Isolation Studies on a Lipoidal Portion of the Bovine Pineal Gland

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Bovine pineal glands were homogenized with chloroform and the filtrates chromatographed on silica columns. Seven major purified fractions were obtained; three of these were characterized. One was shown to be cholesterol; a second was an oil of molecular weight around 1000 which was not identified fully. Examination of a third component—a yellow oil—by infrared, mass spectra, nuclear magnetic resonance, and chemical studies led to an unequivocal structural assignment of dioctyl phthalate. The results raise the question as to whether the ester is of physiological origin or is ingested by the animals from artificial sources and retained by the pineal gland.

Anatomically, the pineal gland has evolved from a protruding position at the top of the skull, where it appears as a third eye in some fish and amphibia, to a position on top of the brain under the skull in the rat. Further evolution has moved it to a central and lower region of the brain in man. This change in location suggests a change in function or mechanism of action for the organ and possible greater integration with the central nervous system. In all classifications, the mammalian pineal organ is considered to be part of the brain. Differences between it and other parts of the brain have been demonstrated in relation to the blood-brain barrier. The rat pineal gland absorbs 18 to 50 times more radioactivity than surrounding brain on injecting intraperitoneally 5-methoxytryptophol, its acetate ester, melatonin, or serotonin (Delvigs and Taborsky, 1966, 1967).

Evidence has been presented by many investigators supporting the fact that the mammalian pineal gland is a functional organ. Some of the functions suggested for it have included stimulation of melanocyte contraction (McCord and Allen, 1917), control of sodium excretion (Machada and da Silva, 1963), gonadotrophic function (Kitay, 1954), thyroid regulation (Baachieri *et al.*, 1963), and recently, that of biologic clock and neuroendocrine transducer (Wurtman *et al.*, 1964). Results of many past physiological studies using crude pineal extracts or pinealectomized animals, however, have been inconclusive (Kitay and Altschule, 1954). Investigations relying on elucidation of the chemistry [enzymatic (Axelrod and Weissbach, 1960) and compound characterization (Axelrod *et al.*, 1965)] have been profitable in helping to describe the pineal and to suggest functions for it. This paper describes further studies using direct chemical approaches of isolation and characterization of substances present in pineal tissue. These initial investigations are concentrated on the chloroform-extractable lipoidal portions of the bovine pineal gland.

EXPERIMENTAL

Bovine pineal glands were quick-frozen at a meat packing concern (Canada Packers, Ltd., Toronto, Ontario, Canada) and shipped frozen by airplane. On arrival, glands were thawed in cold physiological saline, cleaned and sorted by hand to remove extraneous tissue, washed several times, and drained. In one experiment, homogenization in a Waring Blendar for 2 minutes with cold chloroform (86 grams of tissue to 1 liter of chloroform) was carried out. Filtration of the homogenate by vacuum through a Büchner funnel yielded considerable solid material, presumably consisting largely of protein. The solid was homogenized a second time with 500 ml. of chloroform, filtered, and washed with a third 500-ml. quantity of chloroform on the funnel. The yellow filtrates were combined and concentrated to a bright orange solution, about 3 ml. in volume, and applied to a column, 15 mm. in diameter, filled with Baker's silica gel powder to a height of 380 mm. Elution was done sequentially with 200 ml. of hexane, 100 ml. of chloroform-hexane (9 to 1), 100 ml. of chloroform, 100 ml. of chloroform-methanol (9 to 1), 60 ml. of chloroform-methanol (3 to 1), and 100 ml. of methanol; 27 20-ml. fractions were collected. Several drops from each tube were chromatographed on a thin layer of silica on glass (TLC) using chloroform methanol (9 to 1). Spraying with 2*N* sulfuric acid and heating at 130° C. for 10 minutes were used to detect substances on the chromatographic plates.

The hexane elution yielded one fraction seen at the origin on TLC; the chloroform-hexane elution yielded three fractions seen at R_f 0.45, 0.88, and 0.92, respectively. The R_f 0.88 fraction continued to come off with the next chloroform elution, together with others at R_f 0.85 and 0.66. The R_f 0.45 fraction also continued to come off with chloroform. Chloroform-methanol (9 to 1, 3 to 1) continued to elute the R_f 0.83 to 0.85 fraction together with a new one which remained at the origin on TLC. Finally, with methanol, only the R_f 0.0 material continued to come off the column. Initial study was directed at fractions appearing between R_f 0.83 to 0.92 on TLC. In one of the preliminary examinations, two chromatographically similar materials in addition to cholesterol were separated from this fraction. One fraction moved in chloroform-methanol (9 to 1) at about R_f 0.83 and the other at R_f 0.87 to 0.90. The former gave a brown indicator color with 2*N* sulfuric acid (heating to 130° C.), which turned purple. The latter spot remained brown. Some preliminary examination was made of this latter (R_f 0.87 to 0.90) fraction. Since several compounds were in these fractions, including a sizable proportion of cholesterol, extraction on a larger scale was done so that more elaborate separation procedures could be applied. Thus,

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700 grams of cleaned glands were homogenized twice with 500-ml. quantities of chloroform; filtrates were combined and reduced to 35 ml. A column of Baker's silica gel powder, 365 × 40 mm., was used. Elution was done sequentially with 250 ml. of chloroform–*n*-hexane (9 to 1), 1500 ml. of chloroform-benzene (7 to 3), 350 ml. of chloroform-methanol (3 to 1), and 300 ml. of methanol. Two hundred forty 10-ml. fractions were collected. The fraction having an R_f of 0.83 and a purple indicator color on the TLC system was selected for complete study. Contents of tubes comprising this fraction were combined, concentrated, and rechromatographed; this time elution was performed sequentially with 500 ml. of benzene-chloroform (3 to 1), 200 ml. of methanol-chloroform (4 to 1), and 100 ml. of methanol. The fraction was chromatographed a third time in a similar fashion using 200 ml. of petroleum ether–ether (9 to 1), 100 ml. of chloroform, and 100 ml. of methanol. Concentration of selected fractions under vacuum gave a 491-mg. residue of yellow oil which produced a discrete spot in eight different solvent systems on TLC

Infrared spectra of the oil were obtained on an IR8 Beckman infrared spectrophotometer (Figure 1*a*).

Fifty milligrams of the above yellow pineal oil were incubated for 18 hours at 80° C. in 5 ml. of ethanol with 0.5 ml. of 20% aqueous sodium hydroxide; 11 mg. of a sodium salt precipitated on cooling. Formation of volatile substances was indicated by gas-liquid chromatography of the reaction mixture. Five milliliters of water were added to the hydrolysis mixture at pH 12, and the nonacidic products and ethanol extracted with 4–5 ml. portions of ether. The aqueous phase was acidified with concentrated hydrochloric acid to pH 2 and extracted with 10 2-ml. portions of ethyl acetate. Combined portions of the latter were dried over sodium sulfate, filtered, reduced in volume to 4 ml., and added to 80 ml. of *n*-hexane. After several hours of cooling, 14.8 mg. of a solid were obtained which did not depress the melting point of authentic phthalic acid and had an identical infrared spectrum.

RESULTS AND DISCUSSION

At least seven major fractions were obtained when chloroform extracts of bovine pineal gland were chromatographed on silica columns. These ranged from lipid-soluble, possibly nonpolar materials eluted with hexane to more highly polar substances eluted with methanol. By comparison with the authentic substance one of the major fractions was identified as cholesterol. Thus, it produced a spot in chloroform-methanol at R_f 0.88, and gave a characteristic red coloration which turned purple on spraying with 2*N* sulfuric acid and heating at 130° C. A pure crystalline portion did not depress the melting point of authentic cholesterol. Gmelin (1824) recognized cholesterol as a constituent of brain, and its high concentration, in both white matter and myelinated peripheral nerve, has been noted by many workers. A second fraction [R_f 0.93, chloroform-methanol (9 to 1)] was isolated and purified for preliminary examination. It gave an empirical formula of C_8H_8O by combustion, and a molecular weight of 1050 by freezing point depression in benzene. Quantitative hydrogenation showed that it contained five double bonds per molecule. The infrared spectra contained a strong absorption peak at 5.8 microns, indicating the presence of a carbonyl group. A Zeissert determination indicated the possible presence of one methoxyl group per molecule.

A third fraction [R_f 0.83 to 0.85, chloroform-methanol (9 to 1)] was purified and examined. On spraying it with 2*N* sulfuric acid and heating to 130° C., it gave a brown

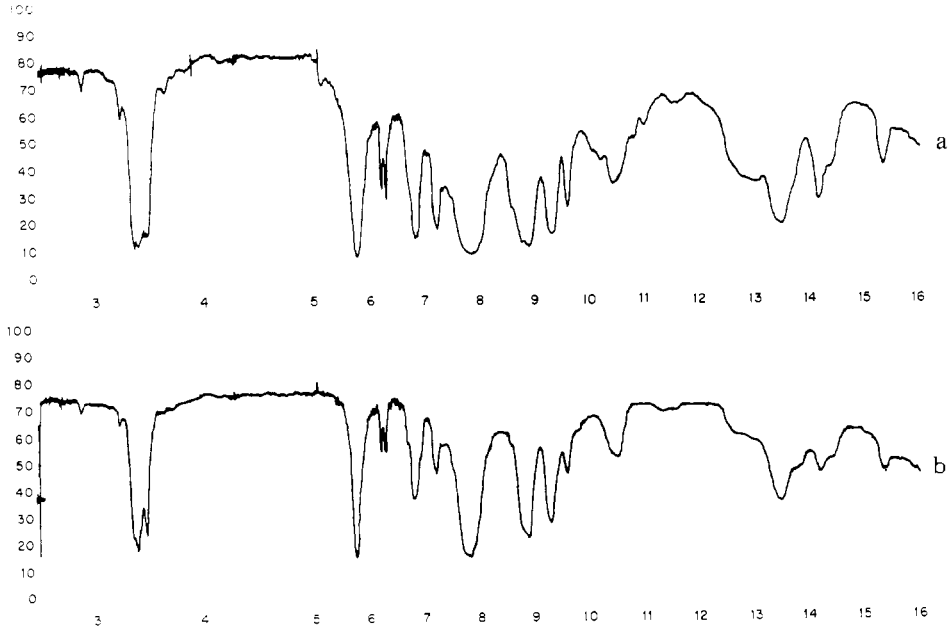

Figure 1. Infrared spectra

a. Oil isolated from bovine pineal tissue
b. Commercial di-*n*-octyl phthalate. Both were in the form of salt block smears. Units on ordinate are % transmission, figures on abscissa are wavelength in microns

color which turned purple after standing for about 30 minutes at room temperature; later it turned brown again. An infrared spectra (Figure 1*a*) of the sample exhibited recognizable peaks at 5.8 microns for carbonyl, aromatic peaks at 6.25 and 6.35 microns, and the asymmetric stretching of =C—O—C at 7.85 microns. The 9- to 14-micron region of the spectra was characteristic of some ortho-disubstituted aromatic compounds. Mass spectrometric determinations gave a parent peak with a mass of 390 giving an empirical formula of $C_{24}H_{38}O_4$. Isotopic analysis of an ion at 167 gave a formula of $C_8H_7O_4$ +, the low H to C ratio indicating an aromatic fragment with four oxygen atoms associated closely with it. Another fragment, C_8H_{17} +, indicated the presence of an eight-carbon atom aliphatic moiety. Nuclear magnetic resonance spectra in deuterated chloroform (Figure 2*a*) showed four types of hydrogens to be present in the molecule. These included an aromatic (7.6 p.p.m.) methine type or those on carbons attached to oxygen (4.2 p.p.m.), methylene (1.3 p.p.m.), and methyl (0.9 p.p.m.).

The assumption was made, based on mass and infrared spectra, that there were four aromatic hydrogen atoms in the molecule. Integration of the NMR peaks, using the above figure, gave a total of 38 hydrogen atoms for the molecule, which coincided with the values obtained already. It was concluded at this point that the aromatic residue was an ortho-disubstituted oxygen derivative of benzene. Chemical studies on compounds representing these classes were made. Basic hydrolysis of the oil in ethanol yielded phthalic acid and a volatile compound.

Commercial di-*n*-octyl phthalate (Matheson Chemical Co.) was treated in an identical fashion to give the same results and stoichiometry. The sodium salt isolated as a result of its hydrolysis had an identical infrared spectrum as the salt from the pineal oil hydrolysis. Gas-liquid chromatography of the volatile product (1-octanol) from authentic di-*n*-octyl phthalate hydrolysis gave retention times identical to that of the volatile product from the pineal oil, and a mixture of the two produced a single symmetrical peak on the chromatogram. Chromatographic characteristics in the TLC systems: methylene chloride, 1-butanol, ethanol, ethyl acetate, benzene, and chloroform-methanol (9 to 1) were identical for the pineal oil and di-*n*-octyl phthalate. Comparison of the infrared spectra of commercial di-*n*-octyl phthalate (Figure 1*b*) showed that it matched that of the pineal oil (Figure 1*a*). Further resolution and integration of the methyl-methylene hydrogen region of the NMR spectra averaged 10 methyl hydrogens, or three methyl groups per molecule of the pineal oil, whereas the NMR spectra of commercial di-*n*-octyl phthalate (Figure 2*b*) indicated two methyl groups as expected.

The NMR data indicated that the aliphatic portion of the pineal substance is largely unbranched. The apparent presence of three methyl groups may be explained by its being essentially di-*n*-octyl phthalate contaminated by more highly branched substances or a mixture of slightly branched isomers. Another possibility is that most of the molecules of the pineal substance do contain three methyl groups, and differ from the authentic substance in that respect. A 2.64% ethanolic solution did not exhibit optical activity. If the pineal derived substance is synthesized naturally, it should be stereospecific. Assuming that most of the molecules are formed naturally and contain three methyl groups, one group must be substituted on the sixth carbon atom of an octanol chain, that being the only position where asymmetry and optical activity would not be produced on branching.

The significance of the presence of dioctyl phthalate in bovine pineal gland revolves around three possibilities: It is an essential physiological substance stored or elicited by the pineal gland; it is nonessential physiologically, and is stored or elicited by the pineal gland; or it, or a precursor, is of exogenous origin, absorbed by the animal during its lifetime. All solvents used during the isolation had been distilled and were shown by concentration and

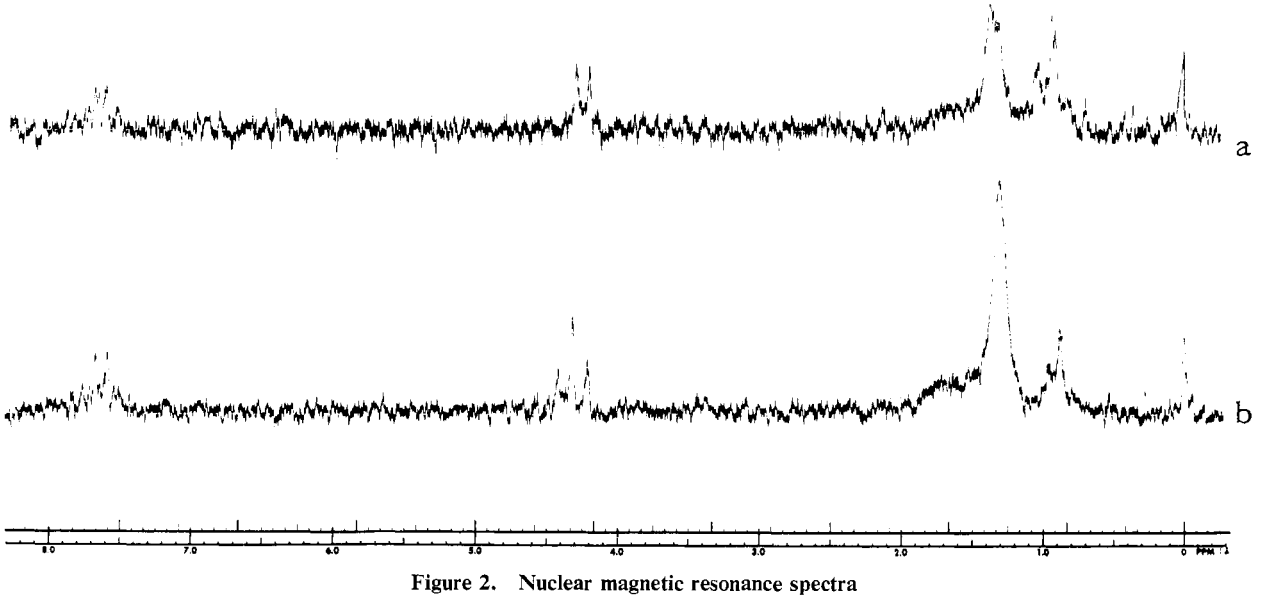

Figure 2. Nuclear magnetic resonance spectra

a. Oil isolated from bovine pineal tissue
b. Commercial di-*n*-octyl phthalate

chromatography to be free of dioctyl phthalate. No other sources of the above ester as contaminant were found.

Source cattle for the pineal glands were of Canadian origin where phthalates are not used for cattle sprays and dips as in the United States (Graham, 1966). However, plastic lines are used for watering, which would contain phthalates as plasticizers. Phthalates possibly might be absorbed by the animals from this source. If that is the case, then pineal glands of animals may serve as valuable loci for determining the amount of absorption of some agricultural chemicals. On the other hand, isolation of phthalides from oats and other plant sources on which cattle feed have been reported (Knight, 1966). Such compounds could be precursors for physiological formation of phthalates by animals. Production of phthalic acid by living organisms (Azotobactor) has been demonstrated (Aso *et al.*, 1932).

That dioctyl phthalate is a commercial synthetic chemical should not exclude the possibility that its occurrence in tissue might be due to its being of natural origin. Because of the known chemical and physical properties of phthalate esters, potential physiological roles can be easily envisaged for them. Functions might include those of being natural plasticizers to render proteins and tissue flexible, and to control the adhesiveness of blood particulate. In the monoester form, these substances could be involved in calcium or other ion transport.

The intriguing question of the origin of dioctyl phthalate in mammalian tissue, however, can be resolved only by the examination of pineal glands and other tissues of various species from differing environments to see whether the ester can be found in other instances.

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