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REVIEW

Cryoprotectants—a new class of drugs

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Living mammalian tissue can survive at temperatures of −196°. Research in cryobiology (low temperature biology) offers new insights to the role of water in living systems as well as offering a method of suspended animation. Survival of frozen animal cells universally depends upon suitably controlling the cooling and warming rates and, in addition, almost always requires specific treatment of the cells with at least one chemical agent.

Techniques which permit cells to survive freezing and thawing are said to afford the cells with cryoprotection. Chemicals which aid in cryoprotection have been called *cryophylactic agents* (Huggins, 1964, 1965, 1966) and *solute moderators* (Karow & Webb, 1964, 1965a, b). The most preferable is the easily understood *cryoprotective agent* (or *cryoprotectant*) as suggested by symposium participants during the second annual (1965) meeting of the Society for Cryobiology.

PHYSIOLOGY OF FREEZING INJURY

The reasons that animal cells are usually killed by freezing have been adequately discussed in several recent articles (Karow & Webb, 1965b; Meryman, 1966; Robertson & Jacob, 1968) and will only be summarized here.

When water solidifies, it may either crystallize or vitrify. Vitrification, the formation of a glassy state, is achieved by ultra-rapid cooling rates such as may be obtained by allowing water vapour to condense on a surface cooled to −200°. When water molecules are cooled this rapidly, their thermal motion is arrested without sufficient time for organization of the molecules in a crystalline array. In 1937, Luyet predicted that if the water in biological systems could be vitrified, viability of the system could be maintained. Complete vitrification of biological systems may be impossible with currently available techniques. Cooling of biological systems, no matter how rapidly, has always yielded some crystals. At rates of 1 to 10°/min all of the ice is composed of hexagonal crystals. At faster rates the ice may form mixtures of hexagonal crystals with increasing proportions of cubic crystals or vitreous ice or both (Dowell, Moline & Rinfret, 1962; Luyet, Tanner & Rapatz, 1962; Bullivant, 1965). Despite this situation, as a rule, slower cooling rates (1°/min) are usually more conducive to survival than faster rates.

Microscopy of frozen cells or tissues reveals that relatively slow (1°/min or less) cooling produces only extracellular crystals which are relatively large in comparison to the cell. As the cooling rate increases, ice crystals become more numerous, very small, and uniformly distributed throughout the intra- and extracellular spaces. The small intracellular crystals formed during rapid cooling can cause extensive disruption to the cellular ultrastructure and cellular death. The membrane damage

is clearly demonstrated in the published electron micrographs of frozen liver (Trump, Young & others, 1965; Stowell, Young & others, 1965), frog erythrocytes (Rapatz & Luyet, 1961), and mammalian erythrocytes (Rapatz, Nath & Luyet, 1963).

Slow cooling may also result in death of the cells because the large extracellular crystals dehydrate the cells. During the formation of these crystals, the water within the cells is super-cooled and therefore at a higher vapour pressure than the ice (Mazur, 1960). This pressure gradient tends to force water from the cells and thus is incorporated into the growing crystals. As extracellular crystallization proceeds, the intracellular concentration of solutes rises (Lovelock, 1957). An increase in electrolyte concentration alters protein conformation and solubility. The precipitation of sparingly soluble buffer salts leads to pH changes. Biomolecules which normally do not interact because of their spatial separation are brought into close approximation. Also possible is the development of toxic levels of urea, dissolved gases and other metabolites.

In an important study, Meryman (1968) has further elucidated the influence of hypertonic salts on mammalian cells, human erythrocytes being the model. He found that, as expected, cell volumes decrease as the extracellular salt concentration increases, but a minimum volume is attained at four times isotonic saline. When the saline concentration is increased above five times isotonic, the cells begin to swell. This swelling was interpreted as representing damage to membrane integrity. The pressure gradient across the damaged membrane allows salt to enter the cells. When these hypertonic cells are then transferred to an isotonic medium, haemolysis occurs. Experimental evidence obtained by Meryman (1968) corroborates the concept that erythrocytes, after exposure to high extracellular salt concentrations, become more permeable to both sodium and potassium.

Perhaps equally as important is the effect of freezing on cellular "bound" water, i.e. water essential to the proper conformation of biological macromolecules. It is generally appreciated that the loss of "bound" water, results in irreversible denaturation of macromolecules (Klotz, 1958; Kauzmann, 1959; Richards, 1963). Hydrogen-bonded water will form hexagonal lattices similar to those of "typical" ice crystals, whereas hydrophobic interactions will cause water to form pentagonal lattices, similar to cubic ice and characteristic of clathrates. Recent crystallographic studies have provided better than 3 Å resolution of the tertiary structure of four proteins: myoglobin (Kendrew, 1962), egg-white lysozyme (Blake, Koenig & others, 1965), ribonuclease (Kartha, Bello & Harker, 1967), and oxyhaemoglobin (Perutz, Muirhead & others, 1968). The results clearly indicate that the exterior of these proteins is encased in layers of hexagonal water lattices while pentagonal, clathrate-like water structures tend to form interiorly. Calculations by Scheraga (1963) based on thermodynamic considerations indicate that even though H-bonds predominate over hydrophilic bonds as structural and stabilizing forces, the contributions of hydrophobic bonds is significant and may even predominate at specific temperatures.

DNA is greatly influenced by its water of hydration (Jacobson, 1953). Falk, Hartman, & Lord (1962, 1963a, b) have been able to study the location of individual water molecules in the rigid first hydration layer of the DNA helix. Additionally, Falk (1965) has shown that the ionic phosphate groups, located on the surface of nucleic acids, are important hydration sites that predictably interact in hexagonal lattice formation. Theoretical calculation (Sinanoglu, Abdulnur & Kestnor, 1964; Sinanoglu & Abdulnur, 1965) based on spectroscopic evidence indicates that the firmly

bound water associated with the purine and pyrimidine bases has clathrate characteristics and remains unchanged when the system is frozen.

The loss of bound water from macromolecules has been related (Karow & Webb, 1965b) to the observation that slow cooling, rather than fast, is generally more conducive to the survival of frozen cells. Even though during slow cooling some of the free intracellular water is incorporated into the extracellular ice crystals, the loss of bound water may be slow enough to allow sufficient time for simultaneous strengthening and growth of protective intracellular water lattices which surround proteins.

When the temperature is lowered quickly, natural groups of 4 to 6 water molecules tend to function as crystal nuclei and give rise to a fantastic number of minute intracellular as well as extracellular crystals. Time is insufficient for the growth of large extracellular crystals which would free cellular water and thereby inhibit intracellular freezing. Considerations of surface-to-volume ratios indicate that in the competition for water, the numerous and small incipient ice crystals of rapid cooling have a distinct thermodynamic advantage over the relatively bulky water lattices of proteins. Therefore during rapid cooling and freezing the water associated with proteins may be removed and subsequently incorporated in the tiny ice crystals. Even though intracellular ice itself is generally a lethal factor in rapid freezing, if protein and other cellular components are maintained structurally intact (Salt, 1959), intracellular ice formation should not be lethal. Salt (1959) has shown that the fat cells in the larvae of the goldenrod gall fly freeze intracellularly without subsequent harm to the cells of the fly. Also, x-ray diffraction demonstrates ice formation within viable human red blood cells during cooling to −196° at the rate compatible with their survival (Rinfret, 1963). A report by Sherman (1962) casts serious doubt about the intrinsic incompatability of intracellular ice with life. He showed by transplantation that slowly frozen (1°/min) parakeet tumours and mouse skin survived the presence of ice within the cytoplasm and nuclear karyoplasm. Rapid freezing (40°/s) was also employed and uniformly produced drastic decreases in survival rates. The morphologic changes studied by Sherman with light microscopy are in general agreement with the findings of Trump & others (1965).

CHARACTERISTICS OF CRYOPROTECTANTS

Table 1 makes evident that a wide variety of chemicals offer cells cryoprotection. For many centuries man has been aware that living cells under certain (ill-defined) conditions could survive freezing and thawing. The use of chemical agents to prevent death of frozen tissues was first fully recognized by the general scientific community when Polge, Smith, & Parkes (1949) published their historic paper. These investigators discovered that Meyer's histological albumin solution allows avian sperm to survive freezing and thawing from −79°. Further investigation revealed that glycerol was the active cryoprotectant ingredient. In 1950, Smith reported that glycerol would also prevent haemolysis in blood slowly frozen to −79°. Subsequently many other cells were shown to be protected from freezing injury by glycerol.

Spurred by the encouraging reports on glycerol, Lovelock (1954a) found that a variety of other mono- and polyhydric alcohols, sugars, and amides protected human red cells during relatively slow cooling and warming. Workers in Luyet's laboratory thoroughly studied the cryoprotective ability of ethylene glycol (Luyet & Keane, 1952) which had previously been shown to protect chick embryonic heart tissue (Gonzales & Luyet, 1950). Keane (1953) later even demonstrated the protective

ability of urea and acetamide for the embryonic chick frozen heart tissue preparation. Bricka & Bessis (1955) observed that polyvinylpyrrolidone (PVP) as well as dextran, both macromolecular polymers, gave cryoprotection to frozen human erythrocytes.

Ten years after publication by Polge & others (1949), Lovelock & Bishop (1959) introduced perhaps the most versatile of all cryoprotectants, dimethylsulphoxide (DMSO). This substance is as effective as glycerol in preserving most tissues and has the added advantage of rapidly attaining osmotic equilibrium across cell membranes. Since the discovery of the cryoprotective ability of DMSO, numerous other compounds noted in Table 1 have been studied for cryoprotective action.

Table 1. *Selected research reports on cryoprotective ability of various compounds*

Compound	Cell or tissue	Reference
Acetamide	Erythrocytes, human	Lovelock (1954)
	Renal cells, human, cultured	Vos & Kaalen (1965)
	Sperm, bovine	Polge & Soltys (1960)
	Trypanosomes	Polge & Soltys (1960)
	Heart tissue, chick, embryo	Keane (1953)
L-Alanine	Bone marrow, mouse	Phan The Tran & Bender (1960b)
Albumin	Erythrocytes, human	Schreiner & others (1962)
Ammonium acetate	Erythrocytes, human	Meryman (1968)
Chloroform	Heart, dog	Connaughton & Lewis (1961)
Choline	Bone marrow, mouse	Bender & others (1960)
Dextran	Erythrocytes, human	Bricka & Bessis (1955)
		deVerdier & others (1965)
	Bone marrow, mouse	Bender & others (1960)
Diethylene glycol	Erythrocytes, human	Lovelock (1954)
	Renal cells, human, cultured	Vos & Kaalen (1965)
Dimethylacetamide	Erythrocytes	Nash (1962)
	Renal cells, human, cultured	Vos & Kaalen (1965)
Dimethylformamide	Erythrocytes, human	Nash (1962)
	Renal cells, human, cultured	Vos & Kaalen (1965)
Dimethylsulphone	*Renal cells, human, cultured	Vos & Kaalen (1965)
Dimethylsulphoxide	Blood, bovine	Rapatz & Luyet (1965)
	Erythrocytes, human	Huggins (1963)
		Lovelock & Bishop (1959)
	Leukocytes, human	Bouroncle (1967)
	Mitochondria, tomato	Dickinson & others (1967)
	Renal cells, human, cultured	Vos & Kaalen (1965)
	Sperm, bovine	Lovelock & Bishop (1959)
	Cornea, canine and human	O'Neill & others (1967)
	Uterus, guinea-pig	Farrant (1965)
	Heart, rat	Karow & others (1965)
Erythritol	Erythrocytes, human	Lovelock (1954)
	Bone marrow, mouse	Bender & others (1960)
Ethanol	Erythrocytes, human	Lovelock (1954)
	*Renal cells, human, cultured	Vos & Kaalen (1965)
Ethylene glycol	Erythrocytes, human	Lovelock (1954)
	Renal cells, human, cultured	Vos & Kaalen (1965)
	Bone marrow, mouse	Bender & others (1960)
	Skin, mouse, rat	Taylor & Gernstner (1955)
Formamide	Renal cells, human, cultured	Vos & Kaalen (1965)
	*Erythrocytes, human	Lovelock (1954)
		Nash (1962)
	*Heart tissue, chick, embryo	Keane (1953)
Glucose	Blood, bovine	Rapatz & Luyet (1965)
	Erythrocytes, human	Lovelock (1954)
		Strumia & others (1960)
	*Bone marrow, mouse	Bender & others (1960)
	Heart tissue, chick embryo	Luyet & Keane (1952)
	Renal cells, human, cultured	Vos & Kaalen (1965)
	Sperm, bovine	Polge & Soltys (1960)
	Trypanosomes	Polge & Soltys (1960)

Table 1. *continued*

Compound	Cell or tissue	Reference
Glycerol	Blood, bovine	Rapatz & Luyet (1965)
	Erythrocytes, human	Lovelock (1954)
		Jones & others (1957)
		Lovelock & Bishop (1959)
		Pert & others (1965)
	Sperm, bovine	Polge & Soltys (1960)
	Tryanosomes	Polge & Soltys (1960)
	HeLa and L cells	Scherer & Hoogasian (1954)
	Renal cells, human, cultured	Vos & Kaalen (1965)
	Bone marrow, mouse	Bender & others (1960)
	Bone marrow, rabbit	Richards & Persidsky (1961)
	Bone marrow, human	Lochte & others (1959)
	Skin, mouse, rat	Taylor & Gerstner (1955)
	Cornea, rabbit	Capella & others (1965)
	Heart, hamster	Smith (1957)
	Heart, mouse, embryonic	Conway & others (1957)
	Kidney, dog	Halasz & others (1967)
	Mitochondria, rat, liver	Greiff & others (1961)
Glycine	Bone marrow, mouse	Phan The Tran & Bender (1960b)
Hydroxyethyl starch	Erythrocytes, human	Doebbler & Rinfret (1965)
Inositol	Bone marrow, mouse	Bender & others (1960)
	Renal cells, human, cultured	Vos & Kaalen (1965)
Lactose	Blood, human	Rinfret & Doebbler (1960)
	Erythrocytes, human	Strumia & others (1960)
		Rinfret (1963)
	Bone marrow, mouse	Bender & others (1960)
Magnesium chloride	Blood	Rapatz, personal communication
	Heart, rat	Karow & others (1967)
		Barner (1968)
Magnesium sulphate	Heart; rat, guinea-pig, rabbit, dog	Karow & others (1967)
Maltose	Blood, human	Rinfret & Doebbler (1960)
Mannitol	Bone marrow, mouse	Bender & others (1960)
	Renal cells, human, cultured	Vos & Kaalen (1965)
Mannose	Renal cells, human, cultured	Vos & Kaalen (1965)
Methanol	Erythrocytes, human	Lovelock (1954)
	*Bone marrow, mouse	Bender & others (1960)
	*Sperm, bovine	Polge & Soltys (1960)
	*Trypanosomes	Polge & Soltys (1960)
	Renal cells, human, cultured	Vos & Kaalen (1965)
Methylacetamide	Erythrocytes	Nash (1962)
Methylformamide	Erythrocytes, human	Nash (1962)
Methylurea	*Heart tissue, chick, embryo	Keane (1953)
Monoacetin	Erythrocytes, human	Lovelock (1954)
	Renal cells, human, cultured	Vos & Kaalen (1965)
Phenol	*Renal cells, human, cultured	Vos & Kaalen (1965)
Polyethylene glycol	*Erythrocytes, human	Lovelock (1954)
Polyoxyethylene	Erythrocytes, human	Doebbler & Rinfret (1965)
Polyvinylpyrrolidone	Erythrocytes, human	Bricka & Bessis (1955)
		Rinfret (1963)
		Doebbler & Rinfret (1965)
	Bone marrow, human	Persidsky & others (1962, 1965)
	Cartilage, human	Braid & others (1966)
L-Proline	Bone marrow, mouse	Phan The Tran & Bender (1960b)
Propionamide	*Heart tissue, chick, embryo	Keane (1953)
Propylene glycol	Erythrocytes, human	Lovelock (1954)
	Renal cells, human, cultured	Vos & Kaalen (1965)
Pyridine *N*-oxide	Erythrocytes, human	Nash (1961)
	Renal cells, human, cultured	Vos & Kaalen (1965)
Resorcinol	*Renal cells, human, cultured	Vos & Kaalen (1965)
Ribitol	Bone marrow, mouse	Bender & others (1960)
Ribose	Renal cells, human, cultured	Vos & Kaalen (1965)
L-Serine	*Bone marrow, mouse	Phan The Tran & Bender (1960b)
Sodium bromide	Bone marrow, mouse	Phan The Tran & Bender (1960a)
Sodium chloride	Erythrocytes, human	Doebbler & Rinfret (1965)
	*Renal cells, human, cultured	Vos & Kaalen (1965)
	Heart tissue, chick, embryo	Luyet & Keane (1952)

Table 1. *continued*

Compound	Cell or tissue	Reference
Sodium iodide	Bone marrow, mouse	Phan The Tran & Bender (1960a)
Sodium nitrate	Bone marrow, mouse	Phan The Tran & Bender (1960a)
Sodium nitrite	*Bone marrow, mouse	Phan The Tran & Bender (1960a)
Sodium sulphate	Bone marrow, mouse	Phan The Tran & Bender (1960a)
Sorbitol	Bone marrow, mouse	Bender & others (1960)
	Renal cells, human, cultured	Vos & Kaalen (1965)
Sucrose	Blood, bovine	Rapatz & Luyet (1965)
	Blood, human	Rinfret & Doebbler (1960)
	*Erythrocytes, human	Lovelock (1954)
	Bone marrow, mouse	Bender & others (1960)
	Skin, rat	Berggren & others (1966)
	Sperm, bovine	Polge & Soltys (1960)
	Trypanosomes	Polge & Soltys (1960)
Triethylene glycol	Erythrocytes, human	Lovelock (1954)
Urea	*Erythrocytes, human	Nash (1962)
		Doebbler & Rinfret (1965)
	*Renal cells, human, cultured	Vos & Kaalen (1965)
	Heart tissue, chick, embryo	Keane (1953)
L-Valine	*Bone marrow, mouse	Phan The Tran & Bender (1960b)
Xylose	Erythrocytes, human	Lovelock (1954)
	Sperm, bovine	Polge & Soltys (1960)
	Trypanosomes	Polge & Soltys (1960)

* Compound when used under conditions reported in the reference provides less than 40% survival.

Attempts to classify cryoprotectants on the basis of their cellular site of action have been unsuccessful. Some of these agents readily penetrate most cells (glycerol, DMSO) but others such as PVP and dextran must remain extracellular if they do not enter by pinocytosis. Sherman (1963) has presented evidence which even indicates that extracellular glycerol confers cryoprotection to nonfertilized mouse eggs or bull sperm while intracellular glycerol is actually toxic to these cells.

The cryoprotective ability of any given compound varies widely from tissue to tissue. It is well known that glycerol is almost ineffective as a cryoprotectant for bovine erythrocytes, but is a good agent for human red cells (Lovelock & Bishop, 1959). Similarly, human bone marrow frozen to -80° in 15% glycerol has 58% survival, while mouse marrow subjected to the same conditions has only 12% survival (Ferrebee, Billen & others, 1957). The wide differences in tissues and their response to any given cryoprotectant is probably due to an interrelation between physical factors and biological variability. Glycerol is impermeable to some cells (Lovelock & Bishop, 1959) but in others may be absorbed by active transport (Zieve & Solomon, 1967). The rate of passive diffusion of a cryoprotectant across cell membranes is related to the solubility of the compound, its electrical charge, viscosity, and the temperature of administration.

The importance of hydrogen bonding between water and cryoprotectants was probably first recognized by Keane (1953). He believed the freezing of "freezable" water could be prevented by bonding the water to protective agents. Nash (1962) has observed that cryoprotection seems to be related to hydrogen bond acceptor capacity (i.e. the presence of lone-pair electrons on the cryoprotectant rather than electron deficient sites). By the use of the parachor, Nash (1966) has been able to develop a "protection coefficient" (Q) for organic non-electrolyte compounds. All the known protective substances for erythrocytes studied by Nash have a Q

value equal to unity or greater, ranging up to 3·5. It will be interesting to observe if Q can be used to predict new cryoprotectants.

Various investigators have noted a similarity in the types of compounds which afford cryoprotection and those which protect cells from drying and radiation or which protect proteins from thermal denaturation. Webb (1965) believes that the reason glycerol, DMSO, and cyclic polyhydroxy molecules, such as sugars, protect bacteria and viruses from dehydration injury is related to the ability of these compounds to form hydrogen bonds. It is commonly known that radiation damage to proteins in aqueous solutions is due to radiolysis of water with subsequent free radical formation. Glycerol and DMSO protect bacteria, yeast, tissue culture cells, mice, and rats from radiation damage (Vos & Kaalen, 1962; Ashwood-Smith, 1962, 1967). Thrombin is readily inactivated by 50° heat, but the enzyme can be stabilized by glycerol (Milstone, 1942) and by various sugars or their derivatives (e.g. xylose, glucose, methylglucosides, methylgalactopyranosides, sucrose) (Seegers, 1944). Glycerol proved to be the most effective agent. Prothrombin, also thermolabile, is stabilized by some of these polyhydroxy compounds.

Perhaps the mechanism of cryoprotection afforded by various chemicals is related in some manner to the pseudo-toxic effect on cardiac muscle observed independently in the laboratories of Karow (Karow & Webb, 1965a; Karow, Carrier & Clower, 1968), Robertson & Jacob (1968), and Almond, Anido & others (1966). When mammalian heart muscle is treated with glycerol, DMSO, dextran, or magnesium—all of which are cryoprotectants—the muscle ceases to contract. If the cryoprotectant is flushed from the tissue within a given period of time, contractions resume. The observations of Farrant (1964) on the reduction of contractility of smooth muscle (guinea-pig uterus and intestine) subjected to various cryoprotectants (DMSO, glycerol, methyl formamide, methyl acetamide, dimethyl acetamide) may be related to the pseudo-toxic phenomenon. Also perhaps related is the reversible reduction of conduction velocity in the DMSO-treated isolated frog sciatic nerve (Sams, 1967). In contrast to this pseudo-toxic effect, true toxicity is manifested by irreversible cardiac arrest when the muscle is incubated with the cryoprotectant for an excessive period of time or in toxic concentrations.

This pseudo-toxic effect may be a reflection of the interaction of the cryoprotectant with cellular macromolecules, especially proteins, or with the water of hydration surrounding these macromolecules. DMSO is readily bound to proteins of tissue and of plasma by a mole-for-mole replacement of water (Gerhards & Gibian, 1967). Rammler (1967; Rammler & Zaffaroni, 1967) has presented evidence that DMSO produces conformational changes in enzymes. These changes are usually reversible when the DMSO is removed. Of course, DMSO can interact with organized water lattices. It is well known that magnesium, an essential constituent of enzymes, probably plays a role in the conformation of these proteins and is also capable of inducing the formation of water lattices by promoting hydrogen bond formation (Kavanau, 1964). In excess, magnesium inhibits several enzyme systems including ATPase (Liebecq, 1953; Noda, Kuby & Lardy, 1954; Webb, Dodds & others, 1966). Dextran, by virtue of its numerous hydroxyl groups, can form hydrogen bonds and adsorb to proteins. Ordinarily, dextran does not penetrate the interstitial spaces, but in the isolated perfused hearts, capillary integrity fails and presumably even dextran of 70,000 *M* is capable of entering the tissue compartment. Certainly, therefore, dextran might influence membrane phenomena of cells.

The true toxicity of an agent must be considered when selecting a cryoprotectant for a specific tissue. As intimated earlier, the effectiveness and the toxicity of compound varies with tissues within an animal and even for the same type of tissue taken from different species. Two types of toxic phenomena are recognized, the intrinsic toxicity of the compound and non-specific toxic phenomena associated with cryoprotectants in general.

The non-specific cryoprotectant toxic effects on tissues are related to concentration, temperature of administration, and duration of exposure. As expected, when the concentration of cryoprotectant increases, toxic manifestation increases. An increase in cryoprotectant exposure time before freezing, and presumably also after thawing, increases the probability of toxic reactions at a given cryoprotectant concentration (Karow, Carrier & Clower, 1968). Cryoprotectant toxicity is decreased at any given concentration as the cryoprotectant is administered at cooler temperatures. This temperature effect has been demonstrated for DMSO on rat hearts (Karow & Webb, 1965a), rat skin (Holst, Feigl & others, 1966), dog cornea (O'Neill, Mueller & Trevor-Roper, 1967), guinea-pig uterus (Farrant, Walter & Armstrong, 1957), and human leukocytes (Bouroncle, 1967), as well as for glycerol on rat hearts (Karow & Webb, 1965a).

A few preliminary investigations have been made into the efficacy of cryoprotectant combinations. Diamond (1964) obtained 30 to 40% viability in cultures of *Entamoeba invadens* treated with 0·24M glucose plus 2·1M DMSO, slowly cooled, and rapidly warmed from liquid nitrogen temperatures. Strumia, Colwell & Strumia (1960) found that dextrose (0·5 to 1·2M), lactose (0·1 to 0·4M), or a mixture of lactose and dextrose (0·3 to 1·0M) allowed 90% or greater survival of rabbit and human erythrocytes cooled at a rate of approximately 100°/s to −65° followed by an equally rapid warming and thawing. Djerassi & Roy (1963) found that rat platelets treated with DMSO and sugar (dextrose, xylose, sucrose) could be frozen by immersion in liquid nitrogen yet upon thawing retain their morphologic integrity and ability to circulate in thrombocytopenic animals. The use of 5% dextrose plus 5% DMSO in plasma permitted a circulating yield of thawed platelets as high as 70 to 87% of the number observed when fresh platelets were injected. The use of either a sugar or DMSO alone provided slight or no cryoprotection. Other workers (Capella, Kaufman & Robbins, 1965) have found that 0·3M sucrose plus 1·4M DMSO provided better cryoprotection for frozen cornea from rabbit and man than either agent alone. Sucrose (0·3M) alone or in combination with DMSO (1·4M) or with glycerol (1·4M) provided frozen rat skin (Berggren, Ferraro & Price, 1966) with cryoprotection superior to that of DMSO or glycerol. Considering the ability of DMSO to assist the penetrability of compounds which are usually biologically impermeable, Robertson & Jacob (1968) have suggested that the combination of DMSO with other cryoprotectants may give cryoprotective synergism. They provide excellent experimental evidence with DMSO plus sucrose which supports this inviting idea. In my laboratory we (Karow, Webb & Stapp, 1965) have found that 6% dextran (*M* 70,000) in saline and 1·8M DMSO provide good cryoprotection to the rat frozen isolated heart when used individually but no protection when used in combination.

MECHANISMS OF CRYOPROTECTANT ACTION

Several attempts have been made to explain how chemicals are capable of offering cryoprotection. Such theories should account for the specificity of cryoprotectants,

i.e. why specific agents are protective and other similar compounds offer no protection (Doebbler & Rinfret, 1962). One of the first theories was proposed by Luyet and associates (Gonzales & Luyet, 1950; Luyet & Keane, 1952, 1953; Luyet & Gehenio, 1952a, b). They observed that one mm^3 fragments of chick embryo heart could survive rapid thawing after direct immersion into liquid nitrogen (−195°) if the tissue was treated before freezing with hypertonic solutions of ethylene glycol (5·3M), glycerol (3·5 to 7·5M), glucose (1·5M), or sodium chloride (1·0M). The evidence presented by these investigators indicates that partial osmotic dehydration of the tissue is of paramount importance in the survival of the rapidly frozen embryonic chick heart tissue. Other characteristics of the chemicals which they believe contribute to cryoprotection are "easy penetration into the tissue, relatively low toxicity, efficiency in binding water (as expressed in the colligative properties) and low eutectic temperature". These workers observed that air dehydration did not provide cryoprotection to the chick tissue but did protect "vinegar eels" (*Anguillula aceti*) frozen at −77° without the benefit of chemical protection (Gehenio & Luyet, 1951). Luyet & Gehenio (1940) have also provided an extensive review of other animals which survive freezing after partial dehydration.

Unfortunately the dehydration theory does not take into account the specificity of cryoprotectants. Additionally, Lovelock (1953b), on the basis of experimental evidence obtained with human erythrocytes, discredits the importance of dehydration. He found that glycerol provides excellent cryoprotection when the cells are immersed in a cold bath at −35° even though these red cells are only transiently dehydrated by suspension in glycerol (2·4M). They are rapidly restored to their original degree of hydration before freezing as the glycerol permeates the cell. If the red cells are treated with copper they are rendered impermeable to glycerol. Even though the copper-treated erythrocytes dehydrate when exposed to glycerol, the glycerol offers them no cryoprotection. In comparing the results presented by Lovelock (1953b), by Luyet & Gehenio (1952a), and by Luyet & Keane (1953) one must consider the important differences of tissue, rates of cooling, and the fact that some chemicals, including glycerol, apparently provide certain tissues with cryoprotection even though the agent is unable to permeate the cells (Bricka & Bessis, 1955; Sherman, 1963). Certainly it is premature to disregard the possible importance of partial dehydration as a means of cryoprotection.

The cryoprotectant theory offered by Lovelock (1953a, b; 1954a, b) has perhaps received the widest acceptance. In a series of experiments with samples of frozen mammalian erythrocytes, Lovelock (1953a, b) related the rise of ionic concentration with the extent of haemolysis. He demonstrated that the degree of haemolysis produced by freezing erythrocytes is equivalent to the haemolysis of red cells exposed to hypertonic saline at a concentration comparable to that found in frozen normal saline. In the presence of glycerol, the development of damaging concentrations of electrolytes was observed to be greatly reduced. Lovelock (1953b) concluded from his experimental results that the mechanism of protection seems to lie primarily in the colligative properties of the non-electrolyte cryoprotective solute which theoretically lowers the effective concentration of salt in equilibrium with ice at any given temperature below freezing. Thus by adding a cryoprotectant to the cells to be frozen, Lovelock (1953b) believed that the increase in salt concentration during freezing would not reach a biologically damaging level before the temperature was so low that the reaction rate of the injurious processes would be negligible. He

predicted that the ideal cryoprotectant should have a low molecular weight, be non-toxic in excessive concentrations, be highly soluble in aqueous electrolyte solutions, and possess the ability to permeate cells (Lovelock, 1953b, 1954a; Lovelock & Bishop, 1959). Experimental observations on the erythrocyte cryoprotective ability of 15 neutral organic solutes seemed to substantiate these ideas (Lovelock, 1954a).

A major triumph of this theory was its use to predict the cryoprotective capacity of DMSO (Lovelock & Bishop, 1959). Additional independent support for the Lovelock hypothesis is the observation that 10% (v/v) DMSO, a standard protective dose for most tissues, imperfectly protects the smooth muscle of the guinea-pig; but the muscle receives excellent protection from the gradual administration of 55% (v/v) DMSO. In the later instance the organ does not freeze even when cooled to −70°. In fact, uteri treated with high DMSO concentrations could be warmed slowly and still function well after thawing. Also, Meryman (1968) has been able to demonstrate that 4M ammonium acetate, a penetrating, relatively non-toxic salt with no apparent eutectic point, will protect human erythrocytes which have been frozen by slow cooling to −70°, and then thawed. Conversely ammonium chloride, with a eutectic point at −15·8°, is useless as a cryoprotectant for red cells.

Other observations have also been made which tend to discredit the colligative theory. Ethylene glycol (*M* 62) and E500M, a mixture of polyethylene glycols (*M* 635, range: 300 to 1,450), are both cryoprotectants for erythrocytes. One would predict from the colligative theory that ethylene glycol would be 10 times as effective as an equal weight of E500M, but Sloviter (1962) showed that both compounds were maximally effective at 40 g/100 ml. In other laboratories, equal cryoprotection was observed to be conferred to erythrocytes by a 10% (w/v) solution of glycerol or of PVP (Doebbler & Rinfret, 1965) and to mammalian cardiac muscle by 1200 mM DMSO, 66 mM Mg^{++}, or 0·86 mM dextran (Karow & others, 1968).

The colligative theory fails to explain other observations such as the cryoprotection afforded by non-penetrating compounds (see Table 1) such as sucrose, PVP, and dextran. Persidsky & Richards (1962) demonstrated that bone marrow cells are protected by PVP (*M* 30,000) during freezing and thawing. Although these cells are capable of absorbing PVP by pinocytosis, cryoprotection is not dependent upon intracellular PVP. The cells are protected even when the pinocytotic process is inhibited. Finally, the colligative theory offers no explanation of specificity; all low molecular weight solutes, regardless of their cryoprotective ability, reduce electrolyte concentration in equilibrium with ice during freezing. Hydrogen bonding could provide an explanation for molecular specificity in cryoprotection (Doebbler & Rinfret, 1962), but in no way does Lovelock implicate hydrogen bonding in his original theory.

Almost all solutes in water have the capability of retarding ice crystal growth. Furthermore, x-ray diffraction studies made in the Union Carbide Linde Laboratories (Dowell, Moline & Rinfret, 1962; Doebbler & Rinfret, 1965) indicate the presence of vitreous ice in rapidly frozen solutions of known cryoprotectants, but only hexagonal crystals in frozen solutions of structurally similar (but nonprotective) compounds. The growth rates of ice in super-cooled aqueous solutions of a variety of solutes have been positively correlated with the mole-equivalent of potential hydrogen-bonding sites provided by the solutes (Doebbler, 1966). The more hydrogen-bonding sites which a solute is capable of providing, the greater the reduction of the rate of ice crystal growth. Doebbler & Rinfret (1962) have shown that multiple hydrogen-bonding sites per molecule markedly increase protective capacity.

Doebbler (1966) suggested that cryoprotectants act by establishing hydrogen bonds with water, reducing the amount of water available for crystallization and thereby promoting the formation of vitreous ice. Thus cellular sites which would be functionally altered by the loss of bound water are protected since the formation of crystalline ice is avoided. Even though the presence of vitreous ice is yet to be resolved definitely (Luyet, Tanner & Rapatz, 1962), the theory of Dobbler has a great deal of merit.

Other theories place major emphasis on the alteration of protein conformation by ice. One of these theories perhaps may be said to have originated with published thoughts of Luyet & Gehenio (1940, 1952a, b) on the relation between dehydration and freezing injury. In essence, these workers contended that death from freezing was ultimately the result of the irreversible loss of cellular water essential to life, the "bound water". Later, Meryman (1956) noted that since glycerol could bind to water, it would limit the amount of water available for crystallization and thereby reduce the extent of cellular dehydration during freezing. In 1962, Doebbler & Rinfret (1962) re-emphasized the importance of hydrogen binding groups on cryoprotective compounds. They stated that "H-bonding sites would serve not only in primarily binding water, but also in forming and stabilizing an extended region of oriented water around each molecule. Disruption of the hydrated structure of the red-cell membrane could be a possible mechanism of freezing injury in addition to the chemical action of salt or the mechanical action of ice. H-bonding protective solutes may act in part to stabilize the surface hydration of the cell". Independently, Karow & Webb (1965b) arrived at a similar conclusion, i.e. that cryoprotectants, by virtue of their ability to create hydrogen bonds with water, stabilize the hydration lattice surrounding proteins and thereby reduce the probability of protein denaturation by desiccation during freezing. In a sense, cryoprotectants, according to this concept, "prefreeze" the cell to prevent the loss of water which serves to maintain structural conformation.

Although there is much evidence to support the concept that protein denaturation, via desiccation, is an important factor in freeze-injury, there is apparently no definitive experimental evidence to support the idea that hydration lattices of macromolecules are maintained intact during freeze-survival. However, Massaro (1967) has presented evidence which may be interpreted as suggesting that cryoprotectants are capable of preventing protein denaturation by preventing disturbance of the shell of water which hydrates proteins. Lactic dehydrogenase is a protein which is hybridized by freezing (Chilson, Costello & Kaplan, 1965) and also by the hydrogen-bond disrupting compound urea (Massaro, 1967). Cryoprotectants such as DMSO and polyhydroxy compounds not only prevent freeze-induced hybridization but also urea-induced hybridization. Also Doebbler & Rinfret (1962, 1965) have reported that urea decreases the amount of cryoprotection afforded by cryoprotective agents. Of course similar results are also predicted by the theories of Levitt (1962, 1966) and of Heber & Santarius (1964).

Levitt (1962, 1966) has proposed, in contrast to the hypothesis that cryoprotectants act on cellular water, that these agents attach directly to proteins. As water is removed from proteins during freezing, glycerol might attach by hydrogen bonds to the hydrophilic sites on proteins and presumably serve to maintain protein conformation. The glycerol would thereby substitute for the bound water and prevent denaturation by forming a protective coat around protein molecules.

Heber & Santarius (1964) also suggested that organic hydroxy-compounds such as

sugars are cryoprotectants by virtue of their ability to retain water or substitute for water in structures sensitive to dehydration. They noted that monosaccharides have been shown to form unstable, hydrogen bonded complexes with proteins in aqueous solutions (Giles & McKay, 1962). Since water firmly bound to disaccharides prevents them from reacting with proteins in the manner of monosaccharides, Heber & Santarius (1962) proposed that during freezing, a portion of the water bound to disaccharides may be lost and thereby permit disaccharide-protein interaction. They also postulated that even sugars larger than disaccharides, presumably polymeric sugars, might provide protection in a manner similar to the disaccharides.

The theories of Levitt (1962, 1966) and of Heber & Santarius (1964) both suggest that protection results from the stability of the hydrogen bonds established between the cryoprotectant and proteins. These hydrogen bonds do not rupture during the freezing process. Just as in the theories previously discussed, there is no good experimental evidence to support the concepts of these authors. As with other theories, a serious objection is that they cannot explain in an entirely satisfying and convincing manner the mechanism by which exogenously administered high molecular weight compounds (e.g. PVP, dextran) serve as cryoprotectants. Nevertheless, in consideration of the apparent importance of water to protein conformation, it may be expected that evidence will be found to support at least one of the theories that relate chemical cryoprotection to an active and direct maintenance of protein conformation during freezing.

In contrast to these theories, Shikama (1963) has suggested, on the basis of experimental data he obtained from freezing myosin A, myosin B, and catalase, that cryoprotectants stabilize the clathrate-like icebergs on native proteins rather than directly modify ice crystal formation. He observed that although cryoprotectants do reduce the extent of denaturation, they are incapable of altering either the time course or the critical temperature (−10 to −80°) of freeze-induced denaturation. Based on Claussen's (1951) report that some clathrates have a cubic pentagonal rather than a hexagonal framework, Shikama assumes that clathrates around nonpolar side chains of amino-acids will also be cubic. Since cubic clathrates have a very low thermodynamic stability, Shikama proposes that their structure might be altered when exposed to hexagonal ice. He noted that several laboratories have reported electron diffraction studies of ice which indicate that only hexagonal crystals form in pure water frozen at temperatures warmer than −80°. Vitreous ice which has been formed by rapid cooling to −180° will transform to cubic ice when warmed to −140°. When further warmed to −80°, the transition from cubic to hexagonal crystals is complete. Shikama believes that the cubic structure of clathrates would be kept stable in the presence of cubic ice. In his experiments, enzymatic inactivation was maximal in the range of −10 to −80° which corresponds to a high yield of hexagonal ice crystals. When he quickly cooled the enzymes to temperatures below this range, the inactivation was minimal. When the enzymes were warmed to any given temperature in the critical range above −80°, the extent of inactivation which occurred coincided with the inactivation obtained by cooling the enzyme directly to that critical temperature.

For Shikama's theory to be seriously considered, its assumptions must be better grounded. All the proteins of known tertiary structure are folded in such a way that clathrate-forming hydrophobic groups are oriented toward the interior of the molecule (Kendrew, 1962; Blake, Koenig & others, 1965; Kartha, Bello & Harker, 1967).

Hydrogen bonding groups compatable with hexagonal ice are found on the "surface" of proteins. Thus hexagonal ice probably might not interfere at all with clathrate-like structures. In any case, by no means do all clathrate hydrates attain a cubic configuration. This is especially true of clathrates around polyfunctional guests (guests with nonpolar groups as well as ionic and hydrogen-bonding sites) such as would be expected of proteins (Frank, 1967, personal communication). Finally, his theory does not explain the well known observation that slow cooling, conducive to hexagonal crystal formation, increases the survival rates of most frozen cells.

Lohmann, Fowler & others (1964) have proposed that glycerol's cryoprotective ability depends upon the chelation of metal ions that are important and necessary to biological activity. They demonstrated by electron spin resonance studies that glycerol forms a complex with certain metal ions including Cu^{++}, and Fe^{+++}. The existence of such complexes in the biological environment would severely limit the ability of glycerol to penetrate cell membranes. The data presented by Lohmann's group (1964), suggests that glycerol would offer cryoprotection when used in concentrations that are lower than those known to be necessary. Perhaps future investigations from Lohmann's laboratory will elucidate further their proposal.

Some of the objections to the discussed theories are obviated if one is willing to concede that it is not necessary to insist upon a single unified comprehensive mechanism of chemical cryoprotection. It may well be true that some—or even all—cryoprotectants operate through more than one of the discussed mechanisms. It cannot be doubted, however, that much more research is required in order to convert the theories of cryoprotection to actual fact.

SUMMARY

Cryoprotectants are a new class of drugs which specifically act to maintain the viability of frozen animal cells. The chemical spectrum of these agents includes certain polyhydroxy alcohols, sugars, inorganic cations, amino-acids, and macromolecules as well as some sulphoxides and amides. All of the cryoprotectants are water soluble. They seem to act by physically or chemically modifying the cellular water before freezing. There is substantial evidence which indicates that cryoprotectants directly interact with the hydration shell of biologically important macromolecules and thereby influencing macromolecular conformation. It is conceivable that future studies on cryoprotectants will not only serve to elucidate their mechanism of action, but also contribute to the design of other types of drugs which generally act by altering the receptor hydration shell. Perhaps even more important will be new insights into the role of water in biological systems.

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A simple infrared spectrum retrieval system

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The retrieval of an unknown spectrum from a medium-sized collection (up to 10,000) of infrared curves has been studied and a system based on a film feature card using microfilm storage developed. Tests of alternative methods of coding resulted in the choice of the six most intense absorption peaks in defined areas of the spectrum. Correct identification within 1–2 min is achieved in over 95% of searches. The system is compact, easy to operate, relatively inexpensive and easily perused.

Infrared spectroscopy is a valuable aid to the forensic scientist for the unequivocal identification of a wide variety of drugs and other "scene of crime" materials. This method of qualitative analysis requires comparison of the unknown spectrum with that of an authentic sample of the material and confirmation that the two spectra are identical. Frequently, the forensic scientist has to identify unknown material and thus it is necessary to select similar curves for visual comparison by searching through reference spectra of a large number of compounds, a laborious and time-consuming task. By utilizing modern information techniques this effort can be minimized (Curry, D. R., 1963; Thomas, 1968).

Rapid identification of unknown compounds by infrared spectroscopy requires the availability of a comprehensive collection of reference spectra and means for the retrieval of individual spectra from the collection by name and by the spectral characteristics of the curves themselves. Every curve must therefore be reduced to a series of parameters or codes and the retrieval system must be capable of rapidly searching these data to select the spectrum identical to that of the unknown under investigation. The chosen method of coding the spectra should give the maximum spread of information, reducing bunching of the curves about major spectral characteristics so that a minimum number of spectra are retrieved. The reference spectra should be in a form which is compact for storage, which can be readily inspected for comparison purposes and which is flexible enough to cope with the continuing stream of new additions to the collection.

The system found by us to be the most suitable for use in a routine forensic science laboratory is described. For our work on retrieval the "Harrogate" infrared collection of the North-East Forensic Science Laboratory (Haddon Lodge, 32 Rutland Drive, Harrogate, Yorkshire) which contains the curves of 1100 compounds of importance to the toxicologist, was used. These spectra had been recorded as Nujol mulls or liquid films using the Perkin-Elmer Infracord 137 instrument.

Infrared spectrum retrieval systems

For the rapid identification of unknowns it is essential to be able to retrieve individual spectra by predetermined spectral characteristics (e.g. by position of major absorption bands) as well as by chemical name. The manual methods for retrieving spectra from reference collections by band position data are (1) absorption band

indexing and (2) punched card systems, including edge punched, body punched and feature card systems. Recently, large libraries of spectroscopic data have been stored on magnetic tape and the collections searched by computer (Anderson & Covert, 1967; Cross, Shields & Stanier, 1966; Erley, 1968). The alternative MIRACODE system for large collections of spectra requires the reference curves with their corresponding coding details to be photographed onto 16 mm film. The resulting library is then automatically scanned by a high speed photoelectric retrieval system. The cost of large systems limits their applicability to large libraries of data with a correspondingly large number of requests for searches and therefore to the central laboratory of an organization, thus restricting immediate availability.

Absorption band indexing

Small laboratory collections of spectra are frequently arranged in order of the peak having the maximum absorbance. If the wavelengths considered in coding are in the "fingerprint region" of the spectrum, grouping of large numbers of curves about a single wavelength is reduced to a minimum (Curry, A. S., 1963). This classification can be further improved by including two major peaks in the coding instead of one. Such simple manual methods of selecting curves are only efficient for infrared collections containing at the most a few hundred curves.

An extension of the major band method of classification is the commercially available Sadtler Spec-finder indexing system (Heyden and Son Ltd., Spectrum House, Alderton Crescent, London, N.W.4, England) for the identification of unknown compounds. In this system the wavelengths of the strongest bands in thirteen 1 μm intervals of the spectrum are arranged in groups depending on the most intense band, all wavelengths being recorded to the nearest 0·1 μm. Identification is by comparing the code of the unknown with numerical lists in the index and retrieving similarly coded curves by spectrum number for final visual comparison. The disadvantage of peak position indexing systems is that the bulky indices are difficult to maintain in the case of a randomly expanding collection, as updating requires the indices to be completely retabulated. This is only practical for the smallest laboratory collection or for large commerical collections where the revised numerical lists can be automatically prepared by computer.

Edge-punched card systems

Edge-punched cards are the simplest of the card systems and have been used extensively (Thompson, 1955; Muir & Hardie, 1962; McArdle & Skew 1965; and others). In an edge-punched retrieval system, coding details which describe each spectrum are represented by a series of holes along the edges of the card. The spectral parameters are incorporated by converting the holes assigned to these characteristics into deep slots. All other necessary information is included in the body of the card.

To search the collection, the complete set of cards is aligned and sorting needles inserted through the holes corresponding to the coding details being searched. All cards which contain the characteristics required fall away from the main collection. This procedure is awkward and tedious with a large collection and machine sorting methods must then be used. The edge-punched card system, however, has one big advantage; each card can be produced and used independently of any other. The disadvantage is the rapid deterioration of the cards with use and punching additional sets is laborious and costly.

Body-punched card systems

Body-punched cards operate on the same principle as the edge-punched system, but coding details of the spectrum are stored by punching holes on the body of the card (Casey, Perry & others, 1958). This enables the card to accommodate more punched data but only at the expense of other scientific information. Frequently, therefore, this system can only refer the searcher to the original spectrum by compound name or serial number. Retrieval using body-punched cards necessitates the examination of all the holes simultaneously and for this electromechanical sorting techniques must be used.

Feature card systems

Feature cards (commonly known as term entry, optical coincidence or peek-a-boo cards) use the principle of assigning one search characteristic to each card and punching holes into the card, corresponding to the number of each spectrum to which the characteristic applies (Curry & Moore, 1963; Schlichter & Wallace, 1963; Kaiser, 1965). The collection of data is searched by selecting the cards having the coding details required and aligning them before a light source and reading off the numbers of the coincident holes through which light appears (Fig. 1). These numbers correspond to spectra possessing all the features selected. Like body-punched cards, feature card retrieval systems require the actual spectrum to be retrieved from a subsidiary storage system.

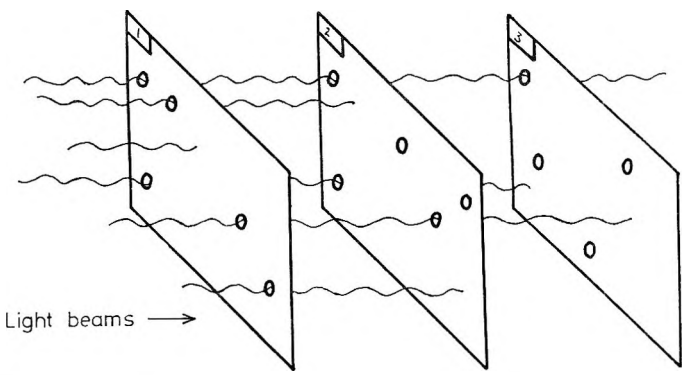

FIG. 1. Principle of operation of the feature card retrieval system.

Comparison of retrieval systems

Small laboratory collections of spectra (<2000 items) to which reference will be made infrequently are most conveniently served by manually operated edge-punched card systems, but the search time increases in proportion to the number of items. Where large numbers of cards are involved a mechanical means of sorting is needed. The cost of electro-mechanical sorters, however, can seldom be justified in small laboratories and for this reason both body-punched and edge-punched cards are not ideally suited to a collection of many thousands of cards. If feature cards are used then simple manual methods of retrieval are sufficient for systems containing up to 10,000 items. Feature card systems are compact, easy to operate, readily updated, easily perused and are undoubtedly superior for medium sized collections of spectroscopic data in small laboratories. For the very large collections of data fully automatic information processing techniques which require the use of computers become economically attractive.

Coding of infrared spectra

The basic data for any infrared retrieval system are derived from the accurately measured wavelengths of the major absorption bands which are characteristic of each spectrum. Frequently, negative search terms such as the absence of absorption bands in specific wavelength regions are included to eliminate unwanted data. The system devised in our laboratory makes use of the major peaks in the 5·0–15·0 μm spectrum range and these are recorded to the nearest 0·1 μm. Those bands appearing in the 6·7–7·6 μm range that are strong enough to be included in the coding are omitted to avoid complications in the examination of the spectra obtained from Nujol mulls. To ensure simplicity in coding procedures we have defined the strongest bands as those bands whose peaks are nearest to 0% transmittance, irrespective of the shape of the background. Shoulders are only counted as bands if they are completely resolved and the point of maximum absorption easily determined. In cases where two or more bands of equal intensity tie for the final coding detail, all these peaks are included in the coding of the spectrum. If the major peak is a hump rather than a sharp band and the point of maximum absorbance not easily determined, or the band too intense as a result of the sample being too thick, the wavelength of the peak mid-point is taken as the coding detail.

In our retrieval system, each 0·1 μm of the wavelength region considered in coding represents a possible coding detail or characteristic feature of the spectrum. This gives a total of 92 features to describe each spectrum. To allow for small variations in band positions resulting from differences in instrumentation and analytical conditions and techniques, it is necessary to include a tolerance of $\pm$ 0·1 μm during searching. In practice this is achieved by coding features 0·1 μm to each side of the coded spectral peak.

Our initial studies employing hand punched Carter-Parrett feature cards (J. L. Jolly and Partners Ltd., Orchard Road, Sutton, Surrey, England) indicated that a minimum of five or six spectral characteristics would have to be coded if the number of false (but equally probable) retrievals per search is to be acceptable for a collection containing several thousand curves. These five or six absorption bands may be selected in two ways. The selection of the six most intense bands in the spectrum is straightforward (six peak method of coding). For some spectra this may restrict the coding to the intense bands originating from the functional groups at the expense of relatively less intense bands in the fingerprint regions. These "fingerprint bands" are important because they exhibit differences between closely related compounds. Alternatively, the most intense bands in five specific intervals of the spectrum can be coded. This method called the five range method of coding ensures that details of the fingerprint spectrum range are included in the coded description of the compound. In our studies the following five ranges were used for this coding method (1) 5·0–6·6, (2) 7·5–8·9, (3) 9·0–9·9, (4) 10·0–10·9 and (5) 11·0–15·0 μm.

To determine the most satisfactory method of coding, the complete "Harrogate" collection of spectra was coded using both the five range and six peak methods. To eliminate the laborious task of punching cards on this scale the coding data were stored on magnetic tape and the spectroscopic library searched by computer (I.C.T. model 1301) in the manner of a retrieval system. The efficiencies of the two coding methods were determined by coding fifty test spectra and the spectroscopic library searched to recover these curves using the procedure which would be employed for the identification of unknown compounds. All the test spectra were coded from written instructions

by an operator not directly involved with infrared work. The first twenty-five of these spectra were copies of reference spectra in the "Harrogate" collection. The remaining test spectra were of compounds for which reference curves were available in the reference library but which had been recorded under different conditions and on different spectrometers. The results of these tests are indicated in Table 1.

Table 1. *Retrieval efficiencies obtained for the 5 range and 6 peak coding methods*

Source of test spectra	Method of coding	No. of features used	No. of spectra correctly retrieved	Average No. of spectra retrieved per search
25 "Harrogate" ..	5 range	4	23	Very large
		5	21	6
	6 peak	5	25	20
		6	25	7
25 "Unknowns" ..	5 range	4	21	Very large
		5	21	8
	6 peak	5	25	15
		6	25	4

The results indicate that of the two methods, the six peak is superior and it is encouraging that this method gave no failure to retrieve the spectrum of interest. The results also emphasize that coding a minimum of six peaks is essential for efficient retrieval from a medium sized collection when this method of coding is used. Detailed examination of the spectra and coding details indicated that the failure of the five range method to select the correct curve was in many cases due to the inclusion (or exclusion) of unreliable peaks of low absorption in the final code. These doubtful bands would not qualify for inclusion with the six peak method. It is of interest that the overall distribution of coding details obtained for the complete "Harrogate" collection by the two methods are not as markedly different as might have been anticipated (Fig. 2A, B).

Spectrum storage systems

All manual methods of spectrum retrieval, excluding edge-punched card systems, yield a serial number which directs the searcher to the location of the standard curve in an independent storage system. For small laboratory collections, the reference spectra are normally stored either numerically or alphabetically in suitable loose leaf folders. This arrangement requires ample storage facilities for the larger collections and suffers from the disadvantage that day to day removal and subsequent replacement frequently results in damage and loss of individual curves. It is, therefore, undesirable that collections should be stored in this manner.

A more suitable and compact system of storing spectroscopic records is microfilm filing. With such a system printed copies of individual curves can be obtained immediately for detailed study using a reader-printer facility while the original microfilmed collection is retained intact. A further advantage is the ready availability of inexpensive microfilm copies of the complete reference collection for distribution to other laboratories.

Infrared retrieval and storage system in use at the Central Research Establishment

This laboratory will soon have over 2,500 spectra of materials which are pertinent to forensic science comprising not only a large number of pharmaceuticals but also substances likely to be met in a routine forensic science laboratory such as butter,

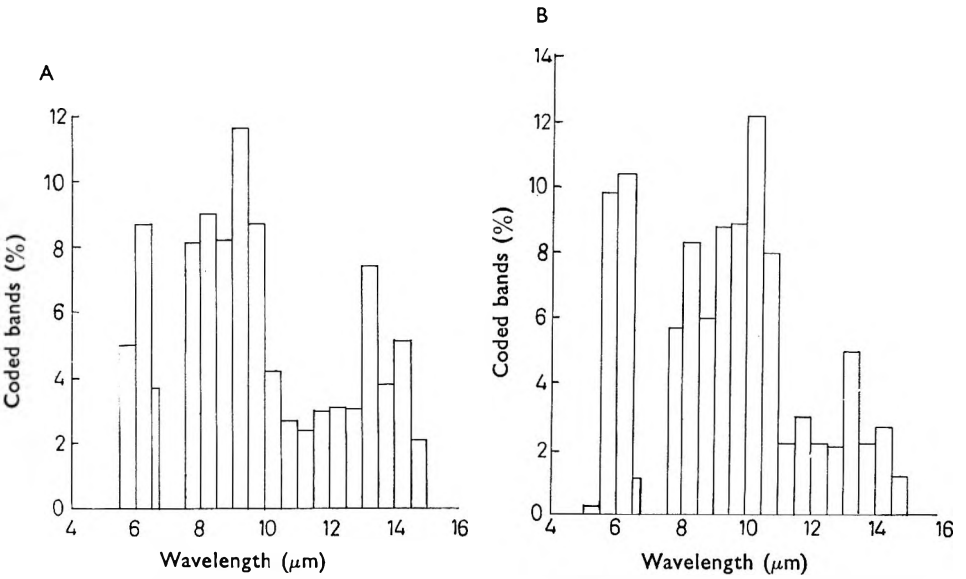

FIG. 2. Distribution of coded bands throughout the infrared spectrum, obtained (A) with the six peaks coding method and (B) with the five range method.

soap and Vaseline. This collection is steadily expanding as new items are encountered and these new additions have to be included in the system. The physical size of this spectroscopic library prohibits its publication for circulation to other workers and these curves are gradually being photographed on microfilm and copies of this film circulated to other forensic science laboratories in the British Isles. At the present time the complete "Harrogate" collection has been microfilmed. This photographic library of curves contains an index which lists the spectra in alphabetical order with an accession number. This accession number is also the same as that obtained from the infrared retrieval system described below.

The most suitable method of retrieving individual curves from a collection of this size is to use feature cards. The coincident card system manufactured by Materials Data Ltd. (19/27 South Street, Farnham, Surrey, England) has several features which make it more attractive than the alternative systems which are available. The MDL feature card can accommodate 3,600 items and the system is normally searched using six cards simultaneously. A larger card able to accommodate twice as many items is being developed. The chief advantage of the MDL system is that the cards are accurately lithographically reproduced on translucent polyester material from punched masters. This not only produces an extremely robust and hard wearing card but also eliminates the costly task of repeatedly punching separate sets of cards. Periodic updating is easily achieved by punching additional holes in the reserved masters with subsequent reproduction of replacement sets of cards.

The MDL system containing all the six peak coding details (see coding of infrared spectra) of the complete "Harrogate" collection of eleven hundred spectra has been in everyday use in our laboratory for several months. Our experience has shown that this collection can be sorted for six spectral characteristics and the spectrum of an unknown identified by matching with the corresponding reference curve in 3 to 5 min. The retrieval efficiency of this system is indicated in Figs 3 and 4. First searches have resulted in the successful identification of 96% of the unknowns which have reference

curves in the collection. The average number of spectra retrieved per search is 2·4. However, the number of false, but also equally probable, retrievals will increase with the population of the collection and it may be necessary to include additional coding features in the system at a later stage. In the few cases where the first search fails to select the correct reference curve, secondary searches are made using combinations of the coded bands. In this context the MDL system is advantageous in that "near-misses" are readily detected by the relative intensities of light appearing through the "holes" or light paths on the card. It is our experience that the correct reference curve

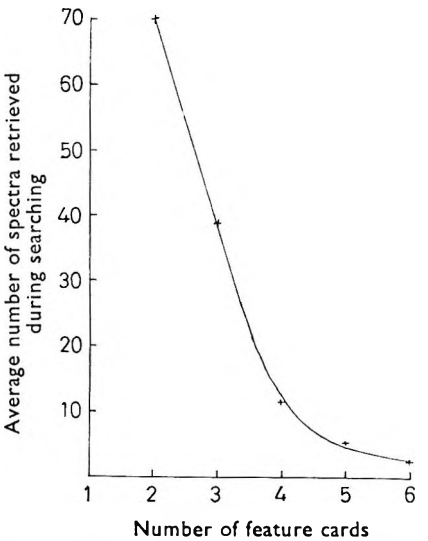

FIG. 3. Fall off in the number of retrievals with increasing number of feature cards for a collection containing eleven hundred items.

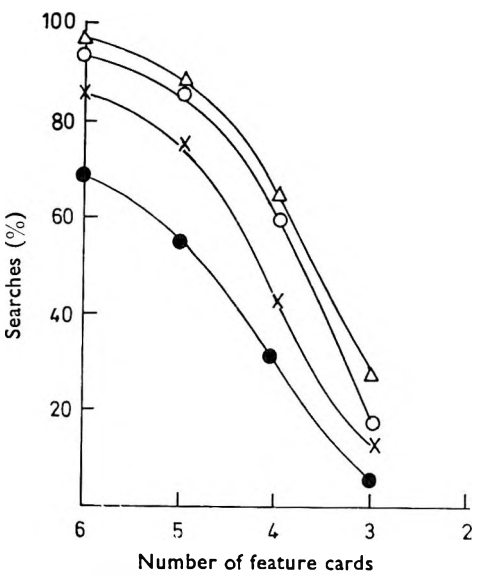

FIG. 4. Percentage of searches yielding x or less retrievals at different stages of searching a collection of eleven hundred items. △ x = 10. ○ x = 8. X x = 4. ● x = 2.

is always selected within two searches. The failure to obtain complete coincidence of coding between the unknown and reference spectra can be attributed to the following factors:

1. Impurities in the unknown or reference samples may introduce additional bands which are strong enough to be included in the coding.

2. Variations in the levels of background absorptions may effect the relative intensities of the absorption bands in the two spectra.

3. Polymorphism in organic compounds may result in the inversion of peak intensities (Mesley & Johnson, 1965; Mesley & Houghton, 1967; Mesley & Clements, 1968 and Mesley, Clements & others, 1968).

4. Grating instruments with high resolution may resolve peaks which appear as shoulders with instruments incorporating only prism optical systems.

It will be obvious that these factors will cause some difficulties with all peak position infrared retrieval systems.

The system described has aroused considerable interest in other laboratories concerned with the problem of drug identification and it is intended to make this system commercially available. We are at the present time considering other possible forensic applications of this type of feature card retrieval system. One such application is an aid to the rapid identification of solid dosage forms for use in hospitals as well as forensic science laboratories.

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Toxicity of ethanol-barbiturate mixtures

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The simultaneous administration of ethanol at doses of either 2, 3, or 4 g/kg intraperitoneally produced a dose-related decrease in the intraperitoneal LD50 for thiopentone, pentobarbitone, amylobarbitone, phenobarbitone and barbitone in rats. The most marked ethanol-barbiturate interaction was with the long-acting, poorly metabolized, less potent barbiturates phenobarbitone and barbitone. Similarly, a non-hypnotic dose of ethanol (3 g/kg, i.p.) produced a much greater prolongation of the sleeping time with non-hypnotic doses of phenobarbitone and barbitone, than with threshold doses of the shorter acting barbiturates. Various postulates are advanced to explain the underlying mechanism of the barbiturate-ethanol interaction.

Many deaths—both accidental and deliberate—occur each year from the coincidental ingestion of alcoholic beverages and barbiturates. Bogan & Smith (1967) in a survey of 85 fatal barbiturate poisonings noted that ethanol was present in 58% of the cases, and that in these the mean blood barbiturate was only 50% of the level found where poisoning was by barbiturate alone. The interaction of these compounds in the living organism has interested many investigators (cf. Gores, 1964) yet the underlying mechanism whereby the potentiation effects are elaborated is still obscure. Since ethanol and barbiturates are both central nervous system depressants, classical pharmacologists have invoked the traditional concepts of drug synergism—potentiation and addition—to explain their observations (Veldstra, 1956).

However their interaction could have a biochemical basis. Ethanol, for example, could suppress barbiturate metabolism, or conversely barbiturates could inhibit the oxidation of ethanol (Whittlesey, 1954; Melville, Joron & Douglas, 1966; Seidel, 1967). It is known that certain barbiturates can inhibit the reoxidation of NADH to NAD (Pumphrey & Redfearn, 1963; Erwin & Heim, 1963). This inhibition might curtail the oxidation of ethanol since both alcohol dehydrogenase and aldehyde dehydrogenase require NAD (Gores, 1964; Lieber, 1967).

Unfortunately there has been little systematic investigation of the ethanol-barbiturate interaction. A review of the pertinent literature reveals a heterogeneous collection of unrelated experiments which employ several species of animals, differences in times and routes of dosing and a variety of response parameters (death, sleeping times, blood decay curves, tissue levels), which makes it difficult to compare the results in different reports. Furthermore, many barbiturates have been used with differing potencies, pharmacologic effects and metabolic fate. Many workers have chosen hexobarbitone for study even though it is rarely employed in current medical practice. For the above reasons a comprehensive systematic investigation of the ethanol-barbiturate interaction is underway in our laboratory.

Since deaths caused by ethanol alone are infrequent, in the first phase of the study, we have investigated the effect of ethanol on the LD50 and threshold hypnotic doses of several commonly used and prescribed barbiturates. Thiopentone, pentobarbitone,

amylobarbitone, phenobarbitone and barbitone were selected because they differ in pharmacological activity and metabolic fate. The results of these investigations are reported in this paper.

EXPERIMENTAL

Male albino rats of the Wistar strain, weighing 180–220 g were housed 10 animals per cage and acclimatized to the laboratory environment for at least one week. They were fasted overnight and then administered simultaneously an aqueous solution of ethanol (15% w/v) and barbiturates by the intraperitoneal route at a volume of 20 ml/kg. After dosing, they were housed in individual cages and observed for 24 h. The number of deaths and time to death were recorded. The LD50 values of the barbiturates were determined in the presence of ethanol at 0, 2, 3, and 4 g/kg and calculated by the moving average method of Thompson (1947) using the tables provided by Weil (1952) for 4 doses of 5 animals each and a dose-interval of 1·5.

The effect of ethanol on barbiturate sleeping time was investigated by treating rats simultaneously with 3 g/kg ethanol and a threshold or subthreshold hypnotic dose of the barbiturate. The drugs were given by the intraperitoneal route. The sleeping time represented the elapsed time in minutes between the loss and the reappearance of the righting reflex. Control studies were made in which ethanol or barbiturates were given separately.

RESULTS

Acute toxicity

The results of the LD50 determinations are shown in Fig. 1. The LD50 (mg/kg) for the five barbiturates were: thiopentone, 53·0 (45·0 to 62·3); pentobarbitone, 69·0

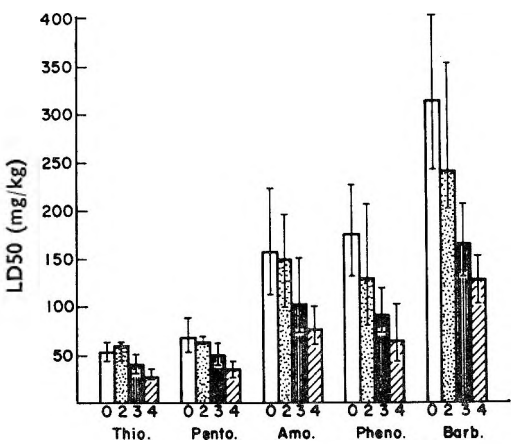

FIG. 1. The effects of ethanol on the LD50 of barbiturates in rats. Figures on the abscissa are doses of ethanol (mg/kg).

(53·4 to 98·1); amylobarbitone, 155 (108 to 221); phenobarbitone, 171 (131 to 224); and barbitone, 312 (241 to 387). These values are in good agreement with published data (Barnes & Eltherington, 1964; Wiberg & Grice, 1965; Coldwell & Peters, 1968).

The presence of ethanol produced a dose-dependent decrease in the LD50 values for all five barbiturates. The decreases were statistically significant ($P < 0·05$) for

phenobarbitone and barbitone at a dose of 3 g/kg ethanol and for thiopentone, pentobarbitone and amylobarbitone at 4 g/kg ethanol. The ratios of the LD50 values without to those with ethanol (4 g/kg) were approximately 2 for thiopentone, pentobarbitone and amylobarbitone, and 3 for phenobarbitone and barbitone.

In our strain of rat the intraperitoneal LD50 of ethanol was 6·5 (6·1–6·9) g/kg. The estimated LD 0 dose was 5·0 g/kg hence it was unlikely that ethanol *per se* produced any fatalities. The cause of death was respiratory failure. Observations on mortality were terminated after 24 h since intraperitoneal injection of ethanol can produce a fatal chemical peritonitis. However, deaths from this cause do not occur within the first 48 h after injection of ethanol (15 to 20% w/v).

The addition of ethanol to the barbiturate reduced the induction time for the appearance of the loss of righting reflex, prolonged the sleeping time of the survivors and shortened the survival time for the fatal doses. The extent of these differences became more marked as the dose of ethanol was increased.

Prolongation of sleeping time

Ethanol (3 g/kg) greatly prolonged the barbiturate sleeping time (Table 1). Where possible, threshold hypnotic doses of barbiturate were employed; this was not feasible

Table 1. *Prolongation of barbiturate sleeping times by ethanol*

Drug(s)	Dose/kg	No. of animals	Median induction time (min)	Median sleeping time + range (min)	Remarks
Ethanol	3 g	20	0	0	Non-hypnotic dose
Thiopentone	30 mg	20	4	24 (5–118)	11/20 did not sleep
Thiopentone + ethanol	3 g	20	2	217 (150–268)	3 fatalities
Pentobarbitone	30 mg	15	4·5	36 (21–42)	3/15 did not sleep
Pentobarbitone + ethanol	3 g	15	2	194 (132–243)	
Amylobarbitone	60 mg	20	5	39 (21–55)	
Amylobarbitone + ethanol	3 g	20	2·5	171 (121–245)	
Phenobarbitone	50 mg	15	0	0	Non-hypnotic dose
Phenobarbitone + ethanol	3 g	15	5	245 (173–359)	
Barbitone	125 mg	20	0	0	Non-hypnotic dose
Barbitone + ethanol	3 g	17	5	426 (264–623)	

with phenobarbitone and barbitone, since the threshold hypnotic dose of these drugs combined with 3 g/kg ethanol was lethal. This effect of ethanol was more marked with phenobarbitone and barbitone than with the shorter-acting barbiturates.

DISCUSSION

Considerable controversy exists as to whether the ethanol-barbiturate interaction is a true potentiation or merely an additive effect. Veldstra (1956) has reviewed the earlier literature and agrees with Gruber (1955) that the interaction is additive and not synergistic. These authors suggest that so-called evidence for a synergism has arisen through failure to consider differences in the characteristics of the barbiturates (long acting, short acting) and the effects of different routes of administration. Nevertheless, considerable experimental evidence accumulating in our laboratory indicates that this interaction involves more than a single additive response of two CNS depressants.

Although ethanol increased the toxicity of all five barbiturates, the interaction was most pronounced with barbitone, the barbiturate least subject to biotransformation. This would suggest that the increase in toxicity did not result from the inhibition of barbiturate metabolism by ethanol but might have been caused by other factors such as: (i) concerted depressant action of ethanol and barbiturate on the CNS, (ii) inhibition of ethanol metabolism by the barbiturates, (iii) altered tissue distribution of the two drugs, (iv) changes in lipid-water partition coefficient of the barbiturates, and (v) changes in protein binding capacity. There is no reason to assume that only one of these proposed mechanisms is involved, it is quite likely that several could operate in concert.

In the present work, since the drugs were given intraperitoneally, the circulating levels of barbiturate and ethanol probably reached their respective maxima rapidly and at approximately the same time. This might not occur after oral administration because of differences in the rates of absorption from the gastrointestinal tract. However, Ramsey & Haag (1946) reported that ethanol ingestion decreased the oral toxicity of quinalbarbitone in mice.

The sleeping time experiments offered some insight to the nature of the interaction. It has been shown that thiopentone is first taken up by the brain and then released for storage in the fat depots, and that the duration of the thiopentone sleep is related to its level in the brain, (Price, Kovnat & others, 1960). Since ethanol prolonged the thiopentone sleeping time it might delay the migration of thiopentone from the brain to the fat depots. Non-hypnotic doses of phenobarbitone or barbitone in the presence of a non-hypnotic dose of ethanol produced very prolonged sleeping times. This did not appear to be an additive effect. Barbitone, which is only slightly metabolized, produced the longest sleeping time which would imply that impairment of barbiturate metabolism is not a major factor in the interaction mechanism.

Ethanol reduced the induction times for the appearance of the loss of righting reflex for all five barbiturates (data from LD50 experiments). The induction times (2–5 min) were too short to be associated with altered rates of ethanol or barbiturate degradation but most likely reflected levels in the brain. There is some evidence that ethanol facilitates the passage of barbiturate into the brain. Seidel (1967) found higher levels of pentobarbitone but not of thiopentone or barbitone in the brains of mice treated with ethanol. We are determining currently the "t-1/2" and rate constants of decay curves for ethanol and several barbiturates alone and in combination in several tissues of rodents and pigs. Although these studies are not completed we have found higher levels of phenobarbitone and barbitone in brains of rats dosed with barbiturate and ethanol than in those given barbiturate only. Ramsey & Haag (1946) noted that in mice barbitone did not change the blood ethanol curves and that the blood barbitone curves were practically identical with and without ethanol. Whittlesey (1954) observed that pentobarbitone decreased the rate of fall of blood ethanol in dogs. Melville & others (1966) reported significant decreases in the rate of disappearance of blood levels of quinalbarbitone and ethanol in dogs given oral doses of these drugs alone and in combination. Seidel (1967) found that the concentration of pentobarbitone in the blood decreased more slowly in mice pretreated with ethanol, but that the rates of disappearance of barbitone and thiopentone were not affected.

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Monoamine oxidase activities in tissues of thiamine-deficient rats*

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The oxidation of monoamine by homogenates of tissues of thiamine-deficient rats was measured with noradrenaline as substrate. Monoamine oxidase activities were depressed in tissues in which the catecholamine content had previously been found raised during thiamine deficiency, with the exception in the cerebral cortex. The impaired enzyme activities were restored to the control level by repeated thiamine injections. An accumulation of pyruvic acid did not inhibit monoamine oxidase activity and thiamine pyrophosphate is not implicated as a co-factor in the oxidation of monoamine substrate.

We have previously reported (Iwata, Fujimoto & others, 1968) that the concentration of catecholamines in the cerebral cortex, in the atria and ventricles of the heart and in the spleen, are significantly increased in thiamine-deficient rats compared with the levels found in these organs of pair-fed control rats and of rats fed the thiamine-deficient diet supplemented with thiamine.

It is well known that catecholamine is mainly inactivated by monoamine oxidase and catechol-*O*-methyltransferase (COMT). It is possible that in the brain, as in the rest of the body, COMT is largely responsible for the degradation of extracellular catecholamine, while monoamine oxidase is responsible for the degradation of the amine existing intracellularly near the site of catecholamine synthesis and storage (Carlsson, 1960; Kopin & Gordon, 1962; Potter, Cooper & others, 1965).

That the raised levels of catecholamine in thiamine-deficient rats could be due to decrease in the activities of these enzymes was therefore considered, and so monoamine oxidase activities in the tissues of thiamine-deficient rats have been measured.

EXPERIMENTAL

Animals. Male Sprague-Dawley rats, 80 to 100 g were used. Thiamine deficiency was induced by feeding a synthetic thiamine-deficient diet *ad libitum*. Control animals were fed the same diet supplemented by 3 mg thiamine hydrochloride per kg of the basal diet. As previously (Iwata & others, 1968), only those animals possessing heart rates less than 70% of those of the control group were considered acutely deficient in thiamine and suitable for use.

Diet. The composition of the thiamine-deficient basal diet was (g): vitamin-free casein 20, soy bean oil 9, cod liver oil 1, McCullum's salt 4, sucrose 64·6, choline chloride 0·1, DL-methionine 0·3 and vitamin solution 1 ml.

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The vitamin solution contained (mg): riboflavin 40, pyridoxine HCl 30, cyanocobalamin 3, ascorbic acid 2000, menadione 50, nicotinic acid 400, calcium pantothenate 400, inositol 1000, *p*-aminobenzoic acid 1000, folic acid 20, biotin 4 and 50% ethanol to 100 ml.

Assay of monoamine oxidase. Tissue homogenates, buffered at pH 7·4 with 0·1M phosphate buffer, were incubated with substrate for 1 h at 37°. (±)-Noradrenaline (Nakarai Chemicals Ltd.) was used at 3×10^{-3}M. The production of ammonia from (±)-noradrenaline was measured by the method of Cotzias & Dole (1951) modified by Tokawa & Takahashi (1966).

Assay of catecholamine. Tissue catecholamine was extracted by the methods of Crout, Creveling & Udenfriend (1961), and measured fluorometrically as described by Euler & Floding (1955), using a Hitachi spectrofluorometer, model 203.

RESULTS

The effects of thiamine deficiency and administration of thiamine to the deficient animals on monoamine oxidase activity of rat tissue homogenates are summarized in Table 1. Noradrenaline oxidase activity was found to be higher in the liver than

Table 1. *Monoamine oxidase activities* (μmol/g h^{-1}) *by homogenates of the tissues of control and thiamine-deficient rats with noradrenaline as substrate*

Tissue	Control (5)	Thiamine deficient (5)	Thiamine deficient †Thiamine (4·0 mg/kg)	Thiamine deficient ‡Thiamine (1·0 mg/kg × 4)
Cerebral cortex	4·85 ± 0·61	4·80 ± 0·57	4·93 ± 0·81 (5)	4·80 ± 0·75 (5)
Brain stem	5·09 ± 0·59	4·98 ± 0·59	5·50 ± 0·73 (6)	5·37 ± 0·52 (6)
Heart atrium	5·32 ± 0·58	3·69 ± 0·52*	3·60 ± 0·55*(6)	4·99 ± 0·51**(7)
ventricle	4·09 ± 0·32	2·90 ± 0·33*	3·14 ± 0·44*(6)	4·30 ± 0·54**(6)
Spleen	4·68 ± 0·59	3·01 ± 0·51*	3·01 ± 0·59*(6)	4·66 ± 0·53**(5)
Liver	8·32 ± 1·01	8·25 ± 0·99	8·31 ± 0·92 (5)	8·72 ± 1·09 (5)

The values are mean ± s.e. Figure in brackets refer to number of experiments.
† Thiamine (4·0 mg/kg): In these experiments, 4·0 mg/kg thiamine was injected subcutaneously 5 h before the animals were killed.
‡ Thiamine (1·0 mg/kg × 4/2 days): 1·0 mg/kg thiamine was injected subcutaneously every 12 h for 2 days and 12 h after the last injection the animals were killed.
* Significant difference from control group ($P<0·05$).
** Significant difference from thiamine-deficient group ($P<0·05$).

in other tissues; the heart and brain appeared to contain one half to two thirds as much monoamine oxidase activity per g of tissue as the liver.

A significant reduction of noradrenaline metabolism was demonstrable in the atria and ventricles of the heart and in the spleen of thiamine-deficient animals.

The rates of oxidation of the amine by homogenates of the tissues of thiamine-deficient rats were unaffected 5 h after subcutaneous injection of 4·0 mg/kg thiamine hydrochloride. Correction of the deficiency required 4 injections each of thiamine, 1·0 mg/kg, at 12 h intervals. This increased the rate of oxidation of noradrenaline by these three organs to control levels.

Table 2 shows the effects of thiamine treatment on catecholamine content in tissues of thiamine-deficient rats. In these rats, catecholamine contents in the cerebral cortex, in the atria and ventricles of the heart and in the spleen were significantly

Table 2. *Catecholamine content* (μg/g) *in tissues of thiamine-deficient rats treated with thiamine*

Tissue	Control¶	Thiamine deficient	Thiamine deficient † thiamine (4·0 mg/kg)	Thiamine deficient ‡ thiamine (1·0 mg/kg)
Cerebral cortex	0·26 ± 0·04 (4)	0·52 ± 0·09*(5)	0·43 ± 0·05*(4)	0·38 ± 0·06**(5)
Brain stem	0·58 ± 0·09 (4)	0·63 ± 0·10 (5)	0·60 ± 0·07 (5)	0·60 ± 0·15 (5)
Heart: atrium	1·31 ± 0·14 (6)	2·60 ± 0·39*(5)	2·50 ± 0·31*(5)	2·02 ± 0·35**(4)
ventricle	0·34 ± 0·12 (3)	0·94 ± 0·12*(5)	0·81 ± 0·10*(5)	0·41 ± 0·06**(4)
Spleen	0·40 ± 0·03 (6)	1·95 ± 0·15*(4)	1·67 ± 0·12*(6)	0·86 ± 0·20**(5)
Adrenal gland	1063·0 ± 107·9 (6)	1107·7 ± 155·0 (5)	1110·3 ± 121·9 (6)	1079·7 ± 74·0 (5)

* Significant difference from control group ($P < 0.05$).
** Significant difference from thiamine-deficient group ($P < 0.05$).
Value: Mean ± s.e., Each group: 3–4 rats, Number of groups are in parentheses.
†‡ See footnotes to Table 1.
¶ The data are those previously reported (Iwata & others, 1968).

increased compared with those of control animals. Catecholamine concentration of tissues from thiamine-deficient rats 5 h after the subcutaneous injection of 4·0 mg/kg thiamine hydrochloride remained unaffected, but, as found with monoamine oxidase activity, thiamine, 1·0 mg/kg, injected 4 times at 12 h intervals, returned catecholamine levels to near control levels in all tissues in which amine concentration was raised by thiamine deficiency.

No significant effects of pyruvate were detected on the rates of oxidation of noradrenaline by homogenates of tissues taken from control rats 5 h after sodium pyruvate, 600 mg/kg, i.p., or 300 mg/kg injected twice daily for 30 days.

To find possible direct effects of pyruvic acid, thiamine and thiamine pyrophosphate on the rates of amine oxidation, experiments were made *in vitro* on control and thiamine-deficient cerebral cortex, brain stem, heart, spleen and liver using sodium pyruvate (1×10^{-4}M), thiamine (1×10^{-6}M) and thiamine pyrophosphate (1×10^{-6}M) added with substrate. But the oxidation rate was not influenced.

DISCUSSION

Gal & Drewes (1961) investigated monoamine oxidase activity in the tissues of thiamine-deficient rats. They found an increase in this activity in mitochondrial fractions isolated from the whole brain and the small intestine of thiamine-deficient animals, but no change in the monamine oxidase activity of liver, spleen and kidney, when it was assayed manometrically (Creasey, 1956) with 5-hydroxytryptamine as substrate. We find that, using tissue homogenate as the source of enzyme and noradrenaline as substrate, thiamine deficiency depresses monoamine oxidase activity in the cardiac atria and ventricles and in the spleen. The reason for this disagreement is not known but may be due to differences in substrate and method. It is likely that tissue homogenates contain enzymes responsible for alternative pathways for the metabolism of amines and therefore give a better picture of the overall effects of thiamine deficiency on amine metabolism. In addition, support for the hypothetical

existence of more than one system capable of metabolizing monoamines in some, at least, of our tissue homogenates has been given by the discrepancies found in the monoamine oxidase activity of the various tissues from normal animals using the different substrates (Iwata, Nishikawa & Fujimoto, to be published).

Overall, except the cerebral cortex, those tissues in which the catecholamine content had been previously found raised during thiamine deficiency (Iwata & others, 1968) are the tissues in which amine metabolism is now found depressed by lack of thiamine. In the accumulation of catecholamine in the tissues, especially in the cerebral cortex of thiamine-deficient animals, other factors also must be taken into consideration such as acceleration of synthesis, enhancement of uptake and inhibition of spontaneous release of the amine. Reduction of spontaneous release of catecholamines from their storage sites was seen and this could be another factor contributing to the accumulation of the amine (Iwata, Nishikawa & Watanabe, 1969).

Previous work has also shown elevation of pyruvic acid levels in the cerebral cortex, cardiac atria and ventricles and spleen during thiamine deficiency (Iwata & others, 1968). Five h after the subcutaneous injection of 4·0 mg/kg thiamine hydrochloride, the concentration of pyruvic acid in all these tissues, except the spleen, fell to normal (Iwata & others, 1968) but the catecholamine content and rates of metabolic destruction of noradrenaline (Table 1) in the tissues were not restored. Two days' treatment with a total of 4·0 mg/kg in 12 hourly doses were required to restore catecholamine levels (Table 2) and amine metabolism to normal. There is therefore no parallel between the changes in the levels of pyruvic acid in the tissues on the one hand and the change in catecholamine levels and amine metabolism, on the other, during recovery from thiamine deficiency. The present work demonstrates that an accumulation of pyruvic acid cannot itself inhibit the catecholamine metabolism and that thiamine pyrophosphate is not implicated as a co-factor in the oxidation of the monoamine.

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A comparison of the effects of (±)-propranolol and (+)-propranolol in anaesthetized dogs; β-receptor blocking and haemodynamic action

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The adrenergic β-receptor blocking activities of (±)-propranolol and (+)-propranolol have been compared in anaesthetized dogs, using isoprenaline as the agonist. (+)-Propranolol possessed less than one fiftieth the potency of (±)-propranolol. Intravenous (±)-propranolol (0·25 mg/kg) produced a six-fold increase in the isoprenaline dose ratio and significantly lowered heart rate, cardiac contractile force, ejection rate and tension time index. The same dose of (+)-propranolol had no effect on the isoprenaline dose ratio nor did it significantly alter these haemodynamic variables. Since both isomers of propranolol have equivalent membrane stabilizing properties it was concluded that the haemodynamic effects of (±)-propranolol at this dose level were due to specific β-blockade and not to any "quinidine-like" properties. Higher doses of (+)-propranolol (1·25 mg/kg) significantly reduced heart rate and cardiac contractile force whilst increasing atrio-ventricular conduction time without raising the threshold to isoprenaline. There was no effect on ejection time, mean ejection rate or tension time index. Extremely high doses of (+)-propranolol slightly raised the isoprenaline dose ratio in intact dogs but not after vagal section. The arithmetic difference between the effects of equivalent doses of (±)-propranolol and (+)-propranolol was approximately constant. The findings suggest that (±)-propranolol reduces cardiac work by blocking the sympathetic drive to the heart at doses up to 0·2 mg/kg (the usual clinical dose range) and that direct depression of the mycardium only occurs at doses well above this.

Many clinical reports demonstrate the utility of (±)-propranolol in correcting or ameliorating a wide range of cardiac arrhythmias (Bath, 1966). The precise mechanism of action of (±)-propranolol in these conditions is not fully understood for two principal reasons. First the aetiology of most arrhythmias is obscure and second (±)-propranolol not only possesses specific competitive blocking properties at adrenergic β-receptors but also marked local anaesthetic activity. Chemical separation of the isomers of propranolol facilitated a detailed study of their respective pharmacological actions (Barrett & Cullum, 1968). The authors concluded that only the (−)-isomer exhibited significant β-receptor blocking activity whereas both isomers possessed equivalent local anaesthetic potency. In arrhythmias associated with adrenergic stimulation, (−)-propranolol was effective in the dose range of 60–100 μg/kg. The (+)-isomer was also effective against these arrhythmias but only at dose levels which also depressed conduction in the myocardium (2–6 mg/kg). Both isomers were effective in reversing arrhythmias produced by ouabain overdosage but only at higher dose levels (2–6 mg/kg). In view of the risk of inducing heart failure with (±)-propranolol many clinicians are reluctant to use this agent for the correction of

arrhythmias where there is evidence of damage to the myocardium. It seemed logical therefore to assess the value of (+)-propranolol as an anti-arrhythmic agent in its own right. This paper compares the degree of β-receptor blockade produced by incremental doses of (±)-propranolol and its (+)-isomer and assesses the haemodynamic effects of those doses to provide background information on the expected effects of (+)-propranolol following intravenous injection in man.

EXPERIMENTAL

Animals and methods

Mongrel dogs (11–16 kg) of either sex were used in these experiments. Anaesthesia was induced with thiopentone and maintained with chloralose in order to maintain a relatively high degree of sympathetic tone. All dogs were artificially ventilated via a cuffed endotracheal tube and in some, bilateral vagotomy was performed. Following thoracotomy through a midsternal incision, a strain-gauge arch was sutured to the epicardial surface of the left ventricle. Heart rate was recorded with a cardiotachometer (Horsfall, 1965) and arterial blood pressure from the left carotid artery using pressure transducer. In some experiments we also recorded Lead II electrocardiogram and aortic flow by placing a probe for an electromagnetic flow-meter (Medicon K 2000) around the root of the aorta.

All responses were monitored on a four channel oscilloscope (Airmec) and recorded continuously on a multi-channel tape-recorder (Ampex SP 300). Permanent records were obtained from the tape on an ink-writing recorder (Minograph 81 B) at the end of each experiment. Accurate measurements of cardiac functions were made from records obtained at a paper speed of 100 mm/s.

Calculations

The following calculations were employed to determine haemodynamic functions:

(1) Cardiac output = aortic flow (ml/min)/body wt (kg). (2) Stroke volume = aortic flow (ml/min)/heart rate (bts/min). (3) Mean blood pressure = Diastolic pressure + ⅓ pulse pressure. (4) Mean systolic ejection pressure was determined by planimetric integration of the area under the systolic portion of the aortic pressure trace. (5) Ejection time was taken between the onset of systole and the dicrotic notch, from the aortic pressure trace. (6) Mean ejection rate = stroke volume/ejection time. (7) Total peripheral resistance = mean blood pressure/aortic flow. (8) Tension time index (pressure time/min) = mean systolic ejection pressure × heart rate × ejection time. Each measurement was taken as the mean for 10 complete cardiac cycles.

Dose-response curves for the positive chronotropic, positive inotropic and vasodilator actions of isoprenaline were obtained in eight dogs. Four dogs each received 0·25, 1·0 and 4·0 mg/kg consecutively of (±)-propranolol or (+)-propranolol intravenously. After each dose the amount of isoprenaline was increased until the maximum response seen before the administration of the drugs was obtained. The results were plotted graphically and the dose of isoprenaline required to produce 50% of the maximum response determined. The experiments were repeated in eight vagotomized dogs.

Eight further dogs received (±)-propranolol or (+)-propranolol only, in the above doses, at 15 min intervals. Changes in haemodynamic functions were measured 13 min after the injection of each dose.

Drugs

The drugs used were (±)-isoprenaline sulphate (Burroughs Wellcome), (±)-propranolol hydrochloride (Inderal, I.C.I.) and (+)-propranolol (prepared by Dr. T. Leigh of the Chemical Research Department at Alderley Park). The (+)-isomer contained less than 0·5% of (−)-isomer as determined by measuring optical rotations.

RESULTS

The effects of (±)-propranolol and (+)-propranolol on the responses to isoprenaline are summarized in Table 1. There was no reduction in the maximum response for the positive chronotropic and inotropic effects with either drug. However, with the highest dose level of (±)-propranolol (5·25 mg/kg) it was not possible to produce a dose-dependent vasodilator response and in some cases a pressor response was observed after higher doses of isoprenaline. Following the 5·25 mg/kg dose of (+)-propranolol the response of the blood pressure to isoprenaline became biphasic, there being an initial rise and a secondary fall. From the data given in Table 1, the dose

Table 1. *A comparison of the doses of isoprenaline necessary to produce 50% of the maximum response in heart rate, heart force and vasodilation before and after various doses of (±)-propranolol and its (+)-isomer*

		(±)-Propranolol		(+)-Propranolol	
Function	Dose (mg/kg)	Isoprenaline for 50% max. (ng/kg)	Dose ratio	Isoprenaline for 50% max. (ng/kg)	Dose ratio
Heart rate ..	0	150 ± 12	1·00	150 ± 9	1·00
	0·25	920 ± 29	6·13	130 ± 12	0·87
	1·25	5000 ± 104	33·4	260 ± 14	1·73
	5·25	20,500 ± 812	137	500 ± 17	3·33
Heart force ..	0	152 ± 14	1·00	89 ± 8	1·00
	0·25	1380 ± 61	9·06	110 ± 10	1·23
	1·25	7000 ± 104	47·0	180 ± 12	2·02
	5·25	28,800 ± 916	189	270 ± 17	3·03
Vasodilator ..	0	36 ± 4	1·00	36 ± 6	1·00
	0·25	520 ± 21	14·5	81 ± 6	2·25
	1·25	6150 ± 219	172	148 ± 14	4·11
	5·25	∞	∞	285 ± 12	7·92

ratio for (±)-propranolol at 0·25 mg/kg was twice that of (+)-propranolol at 5·25 mg/kg. Allowing for a 25-fold difference in dose-level, it could be said that (+)-propranolol was about 50 times less active than (±)-propranolol. However, the dose-response curves were not parallel and the validity of this conclusion is dubious.

Since the highest dose of (+)-propranolol altered the characteristic of the blood pressure response to isoprenaline, it was possible that the apparent degree of β-blockade resulted from the initiation of a reflex bradycardia via the vagal nerves. Accordingly the experiments were repeated in vagotomized dogs, the results being summarized in Table 2. Under these conditions (+)-propranolol did not significantly reduce the positive chronotropic or inotropic actions of isoprenaline but did significantly diminish the vasodilator responses. The results for (±)-propranolol were not significantly different in dogs with intact or sectioned vagi. From these experiments it was concluded that (+)-propranolol was devoid of cardiac β-blocking actions at the dose-levels tested.

Table 2. *A comparison of the doses of isoprenaline necessary to produce 50% of the maximum response in heart rate and heart force before and after various doses of (±)-propranolol and its (+)-isomer in vagotomized dogs*

Function	Dose (mg/kg)	(±)-Propranolol: Isoprenaline for 50% max. (ng/kg)	(±)-Propranolol: Dose ratio	(+)-Propranolol: Isoprenaline for 50% max. (ng/kg)	(+)-Propranolol: Dose ratio
Heart rate ..	0	100 ± 16	1·00	185 ± 17	1·00
	0·25	670 ± 21	6·70	198 ± 21	1·07
	1·25	3200 ± 161	32·0	244 ± 41	1·32
	5·25	15,800 ± 914	158	215 ± 31	1·16
Heart force ..	0	105 ± 46	1·00	142 ± 21	1·00
	0·25	721 ± 60	6·87	131 ± 18	0·92
	1·25	4000 ± 120	38·0	157 ± 20	1·10
	5·25	17,500 ± 600	166	160 ± 41	1·13

The effects of (±)-propranolol and its (+)-isomer on haemodynamic function are summarized in Table 3. Mean control values are given for 8 dogs together with percentage changes from control for the 4 dogs receiving each drug. Since the β-blocking potency of the (+)-isomer is virtually negligible and the local anaesthetic potency of both isomers is equivalent, the difference between the results obtained with (±)-propranolol and (+)-propranolol may be considered to approximate to the effect of β-blockade *per se*. These differences are also given in Table 3.

Table 3. *Haemodynamic effects of (±)-propranolol and (+)-propranolol in anaesthetized dogs*

Parameter	Control value (n = 8)	Dose (mg/kg)	(±)-Propranolol (n = 4)	P	(+)-Propranolol (n = 4)	P	Diff. (±)—(+)	P
Heart rate (bt/min)	167 ± 15	0·25	−23 ± 2·7	0·001	−2 ± 0·3	0·05	−21 ± 2·9	0·001
		1·25	−30 ± 3·1	0·001	−8 ± 1·0	0·01	−22 ± 3·3	0·001
		5·25	−37 ± 4·2	0·001	−20 ± 2·5	0·01	−17 ± 3·9	0·01
PR Interval (ms)	88 ± 4	0·25	+9 ± 3·0	0·05	+3 ± 1·6	N.S.	+6 ± 3·4	N.S.
		1·25	+11 ± 3·2	0·05	+9 ± 1·1	0·01	+2 ± 3·4	N.S.
		5·25	+23 ± 6·4	0·05	+39 ± 4·2	0·01	−16 ± 7·6	N.S.
Cardiac contractile force (% control)	100 ± 0	0·25	−18 ± 3·9	0·01	−6 ± 2·5	N.S.	−12 ± 4·6	0·05
		1·25	−26 ± 4·0	0·001	−15 ± 5·5	0·05	−11 ± 6·8	N.S.
		5·25	−44 ± 3·3	0·001	−32 ± 8·0	0·01	−12 ± 8·7	N.S.
Aortic flow (ml/min)	1610 ± 340	0·25	−20 ± 2·3	0·001	−1 ± 1·3	N.S.	−19 ± 3·0	0·001
		1·25	−34 ± 2·3	0·001	−11 ± 3·1	0·05	−23 ± 3·9	0·01
		5·25	−56 ± 9·0	0·001	−34 ± 7·5	0·01	−22 ± 11·6	N.S.
Carotid blood press. (mean) (mm Hg)	115 ± 9	0·25	−7 ± 3·5	N.S.	+7 ± 3·0	N.S.	−14 ± 4·6	0·05
		1·25	−10 ± 2·3	0·01	+5 ± 4·0	N.S.	−15 ± 4·6	0·05
		5·25	−23 ± 10·5	N.S.	−14 ± 13·2	N.S.	−9 ± 16·8	N.S.
Total peripheral res. (units)	84·8 ± 12	0·25	+17 ± 3·0	0·01	+8 ± 2·4	0·05	+9 ± 3·8	N.S.
		1·25	+37 ± 5·4	0·01	+20 ± 4·0	0·01	+17 ± 6·7	0·05
		5·25	+87 ± 22	0·01	+30 ± 5·8	0·01	+57 ± 22·7	0·05
Stroke volume (ml)	8·2 ± 1·3	0·25	+4 ± 4·4	N.S.	+3 ± 2·3	N.S.	+1 ± 5·2	N.S.
		1·25	−7 ± 2·0	0·05	+2 ± 3·5	N.S.	−9 ± 4·1	N.S.
		5·25	−28 ± 11·4	0·05	−16 ± 9·0	N.S.	−12 ± 14·5	N.S.
Ejection time (ms)	143 ± 15	0·25	+11·0 ± 2·2	0·01	+2·5 ± 2·5	N.S.	+8·5 ± 3·3	0·05
		1·25	+13·5 ± 3·1	0·01	+4·5 ± 2·5	N.S.	+9·0 ± 4·0	N.S.
		5·25	+18·1 ± 3·3	0·01	+19·2 ± 2·0	0·001	−1·1 ± 3·8	N.S.
Ejection rate (ml/s)	70·8 ± 12·1	0·25	−6·7 ± 1·5	0·01	+2·2 ± 2·4	N.S.	+8·9 ± 2·8	0·05
		1·25	−17·7 ± 2·8	0·001	−3·0 ± 3·0	N.S.	+14·7 ± 4·1	0·02
		5·25	−38·2 ± 12·2	0·02	−29·3 ± 8·0	0·01	+8·9 ± 14·4	N.S.
Tension time index (mm Hg sec/min)	2968 ± 225	0·25	−14·8 ± 4·2	0·02	+6·2 ± 4·0	N.S.	+21·0 ± 5·7	0·02
		1·25	−22·1 ± 7·1	0·05	−0·9 ± 2·0	N.S.	+21·2 ± 8·1	0·05
		5·25	−38·3 ± 6·0	0·001	−17·2 ± 6·2	0·05	+21·1 ± 8·6	0·05

Heart rate

Both racemic and (+)-propranolol produced a dose-dependent bradycardia the effect of the former being much greater at equivalent dose levels. The arithmetic difference between the two sets of results was remarkably constant at about 20% and was statistically significant at all 3 doses. This suggests that β-blockade was already complete at 0·25 mg/kg of propranolol and that the greater bradycardia at higher doses resulted from some other action.

Conduction

As judged by effects on the PR interval of the electrocardiogram both drugs significantly increased conduction time between atria and ventricles. At no dose level was the arithmetic difference between the two sets of results statistically significant. However, since 0·25 mg/kg of (±)-propranolol produced a significant effect whereas the same dose of (+)-propranolol did not, the results suggest that conduction time is affected by both β-blockade and local anaesthetic effects. It was interesting to note that the top dose of (+)-propranolol had a greater effect than that of (±)-propranolol (though the difference was not significant) which may reflect the slightly greater potency of the isomer over the racemate in tests *in vitro* for local anaesthetic action (Barrett & Cullum, 1968).

Myocardial contractility

At 0·25 mg/kg (±)-propranolol significantly depressed contractility as reflected by measurements from a strain-gauge arch. Ejection rate, which may be considered as an indirect index of contractility was also significantly depressed. The same dose (0·25 mg/kg) of (+)-propranolol did not exert a significant effect. Higher doses of both drugs produced a progressive reduction of both the direct and indirect indices of contractility. The arithmetic difference between both drugs' responses was again consistent.

Aortic flow

(+)-Propranolol had a significantly smaller depressant action than (±)-propranolol on aortic flow. Again, the difference between the two drugs' action was constant at equivalent dosage. The aortic flow is equivalent to cardiac output minus that fraction going to the coronary vessels which may possibly rise at lower cardiac rates owing to an increase in the duration of diastole.

Blood pressure

Mean blood pressure tended to fall with (±)-propranolol but the effects were small and variable and not of statistical significance. The effects of (+)-propranolol were even less pronounced.

Total peripheral resistance

Since there was a marked fall in cardiac output without much change in mean blood pressure there was a marked rise in calculated peripheral resistance. In the case of (±)-propranolol the increase was proportionately greater than the drop in output. The arithmetic differences were not constant and showed a dose-dependent increase even after allowing for the effects of the local anaesthetic properties.

Stroke volume and ejection time

(+)-Propranolol had no effect on stroke volume whilst higher doses of (±)-propranolol depressed it. Ejection time increased significantly at all dose levels of (±)-propranolol but only at the highest dose of (+)-propranolol.

Myocardial oxygen consumption

No direct measurements of oxygen consumption by the heart were made in this study but it has been shown that oxygen consumption is proportional to the area under the systolic portion of the left ventricular pressure curve (Sarnoff, Braunwald & others, 1958). Calculation of the tension time index (pressure time/min) according to the method of Sarnoff & others (1958) showed that this function decreased in a dose-dependent fashion with (±)-propranolol. The changes correlated well with the changes in myocardial contractility. Only at the highest dose level (5·25 mg/kg) was there any decrease with (+)-propranolol. The arithmetic differences between the effects of the two drugs were again constant, being statistically significant at all doses.

The overall effects of (±)-propranolol (0·25 mg/kg) and (+)-propranolol (1·25 mg/kg) have been presented in histogram form in Fig. 1. The doses selected correspond

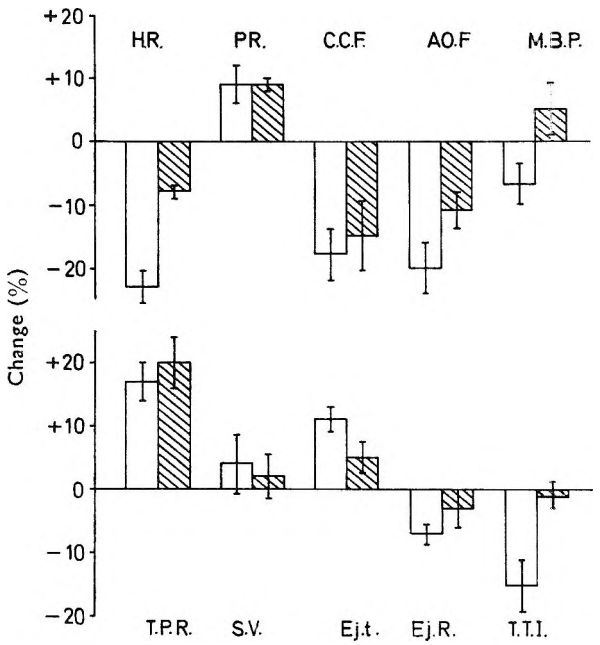

FIG. 1. Haemodynamic effects of (±)-propranolol (0·25 mg/kg) open columns and (+)-propranolol (1·25 mg/kg) hatched columns in anaesthetized dogs. Each bar represents the mean percentage difference from control values for 4 dogs. The vertical lines indicate the standard error of the means: H.R. = heart rate, C.C.F. = Cardiac contractile force, P.R. = PR Interval of ECG A.O.F = Aortic Flow, M.B.P. = Mean blood pressure, T.P.R. = total peripheral resistance, S.V. = stroke volume, Ej.t. = ejection time, Ej.R. = ejection rate, T.T.I. = tension time index.

approximately to the maximum doses likely to be used clinically on the basis of equivalent effects on conduction. After 1·25 mg/kg i.v., (+)-propranolol produced a statistically significant lowering of heart rate, cardiac contractile force and aortic flow. The atrio-ventricular conduction time was significantly raised as was total peripheral resistance. Mean blood pressure, stroke volume, ejection time, ejection rate and tension time index were not significantly altered. In contrast (±)-propranolol, at 1/5th the dose, reduced heart rate significantly more than (+)-propranolol, raised ejection time and diminished ejection rate and tension time index. At 0·25 mg/kg (±)-propranolol raised the dose-ratio for isoprenaline to 6·13 ± 0·7 compared to

1·73 ± 0·7 (means ± s.e.; n = 4) for (+)-propranolol at 1·25 mg/kg. In vagotomized dogs the respective values were 6·7 ± 1·1 and 1·32 ± 0·3.

DISCUSSION

The functional capacity of the myocardium depends mainly on its intrinsic contractile properties but it is also re-inforced by the sympathetic outflow. Competitive inhibition of the sympathetic component by a specific adrenergic β-receptor antagonist will therefore automatically reduce both cardiac rate and contractile force. Whilst abolising the responsiveness of the myocardium to β-receptor agonists, the ability of the heart to respond to calcium, digitalis or xanthine derivatives is not impaired by β-receptor blockade. In contrast, drugs which produce a non-specific depression of the myocardium e.g. barbiturates or local anaesthetics, reduce sensitivity to all forms of cardiac stimulation. (±)-Propranolol possesses both specific β-receptor blocking and local anaesthetic properties. It is not possible to say therefore whether its effects are solely due to β-blockade or to a combination of both actions. Since (+)-propranolol has been shown to be devoid of significant β-blocking properties comparison of the two drugs at equivalent doses permits an evaluation of the two effects as they are equipotent as local anaesthetics. The results strongly support the view that (±)-propranolol at doses up to 0·25 mg/kg produces all its effects by means of β-receptor blockade since an equal dose of (+)-propranolol had no effect on haemodynamics apart from a minor rise in total peripheral resistance. For the effects on heart rate, cardiac contractile force, aortic flow and tension time index the arithmetic difference between the responses to (±)-propranolol and (+)-propranolol were remarkably constant which suggested that complete β-blockade of endogenous sympathetic activity was achieved with the lowest dose of (±)-propranolol (0·25 mg/kg). The additional effects at higher dose levels were therefore presumably due to the non-specific depressant actions of the compounds. Under certain clinical conditions these non-specific effects may be of therapeutic benefit particularly when the myocardium is sensitized to arrhythmogenic influences. The advantage of (+)-propranolol in this context is that it would not be expected to deprive the heart of sympathetic drive.

There are two important implications from these results. First, the observed anti-arrhythmic effects of (±)-propranolol in the clinic (usually at doses of 1–5 mg per 70 kg patient) are almost certainly due to β-blockade and not to any "quinidine-like" action. This is supported by the positive anti-arrhythmic actions of I.C.I. 50,172* which has no local anaesthetic properties whilst being an effective β-receptor antagonist (Gibson, Balcon & Sowton, 1968; Barrett, Crowther & others, 1968). Second, it may be noted that since (+)-propranolol had little effect on myocardial oxygen consumption as shown by estimates of tension time index it would not be anticipated that this isomer would relieve anginal pain due to oxygen deficit.

* 4(2-Hydroxy-3-isopropylaminopropoxy)acetanilide

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The effect of anti-Parkinsonian drugs on oxotremorine-induced analgesia in mice

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The antagonism of oxotremorine-induced analgesia in mice can be used to obtain a quantitative and objective assessment of the central anticholinergic activity of potential anti-Parkinsonian drugs.

The antagonism of the effects of tremorine (1,4-dipyrrolidino-but-2-yne) in laboratory animals is widely used in the examination of potential anti-Parkinsonian drugs. Since the introduction of tremorine by Everett (1956) it has been shown that it is converted to an active metabolite which is responsible for most of its pharmacological properties (Kocsis & Welch, 1960). Cho, Haslett & Jenden (1961) isolated and identified this metabolite as 1-(2-oxopyrrolidin-1-yl)-4-pyrrolidin-1-yl-but-2-yne (oxotremorine).

Cho, Haslett & Jenden (1962) have reported that whereas the effects of tremorine are delayed in onset and may be inhibited by substances such as SKF 525A [2-(diethylamino)ethyl-2,2-diphenylvalerate] and LILLY 18947 [2-(2,4-dichloro-6-phenylphenoxy)-*NN*-diethylethylamine hydrochloride] which are known to be inhibitors fo liver microsomal activity (Axelrod, Reichenthal & Brodie, 1954; Fouts & Brodie, 1955) the action of oxotremorine is immediate in onset, not inhibited by SKF 525A, and quantitatively some thousand times more effective than tremorine.

Leslie & Maxwell (1964) showed that a number of phenothiazine derivatives which were not themselves useful anti-Parkinsonian drugs were able to prevent the effects of tremorine but not the effects of oxotremorine. For these reasons oxotremorine is now replacing tremorine as a means of investigating anti-Parkinsonian activity.

A number of methods have been described for assessing the degree of tremor. Blockus & Everett (1957) used an electro-mechanical recording method. Halliwell, Quinton & Williams (1964) and Farquharson & Johnston (1959) used subjective scoring systems. The subjective methods are much the simpler to use and quantify, but have been criticized as being open to error on the part of the observer. Spencer (1965) compared the activity of a number of anti-Parkinsonian and other drugs against tremorine-induced tremor and hypothermia. He found that drugs with central anticholinergic activity prevented both tremor and hypothermia and that quaternary anticholinergics were inactive against the tremor but did to some extent reduce the hypothermia. However he found that sympathomimetic drugs like amphetamine would also antagonize the hypothermia and to a much lesser extent the tremor.

Chen (1958) reported that tremorine had analgesic properties in mice which could be detected at doses insufficient to cause tremor or parasympathomimetic effects. This analgesic action could be antagonized by anti-Parkinsonian drugs. Oxotremorine also possesses this analgesic (antinociceptive) action which can be antagonized by anti-Parkinsonian drugs, but not by sympathomimetic compounds or

by phenothiazine tranquillizers. When administered subcutaneously to mice, oxotremorine is approximately 3,000 times more potent than morphine as an antinociceptive agent.

EXPERIMENTAL

Male albino mice of the SAS TO strain, weighing 15–20 g, were used. Graded doses of test drugs were administered subcutaneously to groups of 10 mice 1 h before intraperitoneal injection of oxotremorine (50 μg/kg).

The mice were first tested to ensure that they would vocalize in response to electroshocks of 8 V applied at 1 s intervals to flat copper electrodes 1·5 cm apart placed on their tails. Animals which did not respond to five or fewer shocks were rejected. Groups of ten mice were used at each dose level. Forty min after administration of the test compound, the mice were again tested for their response to the electroshocks. Five min later, oxotremorine was administered and after a further 15 min the mice were tested once more. The dose of test compound reducing the antinociceptive effect of oxotremorine by 50% of control values was determined (ED50).

Following this, the mice were individually assessed also for the degree of tremor using a three point scale. This was done by an observer who did not know what drugs the animals had received. The dose of test compound reducing the oxotremorine tremor to 50% of that of the control values was found (ED50). ED50 values were determined on four occasions for both antitremor and antinociception.

RESULTS

The ED50 values for a number of anti-Parkinsonian and other drugs against oxotremorine tremor and analgesia are summarized in Table 1.

Table 1. *ED50 values for some anti-Parkinsonian and other drugs against oxotremorine analgesia and tremor*

Drug	*Subcutaneous dose to reduce by 50%	
	Analgesia ED50 (mg/kg) with s.d.	Tremor ED50 (mg/kg) with s.d.
Atropine (sulphate)	1·2 ± 0·2	1·8 ± 0·4
Benactyzine	14·8 ± 1·7	15·2 ± 1·9
Benzhexol	7·1 ± 0·8	5·3 ± 0·8
Benztropine	2·8 ± 0·4	3·2 ± 0·6
Caramiphen	11·2 ± 1·4	9·7 ± 1·2
Ethopropazine	6·2 ± 0·7	7·1 ± 1·1
Hyoscine	1·0 ± 0·2	1·8 ± 0·4
Promethazine	18·0 ± 2·3	15·4 ± 2·2
Atropine (methyl bromide)	>50	>50
Propantheline	>50	>50
Chlordiazepoxide	>100	40 ± 7
Chlorpromazine	>50	25 ± 6
Dexamphetamine	>40	15 ± 5·5
Diphenhydramine	29 ± 6	38 ± 8
Imipramine	45 ± 8	30 ± 7
Meprobamate	>100	>100
Pentobarbitone	>80	>80
Phentolamine	>10	>10
Phenytoin	>200	>200
Tranylcypromine	>50	>50

* Administered 60 min before the intraperitoneal injection of oxotremorine, 50 μg/kg.

It will be seen that all the centrally active anticholinergic drugs tested were active against both tremor and analgesia and that the correlation between the two series of results is very good.

Whereas chlorpromazine, chlordiazepoxide and dexamphetamine have weak activity against oxotremorine tremor, they are not active in preventing the analgesia.

DISCUSSION

The ability of a drug to prevent oxotremorine-induced analgesia (antinociception) in mice provides a useful objective measure of its central anticholinergic activity. Quaternary atropine-like drugs, e.g. atropine methyl bromide and propantheline, are not active nor are sympathomimetic drugs such as amphetamine, whereas they are active in the test of Spencer (1965) using hypothermia as a measure. The test is not liable to subjective errors in assessment as may occur in tests based on tremor scoring. Compounds which are analgesic would give false positives in this test but their antinociceptive properties would be seen before administration of oxotremorine.

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The relation between blood levels and urinary excretion of amphetamine under controlled acidic and under fluctuating urinary pH values using [^{14}C]amphetamine

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Plasma, blood cell and urine levels of amphetamine were determined after the oral administration of *S*-(+)-[^{14}C]amphetamine sulphate to two subjects under conditions of controlled acidic and fluctuating urinary pH. The decline in plasma concentration of the drug was more rapid under the controlled acidic conditions than under conditions of fluctuating urinary pH. Under controlled conditions, the concentration time profiles of drug and metabolite in urine or plasma (as opposed to body levels), were suitable for kinetic analysis. The apparent rate of urinary excretion of amphetamine was proportional to its plasma concentration only under the controlled acidic urinary conditions. Amphetamine was cleared from blood more rapidly than could be accounted for by glomerular filtration under acid conditions, but when urinary pH fluctuated, clearance of the drug could be accounted for by this route.

Changes in urinary pH in man produce fluctuations in the rate of excretion of amphetamine (Beckett & Rowland, 1965). However, the excretion profile of this drug under normal conditions of fluctuating pH can be predicted accurately from pH values and volumes of urine collected after an oral dose. This has been done by kinetic analysis of the data. For this purpose, distribution and excretion rate constants were obtained by analysis, using an analogue computer, of data obtained previously under controlled conditions (Beckett, Boyes & Tucker, 1968a, b). In these calculations it was assumed that when reabsorption of amphetamine from the kidney tubule was negligible, the rate of urinary excretion was proportional to its plasma concentration, which in turn was proportional to the total amount present in the body (excluding the gut). The prediction of body levels and related plasma profiles of drug-time relations under conditions of changing urinary pH could not be verified at the time, because no suitable, sensitive analytical technique was available to determine amphetamine in human blood after a normal dose.

The purpose of the present work has been to determine the time course of plasma and blood cell concentrations of amphetamine, in addition to urine levels, after an oral dose under controlled and uncontrolled conditions to produce further support for the above assumptions and methodology. An additional objective was to demonstrate the advantage of studies under controlled acid urinary pH in establishing the relative importance of metabolic routes irrespective of whether determined using blood or urine data. It was also proposed to investigate whether amphetamine passed into the urine by routes other than by glomerular filtration.

EXPERIMENTAL

Apparatus and Materials

[^{14}C]Levels were determined on a Tri-Carb liquid scintillation counter (Packard Instrument Company Inc., Model 500D) or by using a radio-chromatogram scanner (Panax Equipment Limited, Model RTLS 1). Chromatographic determinations were made on a Perkin Elmer F11 gas chromatograph with a flame ionization detector and pH values were measured with a Pye Dynacap pH meter.

The *S*-(+)-[^{14}C]amphetamine (α-methyl-[β-^{14}C]phenethylamine) sulphate (3 μCi/mg) was supplied by Smith, Kline and French Research Laboratories, Philadelphia.

Method

Trials. Two male volunteers (21 and 23 years) were given *S*-(+)-[^{14}C]amphetamine sulphate (15 mg; 45 μCi) in aqueous solution by mouth. Urine samples were collected at 30 min intervals for 4 h and then at 60 min intervals for a further 8 h; finally a 24 h sample was collected. Blood samples were taken from the median cephalic vein at times midway between those of urine samples. The blood samples were oxalated and centrifuged (3000 *g* × 10 min) to separate plasma from the blood cells. All samples were stored at 4° until analyses had been completed. Two trials were conducted. In the first, the urinary pH was not modified, whilst in the second, an acid urinary pH (5·0 ± 0·2) was maintained as previously described (Beckett & Tucker, 1966). The pH and volume of each urine sample was accurately measured at the time of collection.

Extraction procedure. The pH of an aliquot (5 ml) from each urine sample was adjusted to pH 1 by adding 0·1 ml 6N HCl. The acidic and neutral metabolites of amphetamine were then extracted from the urine by freshly distilled diethyl ether (3 × 2·5 ml). Analar dioxan (1 ml) was added to the combined ethereal extracts in a Tri-Carb counting vial and most of the ether was removed by evaporation on a water bath at 45°, to give fraction A. The aqueous phase was adjusted to pH 12 by the addition of 0·5 ml 20% NaOH, was re-extracted with ether and the extract treated as described above to give a solution of [^{14}C]amphetamine in dioxan (Fraction B).

The procedure for extracting the drug from plasma (2 ml aliquot) and blood cells (1 ml aliquot) was similar to that described for urine. The blood cells were disintegrated by ultrasonic treatment before extraction.

Radioactive analysis. Scintillation fluid was prepared by mixing naphthalene (60 g), PPO (4 g), dimethyl POPOP (0·2 g), methanol (100 ml), toluene (100 ml), ethylene glycol (20 ml) with dioxan to 1 litre. The scintillant solution (10 ml) was added to the vials before counting. Both fractions (A and B), described above, were counted for each sample of urine, plasma and blood cells. In addition, a sample (0·2 ml) of the aqueous phase remaining after fractions A and B had been removed was counted (fraction C).

Background counting corrections were determined on samples of biological fluid (i.e. urine, plasma or blood cells) which had been extracted and prepared as described above. Corrections for dilution and quenching effects were made by the channel ratio method.

The concentration of metabolites were expressed as the concentration of amphetamine metabolized.

Chromatography. The components giving the activity in the three fractions (A, B and C) were investigated by thin-layer chromatography. Glass plates (20 × 20 cm)

were coated with a layer (0·5 mm) of silica gel G (nach Stahl), activated at 105° for 1 h and stored in a desiccator over silica gel until required. Spots of each fraction and also of [^{14}C]amphetamine in ether as reference were applied and the chromatograms developed in the solvent systems shown in Table 1. The plates were then scanned with a radiochromatogram scanner fitted with a continuous gas flow Geiger tube, and the Rf values of the regions of activity were determined.

Fraction B from several samples of urine was analysed by the gas-liquid chromatographic method of Beckett & Rowland, 1965.

The glomerular filtration rates of both subjects were determined by measuring the clearance of endogenous creatinine from the plasma, and the clearance rate of amphetamine from the plasma was calculated in each trial. Total blood volumes and haematocrit levels were also measured.

RESULTS AND DISCUSSION

Chromatographic studies showed that the radioactivity measured in fraction B was associated entirely with a component having the same Rf value (Table 1) as amphetamine. Determinations of the concentration of amphetamine by gas-liquid chromatography and by the radioactive technique gave similar results. Thus the radioactivity measured in fraction B in subsequent work was interpreted as representing amphetamine levels.

Table 1. *Rf values for the radioactive component present in fraction B and* [^{14}C] *amphetamine after thin-layer chromatography in various solvent systems*

Solvent system	Rf values	
	[^{14}C]amphetamine	Fraction B
Chloroform–diethylamine (9:1)	0·58	0·58
Chloroform–acetone–diethylamine (5:4:1)	0·70	0·68
Chloroform–ethanol–ammonia (100:15:1)	0·08	0·07
Benzene–1,4-dioxan–acetic acid (45:12·5:2)	0·03	0·04

The rate of excretion of amphetamine in urine showed urinary pH dependent fluctuations which agreed with earlier studies made under conditions of fluctuating urinary pH (Beckett & Rowland, 1965). However, as expected, plots of plasma concentration against time, although showing some irregularities, did not exhibit definite fluctuations associated with the changes in urinary pH (Fig. 1). Under controlled acidic conditions, there were no fluctuations in the rate of urinary excretion of the base, and the profiles of rate of urinary excretion and plasma concentration against time were similar (Fig. 2). For instance, peak levels of amphetamine were reached in both plasma and urine $1\frac{1}{4}$ h after administration of the dose; also after absorption was complete (a period of about 4 h being indicated from both urine and plasma data) there was an exponential decrease in both plasma and urine concentrations, as shown by the linear log plots (Fig. 3) in which almost parallel lines were obtained for the two systems. Under uncontrolled conditions, no such relation could be obtained for urine, and the log concentration against time plot of plasma levels gave a completely different relation from that established under controlled conditions (Fig. 3).

The results indicate that under acidic but not under fluctuating urinary conditions the rate of urinary excretion of amphetamine is *directly* proportional to its plasma concentration.

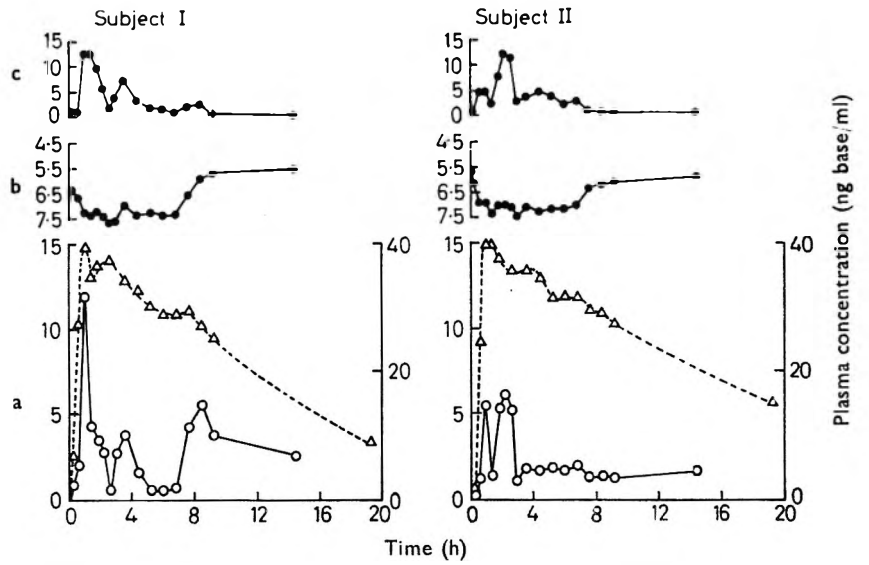

FIG. 1. Urinary excretion and the corresponding plasma levels of [^{14}C]amphetamine after oral administration of 15 mg (+)-[^{14}C]amphetamine sulphate to subjects I and II under conditions of fluctuating urinary pH. a. Rate of excretion. b. Urinary pH. c. Urine flow (ml/min). —○— Urinary excretion. – – △ – – Plasma concentration.

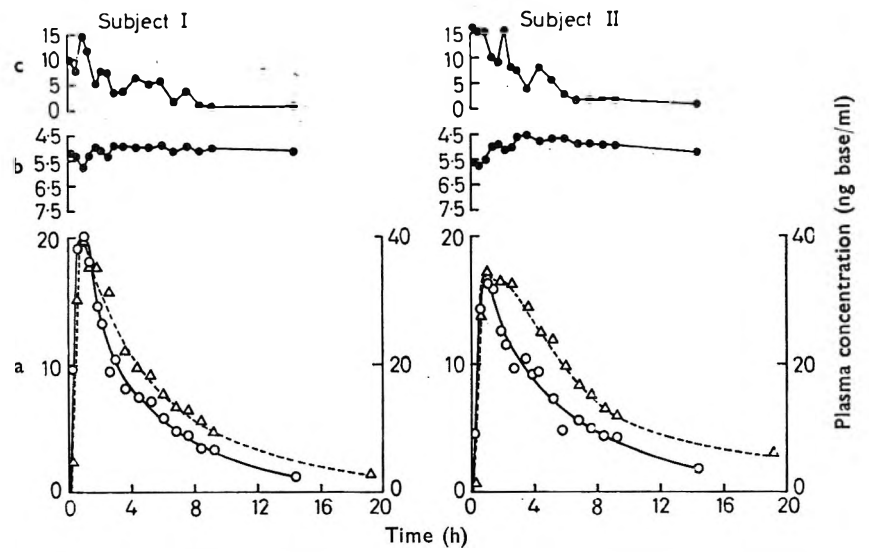

FIG. 2. Urinary excretion and the corresponding plasma levels of [^{14}C]amphetamine after oral administration of 15 mg (+)-[^{14}C]amphetamine sulphate to subjects I and II under conditions of acidic urinary pH. Symbols as in Fig. 1.

The urinary excretion rates of amphetamine, ether soluble acidic and neutral metabolites (Fraction A) and ether insoluble acidic metabolites (Fraction C) gave a very complicated pattern under conditions of fluctuating urinary pH (Fig. 5). However, when an acidic urine was maintained, a clear relation emerged between the rates of excretion of amphetamine and the two above-mentioned metabolite fractions (Fig. 6).

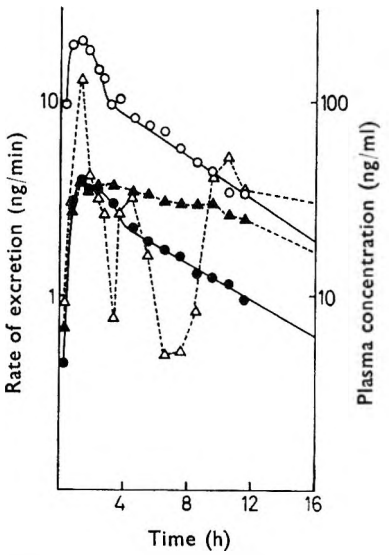

FIG. 3. Urinary excretion and plasma concentration of amphetamine after oral administration of 15 mg (+)-[^{14}C]amphetamine sulphate under conditions of acid controlled and fluctuating urinary pH. Subject I. Controlled urinary pH: —○—Rate of excretion. —●— Plasma concentration. Fluctuating urinary pH: —△— Rate of excretion. - - ▲ - - Plasma concentration. Subject II behaved similarly.

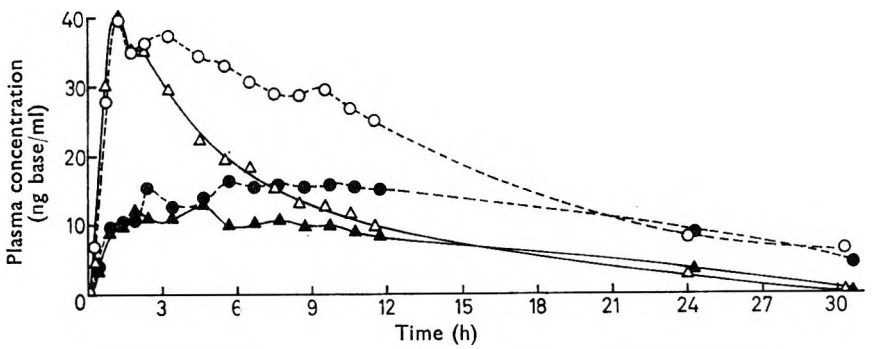

FIG. 4. Plasma concentrations of [^{14}C]amphetamine and its metabolites after the oral administration of 15 mg (+)-[^{14}C]amphetamine sulphate. Subject I. Controlled urinary pH: —△— Amphetamine base. —▲—Acidic and neutral, ether-soluble metabolites urinary pH. Uncontrolled urinary pH: - - ○ - - Amphetamine base. - - ● - - Acidic and neutral, ether-soluble metabolites.

As expected, the recoveries of unchanged drug and metabolite fractions from urine were very different under the two conditions (Table 2), but there was much less difference between the corresponding plasma levels. However, plasma levels fell more rapidly under the controlled acidic conditions (Fig. 4). Under acid conditions there was a lower concentration of neutral and acidic ether soluble metabolites in the plasma (Fig. 4). Ether insoluble acidic metabolites were only detected in urine (Figs 5 and 6).

Calculations of peak blood levels of amphetamine (48 and 40 ng/ml, acidic conditions, and 52 and 47 ng/ml, uncontrolled conditions, for subjects I and II respectively) from the plasma and blood cell data (Table 3) indicate that at peak concentration only 1/40th of the administered dose is present in the blood. This indicates rapid

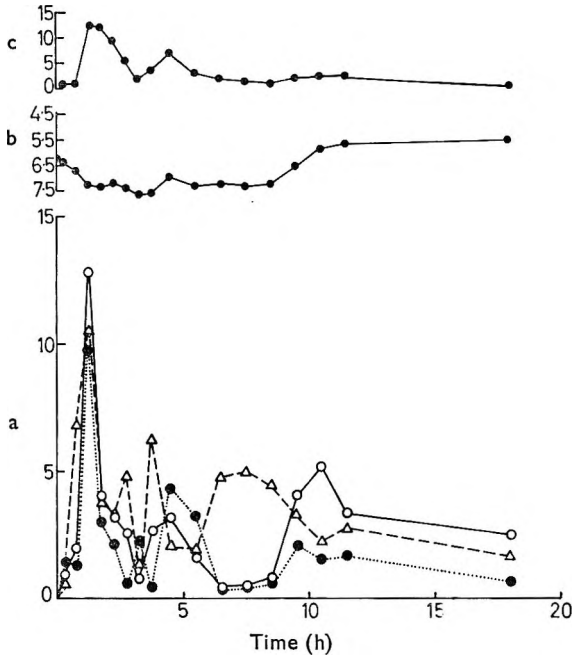

FIG. 5. Urinary excretion of [^{14}C]amphetamine and its metabolites after the oral administration of 15 mg (+)-[^{14}C]amphetamine sulphate under conditions of fluctuating urinary pH. Subject I. —○—Amphetamine base. – – △ – – Ether-insoluble metabolites. ---●--- Acidic and neutral ether-soluble metabolites. Subject II behaved similarly.

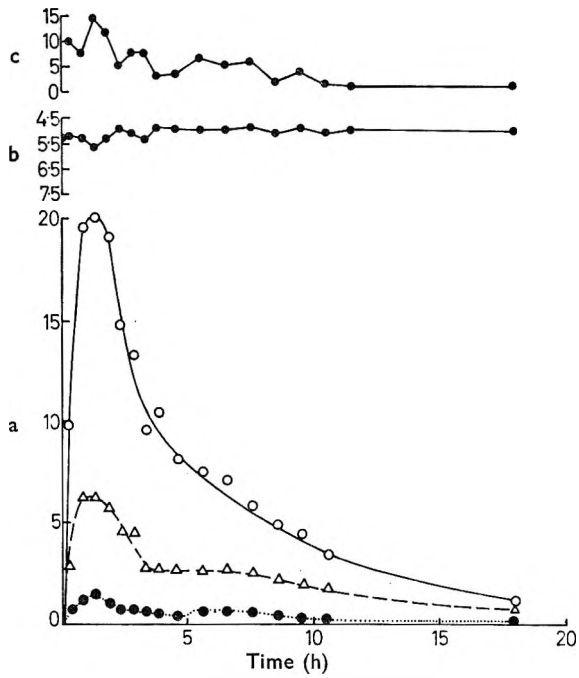

FIG. 6. Urinary excretion of [^{14}C]amphetamine and its metabolites after the oral administration of 15 mg (+)-[^{14}C]amphetamine sulphate under conditions of acidic urinary pH. Subject I. Symbols as in Fig. 5.

Table 2. *Urinary excretion of amphetamine and its metabolites—information from two subjects after each had received an oral dose* (15 *mg*) *of* (+)-[^{14}C]*amphetamine sulphate*

Condition of urine	Subject	Recoveries (%) in 24 h: Amphetamine base extracted into ether under alkaline conditions (pH 12)	Metabolites: Fraction A Metabolites soluble in ether under acidic conditions (pH 1)	Metabolites: Fraction C Metabolites remaining in aqueous phase after alkaline and acidic extractions	Total
Acid control	I	64·9	5·5	20·1	90·5
pH 5 ± 0·2	II	63·1	4·6	23·8	91·5
Uncontrolled urinary pH	I	34·6	17·4	35·5	87·5
pH 5·5–7·7	II	34·9	17·0	19·0	60·9

Table 3. *Results of haematological Investigation*

	Subject I	Subject II
Weight	68 kg	66·5 kg
Erythrocyte volume	1493 ml	2199 ml
Blood volume	4557 ml	5370 ml
Haemoglobin	12·0 g%	15·1 g%
Haematocrit value	36·0%	45%
Mean creatinine excretion in 24 h	1·65 g	1·80 g
Erythrocyte count	3,960,000	4,800,000

extravascular distribution. The maximum plasma concentration of amphetamine occurred 1¼ h after the dose was administered, whereas peak blood cell concentration occurred later. The concentration in the blood cell then remained at a consistently higher level than that in plasma, indicating that equilibrium is established only slowly between plasma and these cells (Fig. 7).

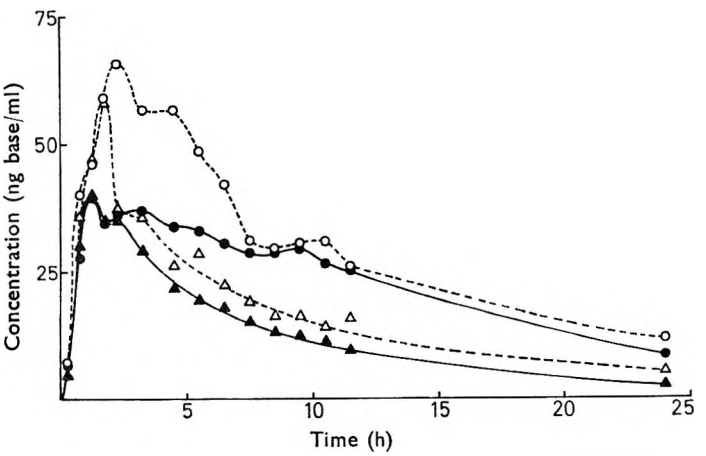

FIG. 7. Plasma and blood cell concentrations of [^{14}C]amphetamine after the administration of 15 mg (+)-[^{14}C]amphetamine sulphate under conditions of acid controlled and fluctuating urinary pH. Subject I. Controlled urinary pH: – – △ – – Blood cell.—▲— Plasma. Uncontrolled urinary pH: – – ○ – –Blood cell. —●— Plasma.

Table 4. *Amphetamine clearance from plasma. Subjects given an oral dose of* 15 mg (+)-[^{14}C]*amphetamine sulphate*

Condition of urine	Clearance of amphetamine from plasma (ml/min)	
	Subject I	Subject II
Acidic control	432–539	242–387
Fluctuating urinary pH	16–115	41–64

Glomerular filtration rate, (measured from creatinine clearance data) for subject I was 125, and for Subject II 126 ml/min.

The plasma clearance of amphetamine under acidic urine conditions was 400–550 ml/min whereas the glomerular filtration rate was only 125 ml/min (Table 4). The amphetamine clearance under uncontrolled conditions could be accounted for by glomerular filtration. The results indicate that about 75% of the drug must be transferred from the plasma into the tubules by routes other than glomerular filtration when the urine is acidic. Probably as urine flows down the kidney tubules, the drug passes from the blood to the urine because of the high concentration gradient of un-ionized drug across the lipid membrane.

Acknowledgements

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The carvone and dillapiole content of dill fruits by gas chromatography without preliminary distillation

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Cold hexane extracts of sixteen specimens of dried dill fruit were examined for their content of carvone and dillapiole by gas chromatography. Dillapiole was absent from "European dill" (even Indian grown), and present in "Indian dill" (including European grown) in about twice the weight of carvone. These two forms of dill are otherwise less distinct than the forms of fennel which are referred to one species.

Growing fruits of European dill, *Anethum graveolens* L., have been observed to produce a 'specific level' of carvone some time before they are ripe (Betts, 1965). Developing fennel fruits were not found to have 'specific levels' of anethole and fenchone (Betts, 1968a), although in mature fruits the proportions of these constituents were found to be reasonably constant for three forms of fennel (Betts, 1968b). Sixteen dried dill fruits of various origins have now been examined by gas-liquid chromatography after cold solvent extraction, to assess the proportion of the terpenoid (+)-carvone to dillapiole, should the latter aromatic substance be present. When found, dillapiole exceeded the amount of carvone in the fruits, even those grown in England. Dillapiole-free dill was found from many geographical sources, including India.

EXPERIMENTAL

Drug material and its extraction. Specimens of dried dill fruit were obtained from the sources indicated in Table 1, where they are also described. Extraction was as previously described (Betts, 1968b) but using n-hexane containing the two paraffins n-eicosane and n-tetracosane as internal standards for the test oil constituents carvone and dillapiole respectively.

Gas chromatography was as previously described (Betts, 1968a, b) on a 15% Carbowax 20M column, but at 190° to enable the dillapiole to emerge within 20 min, t_r relative to tetracosane being 0·81. Carvone emerged within 6 min under these conditions, t_r relative to eicosane being 0·49. The identity of the carvone and dillapiole peaks was confirmed on a column of silicone elastomer E301, on which the paraffins are not suitable as standards owing to their very long retention times.

RESULTS AND DISCUSSION

As with fennel (Betts, 1968b), wide variations were noted in the content of the constituents of the essential oils of different dill fruits (Table 2), these bearing no

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Table 1. *Origin and description of dill fruits*

Reference letter and source	Size (length × width)	Colour	Form	Av. wt in mg of 100 cremocarps
A Retail grocer's proprietary pack	4·5 × 2·5 mm	Grey-brown, buff wings and ridges	Ovoid, rounded cremocarps and mericarps	665
B "Peucedanum sowa" (1897) Pharm. Soc. Museum	5·0 × 2·5 mm	Pale buff-brown lighter wings and ridges	Ovoid-lanceolate rounded cremocarps and mericarps	560
C Madras (Lalah and Sons) India	5·0 × 2·5 mm	Grey-brown, buff wings and ridges	Ovoid, rounded cremocarps and mericarps	550
D "European dill" Stock drug at School of Pharmacy	6·0 × 3·0 mm	Brown, light buff wings and ridges	Mostly flattened mericarps, ovoid	525
E "Indian dill" Stock drug at School of Pharmacy	4·5 × 2·5 mm	Pale brown, lighter wings and ridges	Ovoid-lanceolate, rounded cremocarps and mericarps	515
F Olomouc, Czechoslovakia "A. graveolens"	4·5 × 3·0 mm	Dull brown, paler wings	Ovoid, flat mericarps	400
G Myddelton House, School drug garden, England	5·0 × 2·5 mm	Dark brown, paler wings and ridges	Ovoid, flat, mostly mericarps	395
H Wisconsin, U.S.A. "A. graveolens"	4·5 × 2·5 mm	Dark brown, paler wings	Ovoid-lanceolate, fairly flat mericarps	365
J "Anethum graveolens" Pharm. Soc. Museum	4·0 × 2·5 mm	Grey-brown, paler wings	Ovoid-rounded, fairly flat mericarps	355
K Iasi, Rumania "A. graveolens"	4·0 × 2·5 mm	Brown, pale distinct wings and ridges	Ovoid, fairly flat mericarps	330
L Kaunas, U.S.S.R. "A. graveolens"	4·0 × 2·5 mm	Dull brown, paler wings	Ovoid-rounded, flat, curled mericarps	330
M Jammu, India "A. graveolens"	5·0 × 2·5 mm	Grey-brown, paler wings	Lanceolate, flat, twisted mericarps	300
N Essen, Germany "A. graveolens"	4·5 × 2·0 mm	Light brown, paler wings and ridges	Ovoid, flat, very thin mericarps	295
P Istanbul, Turkey "A. graveolens"	4·5 × 2·5 mm	Grey-brown, paler wings and ridges	Ovoid-lanceolate, rounded, mostly mericarps	295
Q Jammu, India "Anethum sowa"	3·0 × 1·5 mm	Grey-brown paler wings and ridges	Pyriform cremocarps and mericarps	265
R Grown from Q at Myddelton House, School drug garden	4·5 × 1·5 mm	Grey-brown, paler wings and ridges	Pyriform, thin mericarps	(80) unripe

Specimens F, H, K, L, N and P were from Botanical Gardens. M and Q were kindly sent by Dr. C. K. Atal of the Regional Research Laboratory, Jammu, India; and C was supplied by P. Mittulaul Lalah & Sons of Madras, India at the kind request of Dr. J. S. Pruthi of the Central Agmark Laboratory, Nagpur.

relation to the weights of the fruits. However, the various specimens fell into two groups; with or without dillapiole. The first group represents "Indian dill" whilst the second group is "European dill" without necessarily implying geographic origin. Apart from one example in each group (D and Q–R being grown from Q) the Indian dill was heavier than the European dill, weighing over 500 mg/100 fruits (cremocarps) against the 400 mg or less of the latter. Although the carvone content of 100 fruits was similar over part of the range exhibited by the two groups, the assay on fruit

Table 2. *Essential oil constituents of dill specimens*

Reference (see Table 1)	mg/100 fruits (= cremocarps)		Ratio w/w dillapiole: carvone	mg/g fruit wt	
	Carvone	Dillapiole		Carvone	Carvone + dillapiole
B	5·0	9·2	1·8	9	25
A	4·7	7·9	1·7	7	19
E	3·3	4·3	1·3	6	15
C	3·0	6·7	2·2	6	18
R	2·5	4·1	1·6	unripe	
Q	1·8	4·0	2·2	7	22
G	8·2			21	
L	6·5			20	
K	6·2			19	
F	5·3			13	
D	5·3			10	
J	4·8			14	
M	4·4			15	
N	3·9			13	
P	3·9			13	
H	3·7			10	

weight was accordingly distinctive, being 10 mg/g or above for European dill, but less than this for Indian dill. When present, dillapiole occurred in about twice the weight of carvone (except for specimen E), so that the combined content of carvone and dillapiole was equal to, or greater than, the carvone content of the best European dill on a weight basis.

Dill is not an easy plant to grow, often dying before all its fruits are ripe, and some specimens contained many small fruits which were not used in this work. However, the low weight Indian dill Q was not unripe, for it germinated well, unlike many dills. It provided specimen R which showed that the presence of dillapiole is not specific to dill grown in India. Conversely, European dill grown in India (M) is dillapiole free. Plants grown from Q were virtually indistinguishable from European dill plants, so that the validity of the taxon *Anethum sowa* Roxb. for Indian dill appears to rest on slight differences in fruit shape and the presence of dillapiole, although the "*A. graveolens*" fruit of Khafagy & Mnajed (1968) contained dillapiole. The bitter and sweet forms of fennel are much more distinct (Betts, 1968a) yet are referred to as one species.

The carvone content of European dill grown at Myddelton House corresponded to the level per 100 fruits oberved in fresh, ripe material (Betts, 1965). As with fennel, it was possible to examine individual fruits (Betts, 1968b) of a specimen suspected to be mixed because of a dillapiole: carvone ratio of only 0·3. This came from another English school of pharmacy labelled "European dill", and contained some Indian dill.

Although terpene hydrocarbon peaks were observed, their evaluation would have required the gas chromatograph to be run at a lower temperature, and these substances have been observed to decrease during storage of dill (Kalitzki, 1954) which consists of thin structures with a large surface area. The values for Museum specimen B do not suggest any loss of the other essential oil constituents.

Dillapiole is less volatile than carvone and probably more soluble in the aqueous condensate from steam distillation. Nevertheless, dillapiole forms a considerable proportion of Indian dill oil, [19% recorded by Chakravati & Bhattacharya (1954) and about 27% by Khafagy & Mnajed (1968)] though contributing little to the odour.

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Mr. M. Humphrey of Bush, Boake, Allen Ltd. kindly provided the reference dillapiole prepared by gas chromatographic isolation, and the packing for the chromatographic column used. Thanks also to Mr. L. Guglielmi for growing the plants, and to the various botanical gardens and other sources of dill mentioned in Table 1.

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LETTERS TO THE EDITOR

Decrease in homovanillic acid as evidence for dopamine receptor stimulation by apomorphine in the neostriatum of the rat

Substances known for their ability to block the central catecholamine receptors, for example chlorpromazine or haloperidol, increase the concentration of homovanillic acid (HVA) in the neostriatum of various species (Andén, Roos & Werdinius, 1964; Laverty & Sharman 1965; Juorio, Sharman & Trajkov 1966; Sharman 1966). This has been suggested to depend on a feed-back mechanism resulting in an increase in the synthesis of dopamine to compensate for the effect of blocking the receptors (see Carlsson & Lindqvist 1963). The opposite phenomenon, a decrease in HVA after stimulating the catecholamine receptors with some dopamine-like transmitter, i.e. a compound with an effect similar to that of dopamine, has not been reported. However, in 1967, Ernst suggested that the compulsive gnawing induced by apomorphine in rats is not mediated via the release of catecholamines, as it is after treatment with amphetamine, but caused by the action of apomorphine on the dopamine receptors themselves. Further, evidence of a direct stimulating effect of the dopamine receptor in the rat brain by apomorphine was recently presented by Andén, Rubenson & others (1967) and Butcher & Andén (1969). These authors also demonstrated that apomorphine retarded the depletion of dopamine caused by a tyrosine hydroxylase inhibitor (H 44/68) and explained this by a negative feed-back mechanism due to activation of the dopamine receptor. I now report the effect of apomorphine on the level of HVA in the rat brain.

In four experiments, 12 male Sprague-Dawley rats were treated with apomorphine 15 mg/kg i.p. twice, with an interval of 1 h and killed 2 h after the first injection. All rats showed the typical compulsive gnawing. Four groups of 12 rats received no treatment. The level of HVA in the neostriatum of untreated rats was $0{\cdot}47 \pm 0{\cdot}049$ and of treated rats $0{\cdot}10 \pm 0{\cdot}025$ (μg/g; mean $\pm$ s.e.). The decrease in the levels of HVA is statistically highly significant ($P < 0{\cdot}001$, Student's t-test). When this finding is considered in the light of the unchanged levels of dopamine after apomorphine found by Andén & others (1967) in the whole brain of the rat, a pattern, seen in earlier studies with 5-hydroxytryptamine (5-HT) emerges. 5-HT and its corresponding acid, 5-hydroxyindoleacetic acid (5-HIAA), were measured at the same time after lysergic acid diethylamide and there was a decrease in the synthesis of 5-HT (Diaz, Ngai & Costa, 1968). The lack of significant change in the level of the dopamine found by Andén (1967) and the highly significant decrease in the HVA that I found, suggests a reduced activity in the dopamine neurons after apomorphine. If the action of apomorphine were to be mediated by release of dopamine, the level of dopamine might possibly remain unchanged but the HVA level would be increased significantly. Further, inhibition of monoamine oxidase would not leave the dopamine level unchanged. The decrease in HVA after apomorphine that I observed, correlates with the α-methyltyrosine-induced disappearance of dopamine shown by Andén & others (1967), and offers further biochemical evidence for the stimulating effect of the apomorphine on the dopamine receptor.

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January 28, 1969

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Activation of pepsinogen by sulphated glycosaminoglycans: a possible role in peptic ulcerogenesis

Sulphated glycosaminoglycans (SGs) occur in the gastrointestinal tract at lumenal and intracellular levels, and it has been suggested that they may be secreted by gastric zymogen cells (Schrager, 1964; Spicer, 1965; Spicer & Sun, 1967; Gerald, de Graeff & others, 1967). This association of SG and the source of gastric pepsin has led to the suggestion of an antipeptic role for SG at intracellular level (Gerald & others, 1967). Our experiments now suggest that a more subtle relation may exist between the zymogen pepsinogen and SGs.

Back diffusion of hydrogen ion in the abnormally permeable gastric mucosa present in gastric ulcer has been shown (Davenport, 1965; Overholt & Pollard, 1968); there is therefore a distinct possibility that at least regions of specific gastric cells may attain an abnormally low pH. With the chief cell, absorption of hydrogen ion would give rise to a system containing pepsinogen, hydrogen ion and SG. While the intracellular concentration of hydrogen ion may not reach a level sufficient to activate the pepsinogen at a significant rate, any marked increase in the rate of activation could, in conjunction with the autocatalytic nature of the acid activation of pepsinogen (Herriott, 1938), cause significant pepsin production and possibly consequent intracellular proteolysis.

Pepsinogen (Sigma Chemical Co.) was activated at various pH values and constant ionic strength for varying periods of time in the presence and absence of the SGs chondroitin sulphate-A or heparin, and the resulting pepsin was destroyed by raising the pH to 8. The pepsinogen remaining (unactivated) was measured by rapid activation at pH 1·6 followed by assay of the peptic activity using the haemoglobin digestion method. This technique (after Herriott, 1938) allows the effect of the SGs on the activation of pepsinogen, determined by difference, to be observed.

Pepsinogen (10 mg) was dissolved in 0·002 M phosphate buffer pH 6·9 (25 ml), and the varying amounts of SG added* to this solution which was kept at 37° for 10–15 min. Acetate buffer (9 ml) (Long, 1961), ionic strength 0·05, at the pH of activation (pH 3·6, 4·0, 4·3) and 37° was added at zero time to start activation. Aliquots (2 ml) were removed at suitable time intervals, mixed with saturated sodium tetraborate solution (2 ml) which had been diluted so that the pH was thereby raised to 8–8·5, and allowed

* Heparin 0·2–5 mg; chondroitin sulphate 1–20 mg.

to stand 10 min. The pepsin thus destroyed, the remaining pepsinogen was activated by adding 1 ml HCl solution (to adjust pH to 1–2) and the mixture allowed to stand 5 min (solution A). Peptic activity was determined by adding 1 ml of solution A to 5 ml haemoglobin (Armour Laboratories Ltd.) solution at pH 1·6 and measuring the digestion (Northrop, Kunitz & Herriott, 1948). Extinctions were read at 280 nm within 15 min of dilution and units calculated by reference to a standard curve for crystalline pepsin.

From plots of percent pepsinogen remaining *vs* time, the time for 50% activation of pepsinogen was calculated (t50%); the amount of SG required to halve this time was also determined graphically. The results in Table 1 show that this was a fraction of the weight of pepsinogen used.

Table 1. *Ratio of weight of sulphated glycosaminoglycan: weight of pepsinogen which halves the t50% for pepsinogen*

pH of activation	t50% for pepsinogen min mean ± s.d.	Ratio for ½ t50%	
		$\frac{\text{CHS}}{\text{Pg}}$	$\frac{\text{H}}{\text{Pg}}$
3·6	15 ± 2·3	0·18; 0·20; 0·20	0·10; 0·08; 0·11
4·0	51 ± 3·6	0·19; 0·17; 0·20	0·11; 0·12; 0·10
4·3	170 ± 6·4	0·22; 0·19; 0·21	0·07; 0·13; 0·09

CHS = chondroitin sulphate; H = heparin; Pg = pepsinogen; t50% = time for 50% activation of pepsinogen at the stated pH in the conditions of the experiment.

Pepsinogen activation occurs below pH 5 with release of inhibitor (Herriott, 1938, 1941), thus:

$$\text{pepsinogen} \rightarrow \text{pepsin} + \text{inhibitor} + \text{peptides}$$

Even in excess quantity, SGs did not activate pepsinogen above pH 5 in our experiments, indicating that a certain minimum hydrogen ion concentration is required for any activation to occur. Neither SG altered the pH of the solutions.

The results show that chondroitin sulphate-A halved the t50% for five times its weight of pepsinogen, and heparin for ten times its weight, between pH 3·6 and 4·3. Interaction of SG with the cationic inhibitor released during activation is suggested as the mechanism by which SGs accelerate the activation of pepsinogen.

Three points arise. First, if a cell which normally contains pepsinogen and SG absorbs hydrogen ion so that the pH falls below 5, SG will accelerate zymogen activation. Since in most cells a fall to well below pH 5 is unlikely in the presence of some cell integrity, acceleration of activation would assume importance.

Second, this action of SG at very low concentration must be clearly distinguished from the antipeptic activity of sulphated polysaccharides now known to be effected by substrate protection (Anderson & Baillie, 1967).

Third, pepsinogen occurs in the chief cells of the stomach principally encapsulated in granules. These coacervate-like globules will be disrupted by any acid which is absorbed, liberating pepsingoen. Consequent zymogen activation will be speeded up by the free SG in the cell even in the presence of acid sufficient only to lower the pH to just below 5. Thus intracellular proteolytic potential is increased by the presence of SG when small quantities of acid are absorbed. An ulcerogenic tendency of the intracellular pepsin produced is a reasonable assumption. This "pathological" action of SGs would be of most consequence when restricted quantities were present; when excess was available the (normal) antipeptic action of SGs would offset the effects of accelerated pepsin production. Hakkinen (1966) has pointed out that mucosal sulphate is depleted when ulceration occurs.

This concept may help to relate the antisecretory (Anderson, Marcus & Watt, 1962; Eagleton, Watt & Marcus, 1968) and antipeptic actions of SGS in respect of their anti-ulcer action.

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Amphetamine-induced release of [^{3}H]metaraminol from subcellular fractions of the mouse heart

The mode of action of amphetamine is complex and there are many studies reporting various sites of attack for this drug.

Amphetamine has a cell membrane pump blocking action—but it is not as potent as for example protriptyline (Carlsson & Waldeck, 1965; Malmfors, 1965; Carlsson, Lindqvist & others, 1965). It has also been suggested that amphetamine in large doses has a direct releasing effect on the amine storing granules. More recently Carlsson, Lindqvist, & others (1966a) suggested that amphetamine in low doses might act by displacement of amines from hypothetical extragranular binding sites or by an effect on the cell membrane leading to increased release (Carlsson, Fuxe & others, 1966b).

The aim with the present work was to see if studies on subcellular amine distribution could further elucidate the mode of action of amphetamine. This approach has proved useful for clarifying the mechanism of action of other drugs influencing adrenergic mechanisms (Lundborg, 1967). As in much of our previous work [^{3}H]-metaraminol, a noradrenaline analogue resistant to both monoamine oxidase and catechol-*O*-methyl transferase, was used.

Mice in groups of six, were given [^{3}H]metaraminol, 0·04 mg/kg, intravenously. Control groups received no further treatment and were killed by decapitation 30 min later. Other groups were injected with (+)-amphetamine bitartrate 5, 1 or 0·2 mg/kg (calculated as the salt) 15 min after the [^{3}H]metaraminol administration and were killed 15 min later. All animals were kept at an ambient temperature of 30°.

The hearts were removed and homogenized in the cold, using a plastic pestle, in 0·25 M sucrose containing 0·005 M phosphate buffer at pH 7·4 and 0·001 M $MgCl_2$. A coarse fraction was obtained by centrifugation at 4° at 2000 *g* for 10 min. The supernatant was then centrifuged at 100,000 *g* for 60 min in a Spinco model L Ultracentrifuge providing two more fractions, particulate (P) and high speed supernantant (S). After protein precipitation of the various fractions the samples were passed through an ion-exchange column (Dowex 50 W × 4). After elution the samples were analysed by means of liquid scintillation. Further details of the analytical procedure has been previously described (Stitzel & Lundborg, 1967).

When used in a dose of 5 or 1 mg/kg, (+)-amphetamine caused a pronounced decrease in the total content of [^{3}H]metaraminol in the mouse heart (Table 1). In a dose of 0·2 mg/kg the drug did not significantly change the [^{3}H]metaraminol content of the heart.

Table 1. *Effect of (+)-amphetamine bitartrate on the content and subcellular distribution of [^{3}H]metaraminol in the mouse heart.* The animals were killed 30 min after intravenous administration of 0·04 mg/kg of [^{3}H]metaraminol and (+)-amphetamine was given 15 min before death. The doses of (+)-amphetamine bitartrate are calculated as the salt. $P/(P + S) \times 100$ means the amount of [^{3}H]metaraminol in the particulate fraction as a percentage of [^{3}H]metaraminol in the particulate + supernatant fractions.

(+)-Amphetamine dose used mg/kg	No. of exp.	[^{3}H]Metaraminol ng/g	Significance test (Probability)	$\frac{P}{P + S} \times 100$	Significance test (Probability)
5	5	61·9 ± 4·8	< 0·001	17·9 ± 1·0	< 0·025
—	5	100·8 ± 12·5		21·7 ± 1·1	
1	6	63·3 ± 1·7	< 0·025	21·9 ± 1·0	> 0·1
—	6	80·7 ± 5·2		23·3 ± 1·9	
0·2	6	74·1 ± 1·8	> 0·1	23·6 ± 1·6	< 0·01
—	6	78·7 ± 3·0		17·1 ± 0·4	

At the 5 mg/kg dose, (+)-amphetamine caused a pronounced decrease in [^{3}H]-metaraminol levels in all three fractions of the heart (Fig. 1). The decrease was more prounced in the particulate (P) (50%) than in the supernatant (S) fraction (35%), resulting in a decrease in the $P/(P + S)$ ratio (Table 1). Also, 1 mg/kg of (+)-amphetamine caused a decrease in the [^{3}H]metaraminol levels, though now of about the same magnitude in all three fractions. Thus the $P/(P + S)$ ratio did not change significantly. After 0·2 mg/kg of (+)-amphetamine, the [^{3}H]metaraminol content was unchanged in the coarse fraction, increased in the particulate and decreased in the supernatant fraction. The $P/(P + S)$ ratio was significantly increased.

These data imply that the dose used is of importance for the mode of action of (+)-amphetamine. In a dose of 5 mg/kg, (+)-amphetamine caused a release from the particulate (granular) fraction as well as from the supernatant fraction. The fact that the $P/(P + S)$ ratio was decreased suggests that, under these conditions, the effect of (+)-amphetamine on the granular fraction is the dominating effect. Also in a dose of 1 mg/kg, (+)-amphetamine had a releasing effect on the granular fraction; however in these experiments this was of the same magnitude as the supernantant fraction. More interesting is the finding that in the low dose, 0·2 mg/kg, (+)-amphetamine bitartrate did not cause any release from the granules but induced a release of [^{3}H]metaraminol from the supernatant fraction only. This ability to release [^{3}H]metaraminol exclusively from the supernatant fraction has previously been observed for protriptyline, a potent membrane pump blocking agent (Lundborg & Stitzel, 1967). It is, however, doubtful if (+)-amphetamine in the low dose (0·2 mg/kg of the bitartrate) caused release of the [^{3}H]metaraminol by blockade of the cell membrane pump. In fact, the

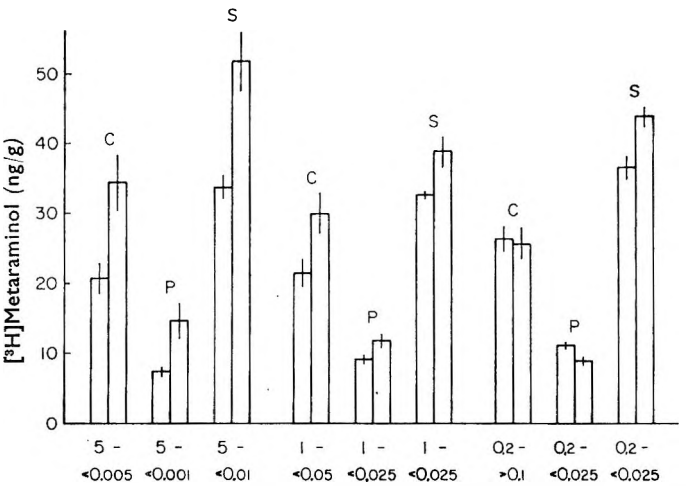

FIG. 1. Effect of (+)-amphetamine on the content of previously administered [³H]metaraminol in subcellular fractions (C = coarse fraction, P = particulate fraction, S = supernatant fraction) of the mouse heart. The dose of [³H]metaraminol was 40 μg/kg. The animals were injected with (+)-amphetamine 15 min after [³H]metaraminol and were killed after another 15 min. Vertical lines indicate standard error of the means. Abscissa: upper scale = amphetamine bitartrate (mg/kg); lower scale = probability.

membrane pump blocking effect of (+)-amphetamine can hardly be seen in this low dosage (Carlsson & others, 1966b and unpublished data). Furthermore, it is well-known that the catecholamine-releasing activity of (+)-amphetamine exceeds that of protriptyline although the latter agent is much more potent in blocking the membrane pump (Carlsson & Waldeck, 1965). This fact also indicates that the mode of action of amphetamine is different from that of protriptyline.

The present data confirm earlier suggestions by Carlsson & others (1966) and Fuxe & Hökfelt (1968) that (+)-amphetamine releases monoamines from extragranular binding sites. The present experimental procedure made possible the demonstration of this type of release more directly and without reserpine pretreatment.

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The resistance of guinea-pigs to cortisone ulcerogenesis

It is generally, though not universally, accepted that the prolonged treatment of man and experimental animals with high doses of glucocorticoids can induce fresh peptic ulcers, reactivate previously quiescent ulcers or exacerbate latent ulcers (Sandweiss, 1954; Gray, 1961; Crean, 1963). We had observed that ulcers did not develop in the stomach of guinea-pigs treated with high doses of cortisone for 10 days, even when animals so treated were also subjected to pylorus ligation at the time of the last cortisone injection (Heisler & Kovacs, 1967a).

We have now subjected guinea-pigs to prolonged cortisone treatment alone or with semi-starvation, restraint and acetylsalicyclic acid administration, to observe whether cortisone produced gastric ulceration under any of these conditions.

Male guinea-pigs, initially 300–340 g, were used. Drugs were cortisone acetate (Cortone; Merck Sharp & Dohme) and acetylsalicyclic acid (Lymans Ltd.). These were always given at 9 a.m.; control guinea-pigs were treated with the same volume of physiological saline. For restraint, the technique described by Brodie & Hanson (1960) was used. Animals were fasted 24 h before the experiment: water was allowed at all times. Cortisone was injected immediately before restraint. Gastric ulceration was evaluated according to the "all or nothing" method of Bonfils & Lambling (1963). In some experiments fasted animals were subjected to pylorus ligation (Shay, Sun & Gruenstein, 1954) and then restrained. The duration of restraint in unligated guinea-pigs was 24 h and in pylorus-ligated animals 16 h. Animals in both groups were injected with physiological saline (3 ml, s.c.) to prevent dehydration. Gastric acidity was measured by titrating the samples, after centrifugation, with 0·1 N NaOH using Töpfer's reagent and phenolphthalein as indicators. The significance of differences between means was calculated by Student's *t*-test and in the "restraint" method the significance was estimated by the χ^2 test.

Prolonged treatment of 10 guinea-pigs with 100 mg/kg of cortisone injected subcutaneously once daily for 14–28 days did not bring about gastric ulceration.

The effect on gastric ulceration of cortisone treatment combined with semistarvation was determined in 12 guinea-pigs. Six guinea-pigs were injected once daily for 10 days with cortisone (100 mg/kg, s.c.), 6 animals received the same dose of cortisone by mouth, since according to Gray (1961) cortisone so given possesses a greater ulcerogenic liability than via the subcutaneous route. The guinea-pigs in both groups were fed for only 5 of every 24 h (12–5 p.m.) except on the last day of treatment when they were fasted all day. Animals were killed 24 h after the last injection of cortisone, the stomachs removed and examined macroscopically. No ulcers were found.

The effect of cortisone combined with restraint on gastric ulceration showed that in the control group (23 animals where restraint was for 24 h) 87% of the animals developed gastric ulceration—no particular anatomical distribution was noted. In the cortisone-treated group (14 animals, cortisone 100 mg/kg, s.c. for 3 days, the last dose before 24 h restraint) the incidence of ulceration was 42·9% which is a significant reduction ($P < 0{\cdot}005$).

Since cortisone stimulates gastric acid secretion in pylorus-ligated guinea-pigs (Heisler & Kovacs, 1967a) we felt that a combination of restraint and pylorus ligation might increase the incidence of ulceration after cortisone administration. This experiment is illustrated in Table 1. Cortisone significantly increased the free and total acid output in comparison with the respective outputs in animals not receiving cortisone. The incidence of ulceration in the latter group was 85% and in the cortisone-treated group 50%. The hyperacidity observed in cortisone-treated guinea-pigs did not increase the incidence of gastric ulceration, rather, there was a definite

tendency for protection against ulceration in the cortisone treated group, though this protection was not statistically significant.

Table 1. *The effect of cortisone on acid secretion and ulcer incidence in pylorus-ligated and restrained guinea-pigs*

Treatment	No. of animals	Volume of gastric juice (ml)	Free HCl m-equiv	Total acid m-equiv	% showing ulceration
Control (pylorus ligated and restrained for 16 h)	7	13·30 ± 2·16	0·37 ± 0·10	0·64 ± 0·12	85·0
Cortisone, 100 mg/kg s.c. for 3 days. Last dose: at ligation	8	17·03 ± 2·10	0·92 ± 0·17	1·18 ± 0·16	50·0
Probability		N.S.*	< 0·02	< 0·05	N.S.

* N.S. = not significant.

Bleeding from the stomach may occur in about half the general population who take aspirin (Smith & Smith, 1966) and gastric erosions after acetylsalicylic acid commonly occur in man (Muir & Cossar, 1955) and experimental animals (Anderson, 1964). To determine whether cortisone becomes ulcerogenic when given with an agent which damages the gastric mucosa, cortisone was administered with acetylsalicylic acid. The results are illustrated in Fig. 1. Acetylsalicylic acid (100 mg/kg orally once daily for 4 days) administered to 4 guinea-pigs caused widespread gastric erosions, but no ulceration. Of the group which received cortisone (100 mg/kg s.c. once daily for 4 days) with acetylsalicylic acid, distinct gastric ulceration was found in 3 of 4 animals in addition to gastric erosions.

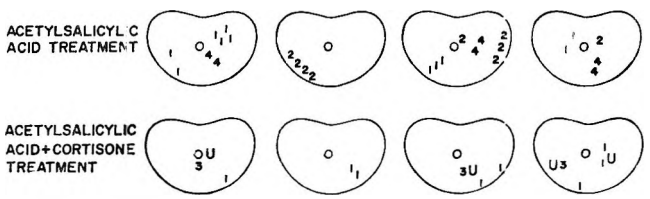

FIG. 1. The effect of acetylsalicyclic acid (100 mg/kg orally, once daily for 4 days) by itself and in combination with cortisone treatment (100 mg/kg s.c., once daily for 4 days) on the guinea-pig stomach. The guinea-pigs were fasted 5 h before and 3 h after treatments and were killed 2½ h after the last treatment.

It is concluded from these results, that in contrast to rats (Ingle, Prestrud & Nezamis, 1951; Robert & Nezamis, 1958) the intact guinea-pig stomach is insensitive to the ulcerogenic action of cortisone and also to some other ulcerogenic stimuli. According to Long (1956) there is a clear-cut species difference to the toxic effects of cortisone; man, monkey and guinea-pig are "cortisone-resistant" while the rat, mouse and rabbit are "cortisone-sensitive" species. Gastric mucosal damage, like that produced by aspirin may be a prerequisite in the guinea-pig, and perhaps also in man, for cortisone-induced gastric ulceration. A protective factor which was found in guinea-pig stomach but was absent in rat stomach (Heisler & Kovacs, 1967b) may also contribute to the relative insensitivity of guinea-pigs to certain ulcerogenic stimuli.

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Prolonged oestrogenic activity in rats after single oral administration of ethinyloestradiol-3-cyclopentyl ether

The observation that relief of symptoms in menopausal patients persisted long after oral treatment with the 3-cyclopentyl ether of ethinyloestradiol had been discontinued was interpreted by Bompiani & Bubani (1961) to indicate its storage in and slow release from body depots.

Since then, Meli, Wolff & Honrath (1963) and Meli, Steinetz & others (1965) have shown that the drug is stored in and slowly released from body fat after oral administration to rats. This they considered the mechanism responsible for its increased and prolonged biological activity. Epstein (1967) also found prolonged oestrogenicity in women treated with the drug. Cohen, Bronstein & Leb (1966) showed a uterine-growth stimulating substance to be present in the fat of women taking the drug by mouth. The metabolic fate of the labelled drug in women indicated that it was stored unaltered in body fat depots (Williams, Layne & others, 1967).

While fat storage and prolonged oestrogenic activity occurs in women after the drug had been taken by mouth, no evidence, other than of fat storage, exists for the rat (Meli & others, 1963, 1965).

We now report prolonged oestrogenic activity in the rat given a single oral dose of the drug.

Female rats, 150–180 g, were ovariectomized two weeks before treatment. Groups of 5 animals each received a single oral treatment of ethinyloestradiol or its 3-cyclopentyl ether dissolved in sesame oil at doses of 1, 10, 100 and 1000 μg/animal.

Vaginal smears were taken daily and the animals were killed when vaginal cornification was no longer present. The uteri were then removed and weighed (after pressing out the intra-uterine fluid) to the nearest 0·1 mg on a torsion balance.

In other experiments, 2 groups of ovariectomized rats similarly treated with the 3-cyclopentyl ether at single oral doses of 100 or 1000 μg/animal were killed at 1, 5 and 13 days after the last day of cornified vaginal smear.

On the basis of vaginal cornification, ethinyloestradiol was ineffective at 1 μg dose (Table 1). The effect of the 10 and 100 μg doses lasted only for 24 h whereas that of the 1000 μg dose was effective for 72 h. Vaginal cornification occurred at all doses of the 3-cyclopentyl ether. Duration of action was proportional to the dose given and,

Table 1. *Effects of oral ethinyloestradiol or its 3-cyclopentyl ether on vaginal smear and uterine weight of mature ovariectomized rats*

Treatment	Dose (μg)	No. of rats	Days of cornified vaginal smear	Uterine weight (mg)
Control		5	0	73·3 ± 4·5
Ethinyloestradiol ..	1	5	0	74·9 ± 6·5
	10	5	1	97·7 ± 6·0
	100	5	1	102·1 ± 9·2
	1000	5	3	114·6 ± 9·3
3-Cyclopentyl ether ..	1	4	2	107·7 ± 4·2 ($P < 0·005$)
	10	5	4	125·6 ± 7·2 ($P < 0·025$)
	100	5	8	154·7 ± 13·2 ($P < 0·025$)
	1000	4	13	158·7 ± 16·1 ($P < 0·05$)

Table 2. *Effects of oral 3-cyclopentyl ether of ethinyloestradiol on vaginal smear and uterine weight of mature ovariectomized rats*

Treatment	Dose (μg)	No. of rats	Days of cornified vaginal smear	Days after oestrogenic smear or ovariectomy	Uterine weight (mg)
Control ..		5	0	24	83·8 ± 4·8
		5	0	28	78·8 ± 8·0
		5	0	36	73·3 ± 4·5
Drug	100	5	8	1	163·2 ± 11·3 ($P < 0·001$)
	100	5	8	5	148·4 ± 11·8 ($P < 0·005$)
	100	5	8	13	119·4 ± 16·9 ($P < 0·05$)
Drug	1000	5	13	1	201·2 ± 29·2 ($P < 0·001$)
	1000	5	13	5	157·4 ± 6·0 ($P < 0·001$)
	1000	5	13	13	153·4 ± 21·6 ($P < 0·01$)

after the 1000 μg dose, lasted up to 13 days. At all doses the ether was significantly more effective than the parent drug in stimulating uterine growth. Even 13 days after the last day of positive oestrogenic smear the uteri were still significantly stimulated over those of controls (Table 2).

These results indicate that as in women (Bompiani & Bubani, 1961; Cohen, Bronstein & others, 1966; Epstein, 1967) the rat is capable of releasing fat stored ethinyloestradiol 3-cyclopentyl ether in an active form over a prolonged time.

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Review

Original Papers

Letters to the Editor