this may be the more appropriate technique for the extraction of a lipophilic substance, although such substances can be extracted by homogenization. In general, solvent extraction of a pre-dried tissue is more specific than homogenization, from which a complex mixture of water-soluble substances inevitably results. Some tissues may be dried by grinding with a solvent such as acetone, depending upon the solubility of the required substance.

Fractionation of intracellular components may be carried out by using a Potter-Elvehjem type homogenizer followed by differential centrifugation.

[1] The survey of literature pertaining to this review was concluded in May 1968.

[2] The following abbreviations have been used: FSH *for* follicle stimulating hormone; LH *for* luteinizing hormone; LRF *for* luteinizing hormone releasing factor; TRF *for* thyrotrophin releasing factor; and 5-HT *for* 5-hydroxytryptamine; and SRS *for* slow reacting substance.

More complex systems of centrifugation may be used (9, 11). The extraction of substances from blood (12) is usually preceded by separation of the erythrocyte fraction from the plasma.

How does the research worker proceed when a crude extract of a tissue is found to possess pharmacological activity? If the nature of the active principle is suspected, the purification procedures used for such a substance previously, and outlined in this review, will provide a guide. If the substance is unknown, rules are not easy to formulate, and Morris's review on choice of methods (4) may assist. Solubility and solvent partition tests, and determination of the stability of the active substance to changes in temperature and pH are suggested as initial experiments, with stepwise checking of the activity of the solution by bioassay. An estimate of the molecular weight of the active substance can be obtained from dialysis, or gel filtration using a suitable grade of molecular sieve or gel (9); if solubility data suggest an ionized substance, electrophoresis may provide further evidence for this.

The crude extract may then be purified by one or more of a number of techniques, progress being followed by bioassay. Differential solubilities may be exploited by partitioning between immiscible solvents, as in the countercurrent distribution technique (1, 8, 13). Dialysis and ultrafiltration through semipermeable membranes (4, 14) or filtration (6, 8, 15) with dextran or polyacrylamide gels are processes frequently used to separate large molecular species such as polypeptides. Chromatography in columns packed with a variety of inert adsorbents or ion exchange resins (1, 4, 16) is applicable to most substances, and liquid ion exchangers (17) may be used in a solvent partition system when an ionized substance is involved. The precipitation of proteins by the addition of suitable agents is common, and some substances may be purified by the reversible formation of insoluble derivatives or complexes. Electrophoresis, a technique adequately reviewed (4, 6, 18, 19), can be used to separate ionized substances, and together with paper chromatography (1, 20, 21) or thin layer chromatography (22) may be useful as the final stage in purification or identification. The active material may be located on a chromatogram by chemical colour tests (23) or ultra-violet fluorescence, or by elution and bioassay of segments of the chromatogram, using standard substances for comparison. Spectrochemical techniques such as spectrophotofluorimetry have been widely used, and eluates from chromatographic columns may be monitored spectrographically using flow cells.

Isolation of Hormones

Hormones are discrete chemical substances produced in special glands or cells, which are transported to exert their effects upon sites of action in other tissues. Recent reviews which include the isolation of hormones are those by Butt (5), for protein hormones by Acher (24) and Law (25), and for hormones from plasma, by Antoniades (12).

Posterior pituitary hormones.—The posterior pituitary or neurohypophyseal hormones are oxytocin and vasopressin, which have a regulatory function. Current methods of isolation of these two hormones have been reviewed (5, 24); in general, posterior pituitary lobes are defatted with acetone and the powder extracted with dilute acetic acid. The hormones are separated by adsorption chromatography and column electrophoresis: oxytocin is isolated by use of triethylaminocellulose columns and electrophoresis on cellulose, and vasopressin by use of DEAE-cellulose and sulphomethylcellulose columns and electrophoresis. Craig (13) reviewed the isolation of active principles by partition, with reference to pituitary extracts.

Pars intermedia hormones.—From the intermediate region of the pituitary in primates and amphibians the polypeptide melanocyte-stimulating hormones or intermedins can be isolated by extraction of the pituitary powder by acetic acid, precipitation by ether, and adsorption of these hormones on oxycellulose, which separates them from corticotrophins (5, 25, 26) to which they are chemically similar.

Anterior pituitary hormones.—Wilhelmi (26) described a useful serial extraction of human pituitaries which yielded gonadotrophins and prolactin as well as somatotrophin. Pituitary extracts have been purified by starch gel electrophoresis (27) and by DEAE-cellulose chromatography (28). Growth hormone or somatotrophin is the most abundant, approximating 4 per cent by weight of the dried gland in man. Its extraction has been reviewed (24, 29, 30). Butt (5), who compared yields and purity of extracts reported by different authors, preferred Raben's method of isolation (30) using acetic acid-acetone. The preparation of pituitary gonadotrophins has been summarized (5, 24). Human follicle-stimulating hormone (FSH) and luteinizing hormone (LH) have been separated from prolactin and growth hormone in primary pituitary extracts by cation exchange chromatography (31). Human pituitary FSH was purified 10,000 times over the crude acetone powder by successively using columns of Sephadex G100, ion exchange resin, electrophoresis on cellulose columns and polyacrylamide gel (32). Homogeneous ovine FSH was recently prepared (33) and the efficiency of various purification methods for ovine FSH has been compared by Butt (5), who also reviewed the purification by ion exchange of gonadotrophins from other sources. LH is best extracted from the pituitaries of horse or sheep, purified by ammonium sulphate fractionation and ion exchange chromatography (34) or gel filtration (5). Purified LH from porcine glands (35) was concentrated from a gonadotrophin-rich extract by fractionation with metaphosphoric acid and ethanol and ion exchange chromatography.

Corticotrophin or ACTH may be extracted from the anterior pituitary by acid acetone and purified (5) by salt fractionation, starch electrophoresis, and ion-exchange chromatography; it is commercially prepared from porcine, ovine, and bovine pituitaries. Early isolation work by Li and his school (36) included the identification of pure α-corticotrophin from sheep pituitary (37). The fractionation of corticotrophic hormones from human

plasma has been reviewed by Munson (12). Corticotrophin release is mediated by a "releasing factor" (CRF), a peptide released by the hypothalamus and which can be extracted by acid acetone (5). Hypothalamic releasing factors for LH and thyrotrophin (LRF and TRF) have also been identified. LRF has been purified (38) from acid extracts of bovine hypothalamus. The isolation of prolactin, the lactation hormone, has been described using ovine pituitary; counter current distribution was used for purification (5). Radio-immune assays using ^{131}I-labelled prolactin showed that extracts of pituitaries from many species gave the same reaction (39).

Methods for the isolation and assay of thyrotrophin have been reviewed (12, 40). It is most readily extracted from preacidified blood or urine using ethanol with sodium chloride, followed by electrophoretic fractionation (5).

Thyroid and parathyroid hormones.—The thyroid hormones have been reviewed by Robbins & Rall (41); they are iodine-containing compounds which occur bound to proteins in the thyroid. Thyroglobulin is such a protein, and is extracted from the thyroid by aqueous solvents (41) and purified by ammonium sulphate precipitation and DEAE-cellulose chromatography. The isolation from thyroid tissue of thyroxine and the related iodine-containing compounds such as triiodothyronine has been described by Barker (42). These hormones are released from combination with protein by the *in vitro* action of pancreatin, trypsin, or pronase at pH 8.4; the iodo-compounds are then extracted with *n*-butanol-saturated *N* HCl or with methanol-ammonia (1:1) and separated chromatographically. The use of labelled thyroid hormones is common in research, since ^{131}I is readily detected. The hormones may be detected physically or chemically by other methods (5, 42). A substance described as a "long-acting thyroid stimulator" has been isolated from serum, purified, and bioassayed on mice (43). Thyrocalcitonin (44), or calcitonin (44a), has been extracted from mammalian thyroid tissue and purified by gel filtration (45).

Difficulties associated with the extraction of parathyroid hormone have been discussed in reviews (5, 46, 47). The phenol extraction method of Aurbach (46) has yielded parathyroid hormone from acetone powder of the gland or from blood, and the hormone has been obtained 90 per cent pure by gel filtration with Sephadex G100 (48).

Hormones of the adrenal gland.—Standard methods for extraction of catecholamines from heart, adrenal glands, or other tissues involve the use of acid alcohol (49) or salt-saturated acid butanol (50, 51). Epinephrine was isolated from adrenal glands by Vogt (52), and was extracted from plasma (53) together with norepinephrine, from which it may be separated by paper chromatography (54) or gel filtration (55). Catecholamines and their metabolites in extracts have been differentiated fluorometrically (53, 56) and electrophoretically (57).

Aldosterone may be isolated from adrenal tissue (58), blood (59, 60), and urine (59) by extracting acidified fluid or tissue with methylene dichloride or chloroform. The adrenal corticosteroids have been extracted from

blood or urine (5, 12, 61) using organic solvents, and have been purified chromatographically.

Androgens have been extracted from testes and adrenal cortex (5), and schemes for extraction from human plasma and from urine have been selected in reviews by Migeon (12) and Dorfman (61). The isolation of estrogens was reviewed by Preedy (61); these hormones may be extracted with organic solvents from urine and purified chromatographically (5, 21, 62). Progesterone has been isolated from corpus luteum by Zander (61) and from blood (12).

Pancreatic hormones.—Insulin was first extracted from dog pancreas in 1922 by Banting & Best (63). It is now produced commercially from bovine or porcine pancreas by a multi-stage process summarized by Butt (5); the review by Acher (24) refers to another method of preparation. Glucagon was isolated in 1955 (64) as a by-product from the purification of crude porcine insulin.

Isolation of Neurohumoral Substances

The occurrence of biogenic amines in brain, including their isolation, has been reviewed (65).

Acetylcholine.—Early methods for the isolation of acetylcholine from brain and other tissues used trichloroacetic acid (66). Separation of acetylcholine from crude extracts by reineckate formation was reported (67), and Whittaker (68) reviewed methods of separating acetylcholine from its homologous esters. Acidified ethanol was used to extract acetylcholine from crushed frozen brain (69); extraction with buffer at pH 4, followed by heating to destroy enzymes (70), is perhaps the most generally useful method—acetylcholine in the resulting extract being purified by paper or thin layer chromatography. Acetylcholine was separated electrophoretically from related compounds in a brain extract (71), and it was identified in the water-soluble fraction from alcohol extracts of optic nerves (72). Cholinomimetic activity has recently been separated from nonbasic material in a brain extract by liquid ion exchange (73). Acetylcholine was identified (74) together with 5-hydroxytryptamine from a sucrose density gradient separation of ganglionic constituents from the clam *Mercenaria,* and was also found in snail and clam ganglia (75). Acetylcholine has been isolated also from nonnervous tissue, for instance from extracts of mammalian urinary bladder (76) and from the heart of *Tapes watlingi* (77).

Norepinephrine.—Together with other catecholamines, norepinephrine may be isolated from brain using acid methanol (78), perchloric acid (79), or acidified butanol saturated with sodium chloride (51). Separation of norepinephrine from epinephrine and its metabolites may be accomplished by ion exchange (79), paper chromatography (54), or electrophoresis (57). Dopamine has been extracted from brain with dilute acid (80) and estimated fluorometrically, or it may be separated from norepinephrine and related compounds by thin layer chromatography (81).

5-Hydroxytryptamine.—5-Hydroxytryptamine was isolated from brain tissue using *n*-butanol (82) or, with better yields, using *n*-heptanol (83). There are good reviews covering its isolation from other tissues (84, 85), and more recent reports of its isolation from plasma (86) and separation from other aromatic amines in urine (87).

There is some evidence that 5-HT acts as a transmitter in the heart of the mollusc *Helix pomatia* since it was identified chromatographically in a perfusate which stimulated a Loewi-type assembly of snail hearts (88); it has also been found in the heart and ganglia of *Tapes watlingi* (89). Erspamer (90) has separated 5-HT and some of its metabolites from extracts of toad skin.

Amino acids.—γ-Aminobutyric acid (91, 92) is widely distributed in the animal and plant kingdoms, and its estimation has been reviewed (93). It has been separated from other amino acids and derivatives in bovine brain by cation exchange resins (94), and identified, with other amino acids, in extracts of molluscan nervous tissue (95). Ergothioneine is an amino acid derivative extracted from brain, and identified as the "cerebellar factor" (96).

Histamine.—Histamine occurs in brain and is also widely distributed in other tissues as a depressor substance and smooth muscle stimulant (97). Methods for its isolation and estimation from various tissues have been summarised by Crossland (98), Blaschko (97), and Adam (65). It was extracted from brain using trichloroacetic acid and purified by ion exchange (65, 99).

Many biogenic amines and other active substances have been isolated from central nervous tissue (65, 91, 92). Toh (100) has described the extraction from brain of several smooth-muscle contracting substances which were not well characterised.

Isolation of Autacoids

Substances P.—Von Euler & Gaddum (101) discovered substance P. It is a polypeptide smooth muscle stimulant and vasodilator which occurs in intestine and brain, and has been isolated from these tissues with acid alcohol or by boiling with buffer at acid pH. These extracts have been purified by ammonium sulphate or ethanol precipitation, solvent partition, alumina chromatography with methanol as eluent, ion exchange chromatography, and electrophoresis (102). Its isolation as two or more active principles in essentially pure form has been reported (103–105).

Other powerful vasodilating hormones related to substance P (106) are eledoisin and physaelamin. These undecapeptides have been studies by Erspamer and his colleagues in Milan; they isolated eledoison from the salivary gland of a mollusc (107) and physaelamin from the skin of a South American amphibian (108).

Other polypeptide smooth muscle stimulants.—There are reviews of the pharmacology, including isolation, of polypeptides (25, 109, 110) and also

of kinins (111, 112)—a group of vasoactive polypeptides produced in plasma from inactive substrates by enzyme action. The enzymes kallikrein or plasmin in the presence of an activator release from an α_2-globulin the kinins bradykinin and kallidin (25). Kallikrein, known since 1930 and described as a depressor substance, was recently isolated from pig serum and purified (113). The first preparation of crude bradykinin was published by Rocha e Silva, Beraldo & Rosenfeld in 1949 (114) after their observation of the release *in vitro* of a potent stimulant in dog's blood upon addition of snake venom or trypsin. Bradykinin was later purified by Elliott, who reviewed the subject (115), using ammonium sulphate precipitation, incubation with trypsin and addition of ethanol, followed by chromatography or paper electrophoresis.

Relationships between groups of plasma peptides were established when bradykinin and kallidin were shown to exhibit crossed tachphylaxis on the guinea pig ileum on one hand, and substance P, physaelamin, and eledoisin on the other, with no crossed tachyphylaxis between the two groups, suggesting two types of common peptide receptor in the ileum (106).

Rocha e Silva, Beraldo & Rosenfeld (114) observed that bradykinin had properties resembling those of "slow reacting substance" (SRS) isolated by Feldberg & Kellaway (116) after observing that egg yolk produced a smooth muscle stimulant when incubated with cobra venom. Vogt (117) studied egg yolk SRS and found it to be a lipid-soluble acid. Another slow reacting substance produced in anaphylaxis (SRS-A) has been investigated by Brocklehurst (118). SRS-A occurs in the perfusate from the lungs of guinea pigs previously sensitized; it has been partially purified by the ion-exchange removal of histamine, and the adsorption of SRS-A on to charcoal and elution with aqueous *n*-butanol. Berry & Collier (119) considered SRS-A, which they prepared by Brocklehurst's method, to resemble the kinins, and they believed it to have a receptor similar to but independent from that of bradykinin. Electrophoresis of Brocklehurst's SRS-A gave a single band, suggesting its homogeneity (120).

Another hypotensive polypeptide is caerulein, which was isolated from the skin of the frog *Hyla caerulea* (121).

The renin-angiotensin system has been reviewed (25, 110, 122). Angiotensin has been isolated from horse serum by a method involving countercurrent distribution and alumina chromatography (123). Renin is a proteolytic enzyme found in crude saline extracts of kidney; it behaves as though it is itself a pressor substance, but is only pressor because it releases angiotensin in plasma. Renin has been purified from aqueous extract of kidney on DEAE-cellulose (124). A renin-like pressor substance has been isolated from the placenta of mammals, (125) for example, and a renin-like activity has been identified in preparations of erythropoietin (126). Erythropoietin is a glycoprotein hormone found in plasma and urine but not yet purified, which is involved in red cell production (127). A renin inhibitor has been extracted from kidney with acetone and purified (128); it is a phospholipid

which is antihypertensive because it prevents angiotensin formation. The antidiuretic effect of angiotensin on the isolated perfused rat kidney is also found with a substance extracted by ethyl acetate from lung tissue (129) likewise by an anionic globulin isolated from plasma (130). A nondialysable pressor substance different from angiotensin and found in serum of several mammalian species has been partly purified (131).

Other Substances

Lipid-soluble smooth muscle stimulants.—A number of lipid-soluble substances which stimulate smooth muscle have been isolated, and some mentioned in reviews by Vogt (117, 132). Darmstoff, a phospholipid, is the name given to a smooth muscle stimulant extracted from intestinal tissue by organic solvents (132). Irin is a similar substance extracted from rabbit iris (132, 133) and is the active principle for contracting the eye muscle. There has been much recent work, particularly by Bergstrom & Horton and their respective groups, on prostaglandins, a group of lipid-soluble carboxylic acids with the ability to contract smooth muscle; they have been isolated from many tissues (134–136). Their extraction, reviewed by Bergstron (134), was reported from blood (137), brain (138), seminal fluid (139) and other tissue homogenates. G-acid, a smooth muscle stimulant with a slow action isolated from plasma (132), may be converted by heat to substance *Q*, a quick-acting smooth muscle stimulant which has been purified (140). A lipid of a different type, carcinolipin, has been purified from rat liver. It is carcinogenic and stimulates protein synthesis *in vitro* (141). Recent reports show that extracts of brain lipid include an acetylcholine-releasing factor (142) and a substance which affects reproduction in the rat (143).

Cardiac stimulants.—There are numbers of naturally occurring substances which stimulate the heart. Early concepts of a heart hormone have been thoroughly reviewed (144); two of such substances are palmitoyl lysolecithin, a cardiac stimulant isolated from blood and identified (145), and cardiolipin, a phospholipid extracted from ox liver (146). Recosen is a commercially available extract of mammalian heart which has an indirect sympathomimetic action on the heart; it is a coronary vasodilator (147, 148). A cardioactive peptide isolated from plasma (149) is kinekard, which has been shown to stimulate toad heart muscle and to behave like epinephrine on isolated smooth muscle (150). A cardiac stimulant substance was extracted and partly purified from spleen and other mammalian tissues (151, 152). Cardenolides such as the bufotoxins, structurally related to the digitalis glycosides, have been isolated from toads (153) and other animal sources (154). Aqueous extracts of the heart of the Pacific hagfish yield a cardiac stimulant, eptatretin (155); and, similarly, extracts of nervous tissue of the mollusc *Aplysia* (156) and of various other mulluscan tissues contain cardiac stimulant substances (74, 75).

Invertebrate tissue extracts.—The extraction of active substances from

invertebrate tissues is a rather special field which has not been treated comprehensively here (157). Whittaker, in one of a collection of reports on active substances of marine origin (158), describes some active choline esters with complex chemical structures isolated from gastropods. Planetocin, a stimulant of uterine smooth muscle, has been extracted from the gut of the cockroach *Periplaneta americana* and partly purified (159, 159a).

There has been recent interest in insect hormones. The isolation of insect attractants or pheromones has been competently summarised by Jacobson (160). The insect moulting hormone, ecdysone, has been isolated from silkworms (157), and recently crustecdysone, the crustacean moulting hormone, has been obtained pure from crayfish (161). Bioluminescent substances have been isolated from *Renilla* (162), and aequorin from the medusa (163). There have been numerous reviews on toxins and venoms (164, 165); methods for their isolation have been omitted through lack of space.

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