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Volume 22 Number 6 June 1970

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REVIEW

The chemistry of dryopteris acylphloroglucinols

ANERI PENTTILÄ AND J. SUNDMAN

The Research Laboratories, Medica Ltd., Helsinki, Finland

The ancient remedies effective against helminthiasis caused by fish tape worm, *Diphyllobothrium latum*, include the powdered rhizomes and extracts of dryopteris ferns which have had, and still have, a widespread use among the parasite infested populations.

Extensive investigations in this field were started around the turn of the century by Boehm (1897, 1898, 1901a,b, 1903a–d) and resulted in the isolation of several dryopteris fern constituents as well as the resolution of the chemical structure of some of them. These studies have served as a basis for the current knowledge of the chemistry of dryopteris acylphloroglucinols.

The basic chemical structure of the dryopteris constituents may be presented as a two-ring construction in which a butyrylfilicinic acid moiety (A-ring) is linked either to another similar moiety or to a *C*- or *O*-methylated butyrylphloroglucinol moiety (B-ring), by means of a methylene bridge. Naturally occurring variations of this basic pattern include substitutions of the A-ring by an acylphloroglucinolic nucleus, enlargement of the molecule by additional acylphloroglucinol units to yield trimer or tetramer structures, substitution of the butyryl side-chain by an acetyl or propionyl homologue, and insertion of a pyrone ring structure instead of a phloroglucinolic one.

CHEMICAL REACTIONS OF THE ACYLPHLOROGLUCINOLS

The general chemical reactions of the dryopteris acylphloroglucinols are those logically derived from their instability under alkaline or oxidative conditions, or both. Thus, the methylene bridge between the two rings is usually easily broken up by alkalis and the acyl side-chain can be removed by the action of either alkalis or strong acids. As phenols these compounds are readily oxidized and especially under alkaline conditions decomposition due to autoxidation is apt to follow if not prevented.

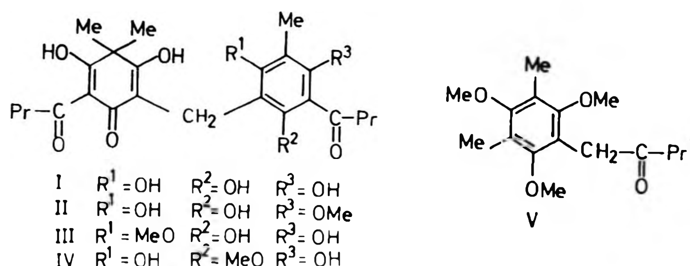
The easily occurring rupture of the methylene linkage leads to a reaction typical for asymmetric polyhydroxydiphenylmethanes of the type R_1–CH_2–R_2. It involves an interchange of the rings and results in an equilibrium between the original asymmetric dimer and the two symmetric dimers, R_1–CH_2–R_1 and R_2–CH_2–R_2. This reaction, known as the rottlerone change, was originally studied on the closely related phenolic constituents of kamala, an Indian colouring matter and anthelmintic drug (Backhouse, McGookin & others, 1948; McGookin, Robertson & Simpson, 1951). In the dryopteris series the rottlerone change was first reported by Birch (1951), and soon it was observed that e.g. flavaspidic acid (I) when heated in carbonate solution undergoes rottlerone change yielding albaspidin (XI) (McGookin, Robertson & Simpson, 1953).

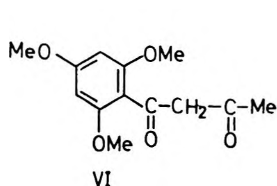
Flavaspidic acid

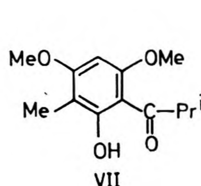
Flavaspidic acid (I) is the methylene linked dimer of butyrylfilicinic acid and methylbutyrylphloroglucinol. This structure was discussed by Boehm (1901b), but

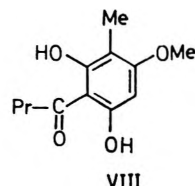
to explain certain decomposition products of flavaspidic acid, Boehm (1901b, 1903b) preferred a slightly modified structure. The latter was commonly accepted until McGookin & others (1953) and Riedl (1954) practically simultaneously synthesized flavaspidic acid and thereby confirmed its structure as (I).

Flavaspidic acid exhibits two tautomeric forms: the enolic α-form is obtained through crystallization from ethanol and has a melting point of 92–95°, while the ketonic β-form, obtainable from benzene or glacial acetic acid, has a m.p. of 156°









(Boehm, 1903b; Aho, 1958). Since the α-form is convertible into the β-form by heat, under gradual increase of the temperature the former actually shows two melting points separated from each other by a crystalline phase.

Aspidin, para-*aspidin and related compounds*

When the B-ring of flavaspidic acid is replaced by its monomethoxyl derivative, three isomeric compounds, aspidin (II), *para*-aspidin (III) and iso-aspidin (IV) are obtained. Aspidin was isolated by Boehm (1897, 1898, 1903c) but the chemical structure then ascribed to it was later shown to be that of *para*-aspidin (III), which was isolated, characterized, and synthesized by Penttilä & Sundman (1962a). Riedl & Mitteldorf (1956) reinvestigated the naturally occurring aspidin and its decomposition products and proved the structure (II) by synthesis. Independently, the same structure was proposed by Aebi, Kapoor & Büchi (1957b).

The third isomer of this group, iso-aspidin (IV), is a synthetic compound (Penttilä & Sundman, 1964b), so far not found in dryopteris ferns.

It would be possible to consider the B-ring to be constituted of a two- or three-methoxyl derivative of methylbutyrylphloroglucinol. Since such related polymethoxyl compounds are common in nature—e.g. torquatone (V), eugenone (VI) and baeckeol (VII)—their occurrence in *Dryopteris* species could well be considered. However, this is not so; the methoxylated constituents are composed of monomers carrying only one methoxyl group.

The mixtures of acylphloroglucinols from *Dryopteris* species almost always contain variable amounts of the mononucleous aspidinol (VIII), first isolated by Boehm (1897, 1901a, 1903a) and synthesized by Karrer & Widmer (1920) and Robertson &

Sandrock (1933a). However, the actual occurrence of aspidinol in nature has seemed questionable and careful investigations have led to the opinion that aspidinol is an artifact arising chiefly from *para*-aspidin (III) (Penttilä & Sundman, 1966). In fact, only those *Dryopteris* species that are devoid of *para*-aspidin, have been shown to yield aspidinol-free concentrates of acylphloroglucinols (Penttilä & Sundman, 1966; Wieffering, Fikenscher & Hegnauer, 1965; Widén, 1967a, 1968). The easily occurring decomposition of *para*-aspidin may also explain why this dimer has escaped detection for so long, in spite of its widespread occurrence in various *Dryopteris* species.

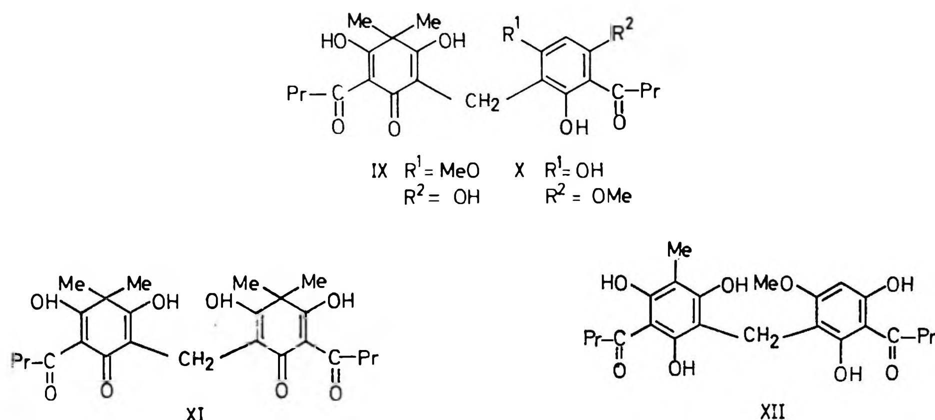
The occurrence in nature of other monomeric acylphloroglucinols, especially methylbutyrylphloroglucinol, has been reported by Stahl & Schorn (1963) but not confirmed.

Desaspidin and ortho-*desaspidin*

The corresponding isomers derived from butyrylphloroglucinol acting as the B-ring are desaspidin (IX) and *ortho*-desaspidin (X). The former, an analogue of *para*-aspidin (IV), was first detected and isolated by Büchi, Aebi & Kapoor (1957). The structure of desaspidin (IX), based on decomposition analyses and spectral studies was later confirmed by synthesis (Penttilä & Sundman, 1962b, 1964a).

Of the variety of acylphloroglucinol derivatives isolated from dryopteris, desaspidin has won the most widespread interest among investigators: it has been shown to be superior in anthelmintic activity to other dimers from the same source (Östling, 1961; Mühlemann & Tatrai, 1969), and its capability of acting as a powerful uncoupler in oxidative phosphorylations has been intensively studied in recent years (Runeberg, 1963; Hind, 1966; Gromet-Elhanan & Avron, 1966).

The occurrence in nature of the dimer corresponding to aspidin, *ortho*-desaspidin (X), was to be expected on analogical grounds. It was synthesized and its chemical and chromatographic properties were examined. Guided by this knowledge, Penttilä & Sundman (1964b) detected and isolated it from natural sources, where it occurs only in trace quantities.



Albaspidin

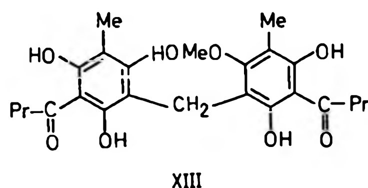
The dimers presented hitherto have been composed of a butyrylfilicinic acid moiety and a methylated butyrylphloroglucinol moiety. If two butyrylfilicinic acid moieties are combined *via* a methylene bridge, the symmetrical albaspidin (XI) is obtained.

It was isolated and characterized by Boehm (1897, 1901b) who also synthesized albaspidin by condensing two molecules of butyrylfilicinic acid with formaldehyde.

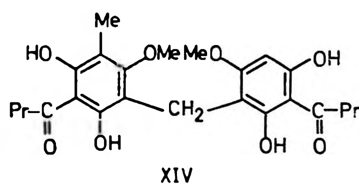
Phloraspin and margaspidin

When the butyrylfilicinic acid rings of desaspidin (IX) and *para*-aspidin (III) are substituted by a methylbutyrylphloroglucinol unit, phloraspin (XII) and margaspidin (XIII), respectively, are obtained. Phloraspin is almost insoluble in benzene and therefore can be easily isolated from dryopteris extracts even though it can only be detected occasionally and in relatively small quantities. Boehm (1903d) proposed the structure (XII) for phloraspin, and this was later confirmed by Penttilä & Sundman (1961c) who synthesized it.

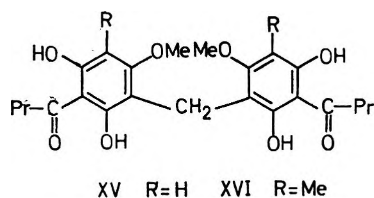
Margaspidin (XIII) is the major compound of *Dryopteris marginalis*, the American substitute for *Dryopteris filix-mas* (Penttilä & Kapadia, 1965). *D. marginalis* is known in commerce as American Aspidium or Marginal Fern, and has been the source of the official drug in the United States. In contrast to the other common acylphloroglucinols, margaspidin has so far been found only in the North American *D. marginalis*, while all the other usual acylphloroglucinols have been reported to occur in both the European and American species (Hegnauer, 1961; Fikenscher & Gibson, 1962; Fikenscher & Hegnauer, 1963a; Wieffering & others, 1965; Penttilä & Kapadia, 1965).



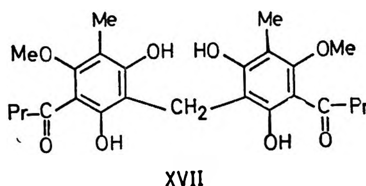
XIII



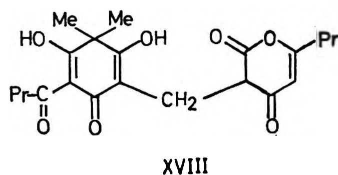
XIV



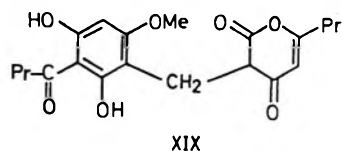
XV R=H XVI R=Me



XVII



XVIII



XIX

Phloraspidinol and methylene-bis-desaspidinol

From dryopteris ferns only two dimers have been isolated with a methoxyl group in each ring: phloraspidinol (XIV) and the symmetrical methylene-bis-desaspidinol (XV) (Penttilä & Sundman, 1963b). The closely related and likewise symmetrical homologue methylene-bis-aspidinol (XVI) (Boehm, 1903a) could well be expected to be a naturally occurring dimer, but so far it has not been detected (Penttilä &

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Sundman, 1963b). Neither has ψ-aspidin (XVII), a dimer easily obtained from aspidin in alkaline conditions (Boehm, 1903c; Riedl & Mitteldorf, 1956).

Phloropyron and phloraspyron

At the first glance it may seem surprising that the structurally rather homogenous group of acylphloroglucinols from *Dryopteris* species is disordered by compounds where an acylphloroglucinol moiety is substituted by a pyronic one as is the case in phloropyron (XVIII) and phloraspyron (XIX) (Penttilä & Sundman, 1961b, 1963b). Biogenetically, this coexistence seems much less confusing, since the two ring structures may be considered to arise from a common precursor: butyrylphloroglucinol from acetate and four malonate units *via C*-acylation, and pyronone from acetate and three malonate units *via O*-acylation (Sundman & Penttilä, 1964; Penttilä, 1967).

Further relations between acylphloroglucinols and pyrones have been examined on a different basis: some pyrones and polypyrones have been proposed as stabilized intermediates in the biosynthesis of acylphloroglucinols and other phenolic compounds (Money, Qureshi & others, 1965; Douglas & Money, 1967; Comer, Money & Scott, 1967). On the other hand, successful biogenetic-type syntheses *in vitro* of both acylphloroglucinols and pyrones have been recently effected by cyclization of polyketoacids or their esters (Harris & Carney, 1967; Harris & Combs, 1968).

An interesting connection between acylfilicinic acid and pyrone structures has recently been reported by Young & Hart (1967a,b). In a study of the photochemical behaviour of acetylfilicinic acid these authors showed that a ready photoisomerization occurred yielding a pyrone compound analogous to the pyrone moieties of dryopteris.

In 1897, Boehm isolated from *Dryopteris* species a colourless crystalline substance, aspidinin. A year later Poulsson (1898) described a similar compound which he called polystichinin. No attempts were made to resolve the chemical structure of either of them until 1961 when phloropyron (Penttilä & Sundman, 1961b) was isolated, characterized and its properties found to be similar to those of aspidinin and polystichinin.

The usual method for resolving the structure of unknown dryopteris constituents comprises a reductive cleavage of the methylene bridge and identification of the two monomeric acylphloroglucinols as themselves or as their one-higher homologues, depending on which side of the methylene bridge the cleavage has occurred. The identification is then completed by synthesis where the two monomers are condensed with formaldehyde and the expected dimer isolated and proved identical with the natural compound.

With phloropyron the major difficulties were connected to the non-phloroglucinolic B-ring: its identification as 2,3-dihydropyran-2,4-dione was preceded by chemical and spectral analyses and partial syntheses (Penttilä & Sundman, 1961b).

Filixic acids

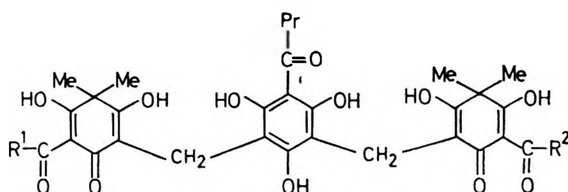
Investigations of filixic acid during the fifty years following its detection (Luck, 1845) revealed little concerning its chemical structure. Luck (1851) found that when filixic acid was treated with acids or alkalies, it liberated butyric acid, and Grabowski (1867) found it to be a derivative of phloroglucinol. Several different structures for filixic acid were proposed and discussed, but the first decisive contribution was made by Boehm (1901b) who found out that filixic acid was composed of three rings of which two were butyrylfilicinic acids and the third was butyrylphloroglucinol. These

fragments were combined by means of methylene bridges to yield the molecule of filixic acid. Riedl (1954, 1955), and later Chan & Hassall (1957), suggested minor modifications in this structure, but an attempted synthesis to prove it, failed (Riedl, 1955).

Recent studies have shown that the naturally occurring filixic acid is not an unambiguous compound but rather a mixture of six homologues which vary in the length of the acyl side-chains of their filicinic acid nuclei (Penttilä & Sundman, 1963a). The major constituents of the mixture are the filixic acids BBB (XXa), PBB (XXb) and PBP (XXc), in which the acyl side-chains attached to the filicinic acid rings are butyryl-butyryl, propionyl-butyryl or propionyl-propionyl, respectively. All cf these have been isolated in pure form and their chemical characterization completed by the syntheses of the symmetrical filixic acids BBB and PBP (Penttilä & Sundman, 1963a).

Probably because of mixed crystal formation, the individual filixic acids cannot be separated by the usual methods although a partial concentration of the butyryl and butyryl-propionyl homologues at the expense of the acetyl homologues, filixic acid ABB (XXd), ABP (XXe) and ABA (XXf), may be attained. Most chromatographic methods also fail to separate the homologues; a fairly good separation can be achieved on buffered papers (Penttilä & Sundman, 1961a, 1963a) and with other special methods (Widén, 1968).

The occurrence of homologous mixtures is not limited to filixic acids but is rather a general phenomenon within certain *Dryopteris* species. Neither are the variations of the side-chain lengths limited to the filicinic acid nucleus, they may also concern the phloroglucinolic ring but those on the side-chains of the filicinic acid nuclei appear to predominate.



XXa R^1 = Pr R^2 = Pr
XXb R^1 = Et R^2 = Pr
XXc R^1 = Et R^2 = Et
XXd R^1 = Me R^2 = Pr
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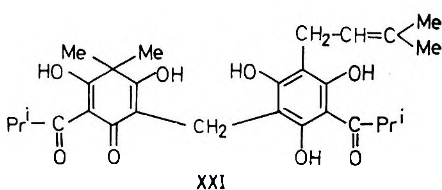
XXf R^1 = Me R^2 = Me



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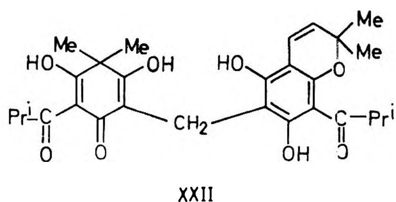


XXII

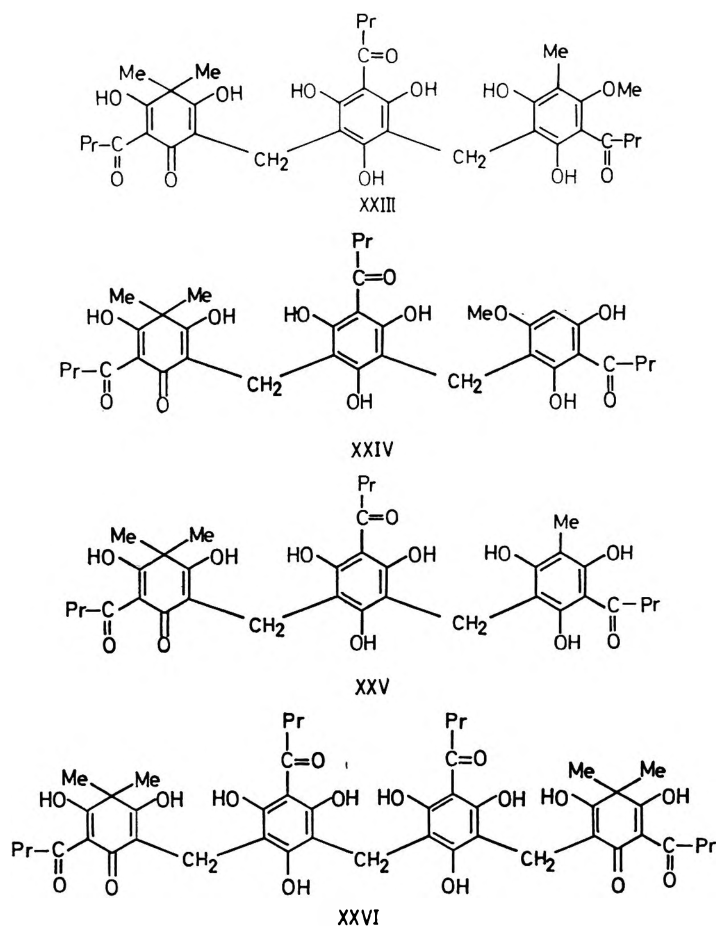
The distribution of the acetyl, propionyl and butyryl side-chains within certain species is constant and conversely offers a valuable tool in chemotaxonomic studies

of *Dryopteris* species and hybrids (Penttilä & Sundman, 1964a; Wieffering & others, 1965; Widén, 1968, 1969).

An analogical case of homologous mixtures has recently been reported by Parker & Johnson, 1968; Parker, Flynn & Boer, 1968). The uliginosins A (XXI) and B (XXII) isolated from *Hypericum uliginosum* are composed of phloroglucinol and filicinic acid residues carrying isobutyryl side-chains. A homologous impurity present in the isolated crystals of these compounds is assumed to carry valeroyl groups instead of isobutyryl groups.

Trisaspidin, trisdesaspidin and trisflavaspidic acid

Besides filixic acids some other three-ring compounds have been isolated. They can be derived from known two-ring components by adding a butyrylphloroglucinol unit between their two rings. Thus trisaspidin (XXIII) has a butyrylphloroglucinol nucleus between the two rings of aspidin (II), correspondingly trisdesaspidin and trisflavaspidic acid have the structures (XXIV) and (XXV), respectively (Penttilä & Sundman, 1963c). These compounds are found only in trace quantities in *Dryopteris* ferns, but according to Widén (1967a) the trimer trisdesaspidin can always be detected in certain species when the corresponding dimer, desaspidin, is present in higher amounts.



Methylene-bis-norflavaspidic acid

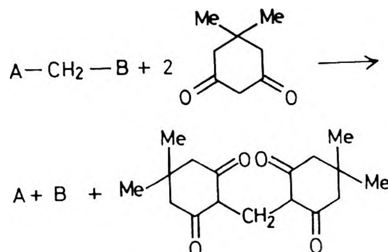
The only four-ring compound isolated from dryopteris is composed of two butyrylfilicinic acid residues separated by two butyrylphloroglucinol residues and is known as methylene-bis-norflavaspidic acid (XXVI) (Penttilä & Sundman, 1963d). Owing to its symmetric structure, methylene-bis-norflavaspidic acid can be synthesized by condensing with formaldehyde two molecules of nor-flavaspidic acid, a dimer derived from flavaspidic acid by omitting its ring methyl group.

Nor-flavaspidic acid is a synthetic compound.

DETECTION AND IDENTIFICATION OF DRYOPTERIS ACYLPHLOROGLUCINOLS

The ferric chloride reaction of 1,3-diketones has been widely used for the detection of dryopteris acylphloroglucinols. A variety of crystalline derivatives with distinct melting points, especially the reaction products with diazoaminobenzene, phenylhydrazine and aniline, have been prepared and have proved useful in the identification of unknown compounds (Poulsson, 1898; Boehm, 1901a,b, 1903c).

Besides the formation of the usual derivatives of phenols the different decomposition products of acylphloroglucinols have been examined. The mild alkaline reductive cleavage on either side of the methylene bridge theoretically results in the liberation of the two rings of which either one may carry the reduced bridge methyl group. This has been developed into the method of choice for identifying unknown dimers, as already mentioned on page 397. However, complications arise because the cleavage occurs preferably on one side of the bridge and because the one higher homologue carrying the bridge methyl group is not always stable and identifiable. A modification of the method has been developed in which an excess of dimedone is added to the alkaline solution of the dimer. Since dimedone has proved capable of substituting the two rings its reaction results in quantitative removal of the methylene bridge whereby the two rings are liberated and can be identified (Scheme 1). This method



Scheme 1

has been used with advantage in biosynthetic studies of labelled dimers (Penttilä, 1967).

Under more destructive alkaline treatments the dimers are deprived of their acyl side-chains as well as their methoxyl groups. This reaction yields a mixture of homologous methyl derivatives of phloroglucinol and filicinic acid (Boehm, 1898, 1903a).

Finally, spectral analyses have been applied in structure studies of fern constituents (Aebi, 1956; Aebi, Kapoor & Büchi, 1957b; Aho, 1958), but the most comprehensive progress in the detection, identification and structural studies of dryopteris acylphloroglucinols has been made with chromatography.

CHROMATOGRAPHY

The first paper chromatographic attempts to separate dryopteris constituents were made by Büchi & others (1957) and Klevstrand (1957, 1960). The former method was based on reversed-phase chromatography using a formamide–water mixture as the mobile phase on ether–pyridine impregnated paper. Klevstrand achieved separation on formamide or formamide-dimethylformamide impregnated paper developed by hexane–chloroform or benzene–chloroform mixtures. Godin (1958) separated some acylphloroglucinols by using sodium carbonate solution as an eluent.

A thorough evaluation and comparison of these methods has been published by Zwimpfer & Büchi (1963a,b, 1964) who improved the technique of Godin to allow the separation of all known acylphloroglucinols and alkaline decomposition products.

Hegnauer (1961) described a two-solvent system, to run in parallel, for identifying phloroglucinols. Later he and his colleagues successfully used a method based on varying concentrations of acetic acid as eluent (Fikenscher, 1962; Fikenscher & Gibson, 1962; Fikenscher & Hegnauer, 1963a,b; Wieffering & others, 1965). Penttilä & Sundman (1961) used buffered papers treated with formamide and developed by benzene–chloroform or cyclohexane-chloroform. All known acylphloroglucinols and their monomeric decomposition products can be separated by this method according to the pH of the buffered papers. Furthermore, at the correct pH the homologous filixic acids migrated yielding distinctly separate spots; other homologues could also be separated on buffered papers (Penttilä & Sundman, 1963a, 1964a). In the study of homologous compounds the method was complemented by the paraffin-acetic acid method of Fikenscher & Hegnauer (*loc. cit.*) which also can be used to separate homologous mixtures.

Thin-layer chromatography was first applied on dryopteris constituents by Stahl & Schorn (1962). v. Schantz (1962a) modified the thin-layer method by buffering the plates to pH 6·0 and this method has later been used as base for the quantitative or semiquantitative estimation of acylphloroglucinols (v. Schantz, 1962b; Widén, 1967a,b; v. Schantz & Widén, 1967).

Further modifications of the thin-layer technique have been reported by Blakemore, Bowden & others (1964), v. Schantz, Ivars & others (1964) and Fish & Kirk (1968).

Column chromatography was applied on dryopteris phloroglucinols by Fichter as early as 1938 but without success. A few years later Mühlemann & Käsermann (1942) likewise attempted separation of raw filicin on a column of alumina, but real progress was made only when acylphloroglucinol mixtures were chromatographed on silica gel, $CaSO_4$, or polyamide powder (Aebi & others, 1957a,c; Penttilä & Sundman, 1963b,c,d; 1964b; Penttilä & Kapadia, 1965; Carelli & Petrangeli, 1961; Widén, 1967a,b, 1968).

Several colour reactions have been employed to identify acylphloroglucinols on paper or thin-layer chromatograms. Zwimpfer & Büchi (1963b) have given a detailed survey of the variety of reagents used for this purpose and they conclude that ferric chloride-potassium ferricyanide and the Folin-Ciocalteu reagent are the most sensitive. Penttilä & Sundman (1961a), however, have found the fast blue salt B (tetrazotized di-*o*-anisidine) superior to all reagents tested.

SYNTHESES OF DRYOPTERIS ACYLPHLOROGLUCINOLS

After deducing the structures of unknown isolated compounds from their chemical and spectral analyses, the task is preferably completed by an unambiguous synthesis.

With symmetrical dimers this could be readily done by condensing two molecules of the monomer with formaldehyde in slightly alkaline solution. Theoretically, the synthesis of any unsummetrical dimer is quite similar, difficulties may arise only from the fact that the reaction product is made up of a mixture of three compounds: two symmetrical ones and the wanted unsymmetrical compound. In certain instances it has been possible to increase the yield of the wanted dimer at the expense of either of the other two (Penttilä & Sundman, 1963b; Penttilä & Kapadia, 1965). This basic method and modifications of the same have been adapted for industrial production of anthelmintically active acylphloroglucinols, especially desaspidin (Andersen, Pentillä & others, 1967a, b, 1968a).

In a broader sense the synthesis of acylphloroglucinols comprises the synthetic preparation of the individual monomers. Thus, Robertson & Sandrock (1933b), Angus, Clark & others (1954) and Riedl & Risse (1954) have synthesized filcinic acid, the latter two authors have also prepared the butyryl derivative of filicinic acid. Recently, the syntheses of acylfilicinic acids have been thoroughly studied (Andersen, 1968; Andersen, Laurén & others, 1968b).

The aromatic moieties of the natural dimers have been synthesized by several investigators, i.e. aspidinol has been prepared by Karrer & Widmer (1920), Riedl & Mitteldorf (1956), and Robertson & Sandrock (1933a). A general method for the syntheses of *para-O*-alkylated polyphenolketones has been reported by Andersen, Penttilä & others (1967a).

BIOSYNTHESIS OF DRYOPTERIS ACYLPHLOROGLUCINOLS

Since Birch & Donovan (1953) presented their study on some possible biosynthetic routes to derivatives of phloroglucinol, the intramolecular *C*-acylation of the four-unit head-to-tail acetate chain to yield acetylphloroglucinol has become a classical example of biosynthetic principles. Both acetylphloroglucinol, as well as its homologue, butyrylphloroglucinol, may be considered as precursors of the naturally occurring dryopteris fern constituents. Results from a biosynthetic study of living *D. marginalis* plants, using a radioactive labelling technique, indicated that butyric acid was the source of the butyryl side-chain, and that the *C*- and *O*-methyl groups were derived from methionine (Penttilä, Kapadia & Fales, 1965; Penttilä, 1967). Furthermore, the methylene link between the two rings was labelled equally with the methyl groups, which suggested that an aromatic methyl was the precursor of the methylene bridge. This aspect was confirmed by enzymatic *in vitro* studies in which appropriate monomers were combined with peroxidase and hydrogen peroxide to yield the naturally occurring dimers (Penttilä & Fales, 1966; Penttilä, 1967).

BIOLOGICAL EVALUATION OF DRYOPTERIS CONSTITUENTS

In the search for effective drugs against the fish tape worm *Diphyllobothrium latum* and related parasites, a variety of techniques and test animals have been applied. The screening of dryopteris extracts and isolated phloroglucinol derivatives has been recently reviewed (Zwimpfer & Büchi, 1962; Fish & Calderwood, 1966) and will not be repeated here.

In spite of countless attempts it appears not to have been possible to create an entirely reliable method by which the anthelmintic activity could easily be evaluated. In the screening work recently performed, the dwarf tape worm (*Hymenolepis nana*) has been used for *in vitro* and *in vivo* tests of mice (Blakemore & others, 1964; Bowden,

Broadbent & Ross, 1965; Steward, 1955; Sen & Hawking, 1960). Unfortunately, results obtained with these methods have proved inconsistent with controlled clinical tests (Rosengård, S., Hackman, C. R., Räsänen, T. A. & Hiltunen, R. A., personal communication). Attempts made so far to cultivate the fish tape worm *in vitro* have shown little promise. Work on this line is being continued, however, and it is to be hoped that a reliable biological evaluation method allowing an easy screening of new anthelmintics will be available soon.

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The effect of sodium salicylate on the binding of long-chain fatty acids to plasma proteins

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Salicylate causes a release of palmitic, stearic, oleic and linolenic acids from their binding sites on human plasma proteins and bovine albumin. The implications of this finding with respect to other effects of salicylate on fatty acid metabolism are discussed.

A large variety of small molecules are transported in the circulation bound to plasma albumin. Any interference by drugs with such binding could increase the availability of diffusible molecules, these would then be able to enter body cells to either change metabolic rates or initiate pharmacological actions. Salicylate can release some normal metabolites, including L-tryptophan (McArthur & Dawkins, 1969) and uric acid (Bluestone, Kippen & Klinenberg, 1969) from their binding sites on plasma proteins and thus affect their rate of entry into the tissues. The present work shows that the drug can also displace long-chain fatty acids from human plasma proteins and bovine albumin *in vitro*.

EXPERIMENTAL

Materials

Pooled human plasma was obtained from the National Transfusion Service, Sutton. Bovine albumin (fraction V) and palmitic, stearic, oleic and linolenic acids were obtained from the Sigma Chemical Company, St. Louis, palmitic acid-1-C^{14} (specific activity 55·2 mCi/mmol), stearic acid-1-C^{14} (specific activity 48·4 mCi/mmol), oleic acid-1-C^{14} (specific activity 57·8 mCi/mmol) and linolenic acid-1-C^{14} (specific activity 41·5 mCi/mmol) from the Radiochemical Centre, Amersham and 2,5-diphenyloxazole (PPO) from the Packard Instrument Company, London. All other chemicals were of analytical grade and distilled water was used throughout.

Measurement of binding of fatty acids to protein

The technique of partition analysis (Goodman, 1958a) was used to measure the concentrations of unbound fatty acids in solutions of bovine albumin and in human plasma. The method depends on the determination of the distribution of varying quantities of the fatty acids between n-heptane and either an aqueous buffer, a solution of bovine albumin in the buffer or human plasma. The use of trace amounts of labelled fatty acids mixed with the non-radioactive fatty acids enabled accurate measurements to be made at low concentrations. The presence of salicylate did not alter the partition of the fatty acids between the n-heptane and aqueous phases in the absence of albumin nor did the salicylate enter the heptane layer.

In the experiments using bovine albumin, a series of mixtures, each of 20 ml volume, were prepared using 0·1M phosphate buffer, pH 7·4, to contain the unlabelled

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fatty acid; 0·001 to 0·5 mM, the corresponding radioactive fatty acid, 0·5 μCi; bovine serum albumin, 0·2 mM (1·36% w/v) and sodium salicylate, 0 to 5 mM. The individual components were added in the order given above. In the experiments with human plasma 0·4 ml of a solution, containing 0·5 μCi of each of the four radioactive fatty acids plus sufficient sodium salicylate to give final salicylate concentrations ranging from 0 to 5 mM dissolved in the 0·1M phosphate buffer, pH 7·4, was added to 19·6 ml of pooled human plasma.

Each mixture was placed in a 25 ml round bottom glass-stoppered flask, 2 ml of n-heptane were added, and the flask and contents shaken for 48 h at 125 rev/min in a Luckman rotary shaker at room temperature (22°). The results of preliminary experiments using incubation periods up to 96 h showed that equilibrium was reached within 48 h. The flasks were inverted to prevent evaporation of the heptane. At the end of the equilibration period the contents of each flask were centrifuged at 2000*g* for 20 min and duplicate aliquots (0·25 ml) of the heptane layers were mixed with 5 ml of 0·5% (w/v) PPO in toluene. After complete removal of the remaining heptane, duplicate samples (0·1 ml) of the aqueous layer in the bovine albumin experiments were transferred by pipetting on to 2·1 cm diameter glass fibre discs. The discs were allowed to dry in air for 12 h and then added to 5 ml of the PPO–toluene phosphor. In the experiments with human plasma the material remaining after removal of the heptane was diluted 1 to 5 with distilled water before the 0·1 ml aliquots were transferred to the glass fibre discs. This was done to avoid subsequent quenching of the phosphor by a high concentration of protein. The radioactivity in the final toluene–phosphor mixtures was counted in a Beckman LS 200B liquid scintillation system, at least 10000 counts being recorded for each sample.

The total concentration of fatty acid in the heptane divided by the total concentration of fatty acid in the phosphate buffer gives the distribution ratio of each fatty acid within the system after equilibration (Goodman, 1958a). In the experiments with bovine albumin and human plasma the concentrations of unbound fatty acid in the aqueous protein phases were calculated by dividing the total concentration of fatty acid in the corresponding heptane phase by the appropriate distribution ratio for the fatty acid.

RESULTS

The effects of salicylate, in concentrations ranging from 0·5 to 5 mM, on the concentrations of unbound palmitic, stearic, oleic or linolenic acids present in solutions containing 0·2 mM bovine albumin are given in Table 1. In every experiment the presence of increasing concentrations of salicylate increased the concentrations of each fatty acid in free solution. Similar results were observed in the experiments with human plasma in which the four radioactive fatty acids were added as a mixture to the pooled plasma. For concentrations of salicylate of 0, 3·0 and 5·0 mM, the per cent unbound radioactive fatty acid (as a percentage of the total of the labelled acid added) was: 0·035 ± 0·007 (s.d., 8 det.), 0·078 ± 0·008 (s.d., 8 det.) and 0·114 ± 0·007 (s.d., 8 det.) respectively. In solutions of bovine albumin the concentration of an individual fatty acid in free solution depends on the presence or absence of other fatty acids. This is illustrated by the results in Table 2 which show that the final unbound concentration of a radioactive fatty acid in a bovine albumin solution is increased if a different non-radioactive fatty acid is added simultaneously. Presumably this effect is due to the various fatty acids competing for the same binding

Table 1. *The effect of salicylate on the concentrations of unbound fatty acids in the presence of bovine albumin.* Each value represents the mean of three separate observations

Salicylate concn (mM)	Fatty acid concn (μ-equiv/litre) added to albumin solution	Unbound fatty acid concn (n-equiv/litre)			
		Palmitic	Stearic	Oleic	Linolenic
0·0	1·0	0·09	0·09	0·44	0·11
0·5		0·12	0·15	0·57	0·17
2·0		0·19	0·32	1·35	0·33
5·0		0·32	0·66	2·70	0·59
0·0	10·0	1·00	0·90	7·20	1·20
0·5		1·50	1·40	6·90	1·50
2·0		2·40	3·20	17·10	2·80
5·0		4·30	6·90	29·50	6·30
0·0	100·0	11·0	15·0	58·0	22·0
0·5		15·0	20·0	97·0	25·0
2·0		29·0	55·0	215·0	39·0
5·0		59·0	130·0	425·0	63·0
0·0	500·0	140·0	245·0	695·0	145·0
0·5		250·0	590·0	1160·0	215·0
2·0		640·0	1495·0	2465·0	320·0
5·0		1365·0	3280·0	6110·0	575·0

Table 2. *Effect of salicylate on the concentration of unbound radioactive fatty acid in a bovine albumin solution to which was added either a radioactive fatty acid or a mixture of one radioactive and one non-radioactive fatty acid.* In each experiment the bovine albumin concentration was 0·2 mM, radioactive fatty acid was added to give a final concentration of 0·5 μ-equiv/litre, non-radioactive fatty acid to give a final concentration of 500 μ-equiv/litre and salicylate, when present, to produce a final concentration of 5 mM. Each experimental value represents the mean of three separate determinations and is expressed as n-equiv/litre of radioactive fatty acid in free solution

Radioactive fatty acid	Non-radioactive fatty acid	Unbound radioactive fatty acid concn	Increase in unbound radioactive fatty acid concn with salicylate
Palmitic	None	0·045	0·115
	Linolenic	0·105	0·455
Linolenic	None	0·055	0·240
	Palmitic	0·110	0·650
Palmitic	None	0·045	0·115
	Stearic	0·145	1·255
Stearic	None	0·220	1·130
	Palmitic	0·170	1·675

sites on the protein. Table 2 also shows that the effect of salicylate in displacing the albumin-bound radioactive fatty acid is substantially enhanced if a second non-radioactive fatty acid is added to the albumin solution at the same time as the labelled fatty acid.

DISCUSSION

The greatest net transport of fat in the plasma from the adipose to other tissues occurs as long-chain fatty acid anions although these represent only a few per cent

of the total plasma lipids (Fredrickson & Lees, 1966). The normal level of these fatty acids in human plasma is of the order of 0·5 m-equiv per litre (Thorp, 1963) and oleic, palmitic, stearic and linolenic acids comprise over 80% of the fatty acid fraction (Robertson, Sprecher & Wilcox, 1968). In the circulation these fatty acids are largely complexed with albumin and *in vitro* determinations (Goodman, 1958b) suggest that this binding amounts to over 99% of the fatty acid fraction in normal human plasma. Only a very small fraction of the plasma fatty acids are therefore available to enter body cells at any one moment of time. Steinberg (1966) has proposed a series of equilibria representing the transfer of fatty acids from albumin via unbound fatty acid anions in free solution to the cell surface, followed by transfer into metabolic pathways. With ascites tumour cells his experimental data suggest that the concentrations of the fatty acids at or near the cell surface governs their subsequent rate of utilization inside the cell. The present results show that salicylate displaces several long-chain fatty acids from their combination with purified bovine albumin and human plasma proteins *in vitro*. The magnitude of the effect is small in relation to the amount of protein-bound fatty acid but represents a ten-fold rise in the concentrations of fatty acids which are free in solution. If this effect occurs *in vivo* it may explain, at least in part, several reported actions of salicylate on fatty acid metabolism in man and in experimental animals.

The first of these concerns the changes in the plasma non-esterified fatty acid concentrations. It is generally agreed that in the rat the acute administration of sodium salicylate causes a marked, if transient, decrease in this component in the plasma (Bizzi, Garattini & Veneroni, 1965; Torsti & Mattila, 1966; Wooles, Borzelleca & Branham, 1967). Salicylate has been differentiated from other drugs, such as 3,5-dimethylpyrazole and nicotinic acid, that lower plasma fatty acid concentrations in the rat in that it shows activity in fed and noradrenaline-treated animals but not rats fasted up to 48 h (Garattini & Bizzi, 1966). The published work on the effects of salicylate on plasma fatty acids in man is more confusing. Gilgore, Drew & Rupp (1963) claimed that the infusion of sodium salicylate caused a 20% rise in the plasma fatty acids in normal subjects and a 50% rise in diabetic patients. These results have been criticized (Bizzi & others, 1965) on the basis that the presence of salicylate in the plasma interfered with the measurement of the fatty acids. Field, Boyle & Remer (1967) observed no significant effect on plasma fatty acids in normal subjects and diabetic patients who received an infusion of sodium salicylate. However, all the subjects showed a significant increase in plasma-insulin levels and the authors themselves comment that this combination of results appears difficult to explain in view of the well-documented ability of insulin to decrease the plasma fatty acid concentrations. In contrast, Carlson & Ostman (1961) reported that the oral administration of acetylsalicylate to normal man and to mildly diabetic subjects decreased the plasma fatty acid concentrations. In all the human experiments described above the subjects were fasted either overnight or for 12 h before the salicylate was given. The influence of more prolonged fasting was studied by DeFelice & Gilgore (1968) who found that infusion of sodium salicylate decreased the plasma fatty acids in obese non-diabetic subjects after a 72 h fast but not before the fasting period.

Some consideration has been given to the mechanism by which salicylate could induce decreased levels of plasma fatty acids. An increased transfer of the acids from the circulation to the tissues was apparently excluded by the results of Carlson & Ostman (1961). These workers infused diabetic patients with a solution containing

human albumin and C^{14}-labelled palmitate at such a rate that a constant amount of radioactivity/ml of circulating plasma was maintained over several hours. The subsequent intravenous administration of calcium acetylsalicylate did not affect the concentration of radioactivity in the plasma although the level of circulating fatty acids was markedly decreased. Carlson & Ostman used only trace amounts of radioactive palmitate, approximately 1 μmol per 200 μmol of protein, to label the human albumin before infusion. It has been shown by Goodman (1958b) that there are several different types of binding sites for fatty acids on human albumin. These sites differ in their affinities for palmitate. A trace (radioactive) dose of the acid would become attached to the sites possessing the greatest affinity and would therefore be less likely to become dissociated than the non-radioactive fatty acid molecules which bind to the albumin after its introduction into the circulation. Thus the subsequent administration of salicylate may have preferentially displaced fatty acids other than the labelled palmitate. An expected result of this displacement would be increased concentrations of the fatty acids in free solution in the plasma which in turn would cause decreased mobilization of fatty acids from depot fat in adipose tissue. Thus the lipolytic effect of salicylate in the rat and in man may be caused by a diminished rate of entry of fatty acids into the plasma and mediated by the drug displacing a small, but important, percentage of the fatty acids from their binding sites on circulating albumin.

Some support for this view is provided by the results of experiments with adipose tissue preparations *in vitro*. Carlson & Ostman (1961), using rat epididymal fat bodies, and Stone, Brown & Steele (1969), using isolated fat cells, found that salicylate possessed antilipolytic effects *in vitro* if the incubation mixtures contained either human or bovine albumin. It has been shown (Campbell, Martucci & Green, 1964) that fatty acids are only released from adipose tissue if a substance with special properties as an acceptor of fatty acids is present and that plasma albumin performs this function most effectively. The lower degree of binding of the fatty acids to the albumin in the presence of salicylate would in turn decrease their release from the triglycerides of the adipose tissue.

An increased rate of entry of fatty acid molecules into body tissues from the plasma as a response to the acute administration of salicylate may also be related to the development of metabolic acidosis in salicylate intoxication in children (Winters, 1963). The principal cause of the acidosis seems to be an accumulation of anions of organic acids, including acetoacetate. It has been suggested (Smith, 1968) that a combination of the uncoupling action of salicylate together with its inhibitory effects on aminotransferase and dehydrogenase enzymes would lead to an increased formation and a restricted metabolism of acetyl-CoA so that there is an increased conversion of this intermediate to acetoacetyl-CoA and hence acetoacetate.

The formation of acetoacetate in the liver would be exacerbated by the increased rates of entry and catabolism of the fatty acids in the presence of salicylate. It has been shown by Ontko & Zilversmit (1966) in the rat and Steinberg (1966) in man that there appears to be a direct relation between the concentrations of fatty acids and ketone bodies in the plasma. These results indicate that an increased level of plasma fatty acids and hence an increased entry of fatty acid anions into the liver can accelerate hepatic ketogenesis. The results of Numa, Bortz & Lynen (1965) suggested that the metabolic pathways for the utilization of acetyl-CoA may be further restricted if fatty acids accumulate in the tissues. These workers discovered that long-chain

fatty acids and their acyl-CoA derivations inhibit acetyl-CoA carboxylase [acetyl-CoA: carbon dioxide ligase (ADP) EC 6.4.1.2] activity *in vitro* and thus decrease fat synthesis. However, Pande & Mead (1968) observed that fatty acids inhibit several enzyme activities *in vitro* by an unspecific mechanism depending on their detergent properties and consider that the extrapolation to *in vivo* situations must be viewed with caution. Salicylate itself inhibits acetyl-CoA carboxylase activity in cell-free systems (Goldman, 1967) and if this effect occurs *in vivo* it may reinforce the development of ketosis in acute salicylate intoxication in children.

A further interaction of salicylate and fatty acid metabolism is the fatty infiltration and degeneration of the liver observed either in acute salicylate poisoning in man (Gross & Greenberg, 1948) or after the chronic administration of the drug to experimental animals (Janota, Wincey & others, 1960; Niederland, 1963). Such effects may result from an increased entry of fatty acids into the liver as a consequence of increased concentrations of fatty acids in free solution in the plasma.

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Colorimetric determination of amphetamine salts in dosage forms

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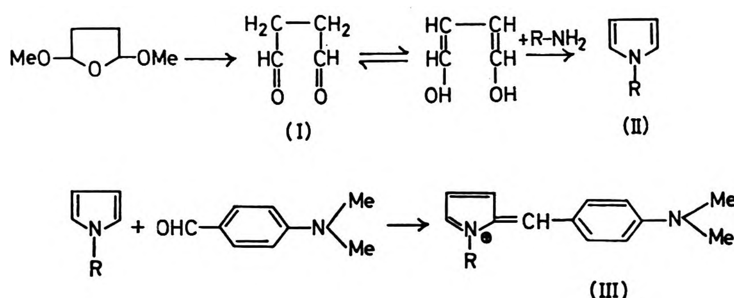
A colorimetric method for the determination of primary amines has been applied to the assay of amphetamine salts in dosage forms. Aqueous solutions of amphetamine salts heated in acidic medium with 2,5-dimethoxytetrahydrofuran and subsequently reacted with *p*-(dimethylamino)benzaldehyde, give a red colour. This reaction is useful for photometric determinations at 557 nm. Secondary and tertiary amines do not interfere.

Amphetamine salts in dosage forms may be determined by spectrophotometric methods, by chromatographic procedures or by steam distillation from alkaline medium and titrimetric assay of the distillate. In this last method (British Pharmacopoeia, 1968) a large sample is required and volatile bases interfere. Rarely are the spectrophotometric methods (Ebstein & Van Meter, 1952; Milos, 1960) useful in the assay of amphetamine dosage forms, because the molar extinction of the drug is low and many other drugs associated with amphetamine, cause interference.

Although chromatographic methods (Beckett & Rowland, 1965; Cardini, Quercia & Calò, 1967; Choulis & Carey, 1968) can be used successfully in analyses of pharmaceutical preparations, a simple and fast colorimetric method would also be very useful for routine amphetamine assay in dosage forms. Our attempts to use the procedures proposed by Wachsmuth & Van Koeckhoven (1964; 1965) were unsuccessful since the sensitivity and the reproducibility of the methods were not good enough. Sawicki & Johnson (1966) reported a colorimetric method for the determination of primary amines. In this procedure $R–NH_2$ reacts with succinaldehyde (I) generated *in situ* from 2,5-dimethoxytetrahydrofuran to give the *N*-substituted pyrrole (II). The reaction between this compound and *p*-(dimethylamino)benzaldehyde produces the chromogen (III).



We have applied this method to amphetamine assay in dosage forms and investigated the variables in the determination.

EXPERIMENTAL

Reagents. 0·5% solution of 2,5-dimethoxytetrahydrofuran (Aldrich-Chemical Co.) in glacial acetic acid. 2·0% solution of *p*-(dimethylamino)benzaldehyde (Merck) in glacial acetic acid–concentrated hydrochloric acid (85:15 v/v). 0·025 mg/ml standard amphetamine sulphate aqueous solution.

A "Densitronic" (Optica, Milan) spectrophotometer with sample path length of 10 mm was used.

Sample preparation. All the samples are diluted so that the final solutions contain about 0·025 mg/ml of amphetamine salt.

Tablets (amphetamine sulphate = 5·0 mg): powder 20 tablets, add water to a weighed amount of powder and dilute to volume with water. Mix and filter.

Injections (amphetamine sulphate = 10·0 mg/ml): dilute a measured volume of sample to volume with water.

Suppositories (amphetamine sulphate = 5·0 mg + dimenhydrinate 100 mg): cut 10 suppositories into small pieces and dissolve a weighed amount in chloroform, extract with three equal quantities of water, combine the aqueous extracts, dilute to volume with water, mix and filter.

Assay

Into three glass stoppered tubes (20 × 100 mm) introduce respectively 2 ml of sample solution, 2 ml of standard solution and 2 ml of water. Add 1 ml of 2,5-dimethoxytethrahydrofuran solution to each tube. Place the tubes simultaneously in a boiling water bath and, 30 s later, stopper the tubes. Heat for 20 min. Remove the tubes from the bath and cool them in iced water. Add 2 ml of *p*-(dimethylamino)-benzaldehyde solution to each tube, stopper again and heat in boiling water bath for 2 min. Gently shake the tubes, place them in cool water and, 10 min later, read the extinctions of standard and sample solutions at 557 nm against the blank.

RESULTS

The extinction spectrum of chromogen solution shows maxima at 517 and at 557 nm. A linear relation exists at 557 nm between extinction value and drug concentration but the values are not exactly reproducible and an assay on a standard solution must be run alongside each sample.

The recoveries obtained in ten repeated analyses of a standard solution of amphetamine sulphate containing 0·025 mg/ml, gave a mean value of 0·0256 with a relative standard deviation of 0·0007 (2·73%). The results obtained in comparative analyses of different lots of dosage forms (Table 1) show that the proposed colorimetric procedure yields results which are in agreement with those obtained using volumetric or gas chromatographic methods.

The optimum conditions for the method were sought with the following findings. (i) The specific absorption values at 557 nm were more constant than those at 517 nm. (ii) Optimum results were obtained with a 0·5% solution of 2,5-dimethoxytetrahydrofuran. Higher concentrations gave lower results. (iii) By heating the sample in boiling water for 20–30 min the transformation of amphetamine into *N*-substituted pyrrole took place with maximum yield (10 min of heating gave 3/4 of maximum yield). (iv) Maximum colour development took place when 2% solution of *p*-(dimethylamino)benzaldehyde in 85:15 mixture of glacial acetic acid and concentrated

Table 1. *Comparative assays of amphetamine sulphate carried out in duplicate with the proposed procedure (A) and with published procedures (B, C, D) on homogenized samples obtained from different batches of dosage forms.*

Samples	5 mg Tablets A mg/t	5 mg Tablets B mg/t	10 mg/ml Injections A mg/ml	10 mg/ml Injections C mg/ml	5 mg Suppositories (+ 100 mg dimenhydrinate) A mg/s	5 mg Suppositories (+ 100 mg dimenhydrinate) D mg/s
Lot I	4·70	4·88	10·03	9·80	5·00	4·98
Lot II	5·10	4·90	9·86	10·30	5·03	4·86
Lot III	4·90	5·10	10·03	9·81	4·96	5·04
Lot IV	4·87	4·74	10·08	9·64	4·90	4·95
Lot V	4·97	4·80	9·86	10·30	5·19	4·98
$\bar{x}$	4·908	4·884	9·972	9·970	5·016	4·962
s.e.	0·065	0·061	0·046	0·138	0·048	0·029
Average of differences* ..	—0·024 ± 0·088		—0·002 ± 0·184		—0·054 ± 0·058	

A = Proposed colorimetric procedure.
B = Volumetric B.P. 1968 (p. 45) method.
C = Volumetric U.S.P. XVI (p. 55) method.
D = Gas chromatographic procedure proposed by Cardini, Quercia, & Calò (1967).

* = The standard error refers to the average of differences obtained in each pair.

hydrochloric acid was used. An increased percentage of acetic acid in the mixture (i.e. 95:5) gave negligible variation, but if the amount of hydrochloric acid was increased, results were lower (60:40 mixture gave half maximum colour). (v) By heating the sample from 1 to 3 min in boiling water after the *p*-(dimethylamino)-benzaldehyde was added, the maximum colour was obtained. Longer heating times gave low colours (80% after 5 min and 60% after 10 min). (vi) The colour stability is good between the 5th and the 12th min after heating. In this period the highest extinction is read. Afterwards the colour fades slowly at a rate of about 6% of absorbance per 10 min during the first hour. The colour of the sample and of the standard fade at the same rate, preventing biased results. The colour is more stable if the solutions are put on ice rather than in cool water. Storage in the dark did not affect the fading.

The following compounds, sometimes associated with amphetamine in dosage forms, gave no interferences in the assay: dimenhydrinate, diphenhydramine hydrochloride, phenylephrine hydrochloride, methamphetamine hydrochloride, adrenaline bitartrate, diphenylhydantoin, mephobarbital.

Imidazole and purine derivatives and secondary and tertiary amines do not undergo the reaction and do not interfere. Primary aliphatic and aromatic amines, including amino-acids, do interfere.

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The determination of sodium aminosalicylate in cachets of sodium aminosalicylate and isoniazid by ultraviolet absorption

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Sodium aminosalicylate can be determined in the presence of isoniazid by its ultraviolet absorption at 299 nm. The method is at least as accurate as the B.P.C. 1968 bromination method for cachets of sodium aminosalicylate and isoniazid. Analysis of a powder mix containing sodium aminosalicylate (2 g) and isoniazid (50 mg) gave a mean value of 2·02 $\pm$ 0·01 (s.d.) for ten determinations using the proposed method. The B.P.C. method gave a mean value of 2·03 $\pm$ 0·33 (s.d.). The proposed method has the advantage that the determination is independent of the isoniazid content and is more rapid than the official method. It can be easily adapted for automatic analysis.

The British Pharmaceutical Codex (1968) method for the determination of sodium aminosalicylate (NaPAS) in cachets of sodium aminosalicylate and isoniazid has the disadvantages that it is not independent of the isoniazid content, breakdown products interfere and it is not easily adapted to automatic methods of analysis.

The method is based on the bromination techniques of Simmonite (1949). As isoniazid also absorbs bromine, a correction factor must be applied after the isoniazid content has been determined by the method of Elliston & Hammond (1965). 3-Aminophenol, a breakdown product of NaPAS also absorbs bromine, but no correction for this is applied in the B.P.C. method.

Several methods have been described where NaPAS and isoniazid are separated on ion-exchange columns and determined independently (Fan & Wald, 1965; van Pinxteren & Verloop, 1965). Colorimetric methods have also been proposed for the determination of NaPAS in the presence of isoniazid (Fried, 1962). Wray, Smith & others (1948) found NaPAS at pH 7 to give two ultraviolet absorption peaks, one at 299 nm and a larger one at 265 nm. An assay method, based on the absorption at 265 nm was used in New and Non-official Remedies (1951) for the assay of aminosalicylic acid tablets. A spectrometric method for the determination of aminosalicylic acid (PAS) in the presence of isoniazid was reported by Welsh (1957, 1958). He found that although interference due to isoniazid at 265 nm was small in the ratios of PAS: isoniazid normally found in tablets, the interference could be reduced further by using the peak at 299 nm. Absorption of PAS (pH 7) at 299 nm was only two-thirds that at 265 nm, but interference due to isoniazid was cut to one tenth at 299 nm and could be discounted.

This paper describes the adaptation of the method of Welsh (1957) to the determination of sodium aminosalicylate in cachets of sodium aminosalicylate and isoniazid B.P.C., and a comparison with the official method.

EXPERIMENTAL AND RESULTS

The NaPAS and isoniazid were to British Pharmacopoeia (1968) specification. 3-Aminophenol was obtained commercially and was further purified by sublimation.

The method of Welsh (1957) for PAS necessitated an initial extraction with sodium bicarbonate solution with a final adjustment of the solution to pH 7. As NaPAS is essentially neutral, it was found that direct solution into distilled water was sufficient. Preliminary studies indicated that the ultraviolet absorption of NaPAS was independent of pH between pH 5–9 and that Beer's law was observed at 265 nm and 299 nm in solutions containing 5–40 μg/ml NaPAS.

Interference from isoniazid and 3-*aminophenol*

Solutions of NaPAS, isoniazid and 3-aminophenol (20 μg/ml) were prepared and the ultraviolet absorption curve for each compound was determined on a Unicam SP800 spectrophotometer using 1 cm cells. Isoniazid interference is markedly reduced at 299 nm compared to that at 265 nm. At the lowest NaPAS : isoniazid ratio in the B.P.C. cachets (30 : 1) absorption due to isoniazid at 299 nm produces an overestimate of 0·1% in the NaPAS content and can be neglected for all practical purposes. Interference from 3-aminophenol is negligible.

Determination of E (1%, 1 *cm*) *for NaPAS at* 299 *nm*

Approximately 200 mg NaPAS were weighed accurately and made up to 1 litre with distilled water. 5 ml of this solution was diluted to 100 ml with distilled water and the extinction at 299 nm measured on a Unicam SP500 spectrophotometer using 1 cm cells. The experiment was repeated ten times, a mean value of 407·2 ± 2·85 (s.d.) was obtained for *E* (1%, 1 cm).

Assay of a known NaPAS: isoniazid powder mix

The mix was prepared by diluting isoniazid (1 g) with NaPAS (40 g). The mix was blended for 2 h in a cube mixer after a preliminary mix with a mortar and pestle. The powder is equivalent to that found in NaPAS (2 g) isoniazid (50 mg) cachets of the B.P.C.

Proposed method. Approximately 200 mg of powder mix were weighed accurately and made up to 1 litre with distilled water. 5 ml of this solution was diluted to 100 ml with distilled water, and the extinction at 299 nm measured as before. By using an *E* (1%, 1 cm) of 407, the amount of NaPAS in 2·05 g powder mix was determined. Ten such determinations were made, a mean value of 2·02 g ± 0·01 (s.d.) was obtained.

B.P.C. method. Approximately 200 mg of powder mix were weighed accurately and made to 100 ml with distilled water. 25 ml of this solution was used for the bromination part of the assay and 5 ml solution for the isoniazid determination. 5 ml quantities of the other reagents were also used and the final volume made to 25 ml. Ten determinations were made, a mean value of 2·03 g ± 0·03 (s.d.) was obtained for NaPAS content and 49·38 mg ± 0·70 (s.d.) for isoniazid content.

DISCUSSION

The direct ultraviolet absorption method of analysis for NaPAS in the presence of isoniazid is at least as accurate as the B.P.C. bromination method described under

cachets of sodium aminosalicylate and isoniazid. The ultraviolet method has the advantages that it is independent of the isoniazid content normally found in pharmaceuticals, it is more rapid and can be easily automated.

It is recommended that the ultraviolet method of analysis should be considered as a replacement for the B.P.C. bromination method in official standards for the estimation of sodium aminosalicylate in cachets of sodium aminosalicylate and isoniazid.

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The transfer and penetration of fatty alcohols and fatty acids in relation to their self-bodying action in emulsions

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The transfer of fatty alcohols and acids from an oil phase to an aqueous solution of surfactant has been examined. Also, penetration of the solid amphiphile by these surface-active agents has been observed by optical microscopy. The findings have been correlated with the flow behaviour of emulsions and gels containing these materials. The results indicate that before a fatty alcohol or acid, in conjunction with a surfactant, will have any self-bodying action in an emulsion two conditions must be fulfilled. Firstly, a portion of the alcohol or acid must migrate from the oil to the aqueous phase and secondly, the surfactant molecules must be of such size or shape or present in sufficient concentration to permit penetration of the amphiphile crystal lattice and the formation of a ternary liquid crystalline phase.

Talman, Davies & Rowan (1967, 1968) have postulated that the flow properties of some of their emulsions were related to the migration of the oil-soluble component and the subsequent formation of a gel in the aqueous phase. Some microscopical evidence for the migration of cetostearyl alcohol has been presented (Talman & Rowan, 1968). This paper reports the results of further investigations into the transfer of the oil-soluble component to the aqueous phase and the role of the water-soluble surfactant.

EXPERIMENTAL

Materials. These were as previously described (Talman & others, 1967, 1968; Talman & Rowan, 1970).

Methods

Transfer experiments. An aqueous solution of the surfactant (0·5% w/w unless otherwise stated) and a liquid paraffin solution of the fatty alcohol or acid (usually 7% w/w) were separately heated to 60°. The aqueous phase was placed in a pear-shaped separating funnel and the oily solution immediately poured on top with minimal disturbance of the lower layer. A series of controls, omitting either the surfactant or the fatty amphiphile was also prepared. The systems were stored undisturbed at room temperature for 3 days. Transfer was reported to have occurred if a cloudiness or white amorphous mass appeared in the aqueous phase during this period.

Penetration experiments. A small amount of coarsely powdered fatty alcohol or acid was placed on a microscope slide and a few drops of the surfactant solution (0·5% w/w unless otherwise stated) was added. This system was observed as the

temperature was slowly raised by a Leitz hot stage attached to a Conference photomicroscope, Mark I (Gillet & Sibert Ltd, London). If penetration took place some of the crystals became striated and the edges indistinct and crenulate due to the formation of an envelope of liquid crystals. In some instances, where penetration was extensive, whole crystals disintegrated to form a mass of filamentous structures.

RESULTS AND DISCUSSION

Table 1 correlates the results of the transfer and penetration experiments with the flow behaviour of corresponding gels and emulsions. Detailed descriptions of the latter have appeared previously (Talman & others, 1967, 1968; Talman & Rowan, 1970).

Transfer of amphiphile

There was no evidence of transfer in any of the control systems. Where transfer occured in the test systems, sometimes it was not apparent until about 24 h had elapsed (cetostearyl alcohol–cetomacrogol 1000 systems). On other occasions a cloudiness became visible within minutes of the two layers being brought into contact (cetostearyl alcohol–cetrimide systems). Amorphous masses developed near the

Table 1. *Comparison of transfer, penetration, gel and emulsion data for preparations containing a range of fatty alcohols and fatty acids together with some surfactants*

Fatty amphiphile	Surfactant	Transfer	Penetration	Gels	Emulsions*
Oleyl alcohol	Cetomacrogol 1000	0		0	Thin fluids
	Sorbester Q 12	0		0	Thin fluids
	Sodium lauryl sulphate	+		0	Thin fluids
	Cetrimide	+		only at 5% w/w surfactant	Thin fluids except at 5% w/w surfactant
	Solumin FX170SD	0		0	Thin fluids
	Texofor FX170	0		0	Thin fluids
Lauryl alcohol	Cetomacrogol 1000	0		+	Thin fluids
	Sorbester Q 12	0		+	Thin fluids
	Sodium lauryl sulphate	+ +		+ +	Semi-solids
	Cetrimide	+ +		+ +	Semi-solids
	Solumin FX170SD	0		0	Thin fluids
	Texofor FX170	0		0	Thin fluids
Cetostearyl alcohol	Cetomacrogol 1000	+ +	+ +	+ +	Semi-solids
	Sorbester Q 12	+ +	+ +	+ +	Semi-solids
	Sodium lauryl sulphate	+ + +	+ + +	+ + +	Semi-solids
	Cetrimide	+ + + +	+ + + +	+ + + +	Semi-solids
	Solumin FX170SD	0	0	0	No emulsion
	Texofor FX170	0	0	0	No emulsion
Myristyl alcohol	Cetomacrogol 1000	+ +	+ +	+ +	Semi-solids
	Cetrimide	+ + +	+ + +	+ + +	Semi-solids
Cetyl alcohol	Cetomacrogol 1000	+ +	+ +	+ +	Semi-solids
	Cetrimide	+ + + +	+ + + +	+ + + +	Semi-solids
Stearyl alcohol	Cetomacrogol 1000	0	0	0	Contained waxy particles
	Cetrimide	+	+	+	Contained waxy particles
Lauric acid	Cetomacrogol 1000	0		lumpy	Thin fluids
	Cetrimide	+		lumpy	Thin fluids except at 5% w/w surfactant

Fatty amphiphile	Surfactant	Transfer	Penetration	Gels	Emulsions*
Myristic acid	Cetomacrogol 1000	0	0	0	Unstable, waxy particles
	Cetrimide	+	0·5% w/w 0	+	0·5% w/w unstable, waxy particles
			5% w/w +		5% w/w semi-solids
Palmitic acid	Cetomacrogol 1000	0	0	0	0·5% w/w unstable, waxy particles 5% w/w some pseudo plastic and some as above
	Cetrimide	+	0·5% w/w 0	0	0·5% w/w unstable, waxy particles
			5% w/w +		5% w/w semi-solids
Stearic acid	Cetomacrogol 1000	0	0	0	Unstable, waxy particles
	Cetrimide	+	0·5% w/w 0	0	0·5% w/w unstable, waxy particles
			5% w/w +		5% w/w semi-solids
Behenic acid	Cetomacrogol 1000	0	0	0	Unstable, waxy particles
	Cetrimide	0	0	0	Unstable, waxy particles
Erucic acid	Cetomacrogol 1000	0		0	Thin fluids
	Cetrimide	+		0	Thin fluids

All experiments were made with 0·5% w/w surfactant unless otherwise stated.
* Emulsions refer to those containing high concentrations of fatty amphiphile.

interfaces and were extremely difficult to separate from their respective aqueous phases. They could not be removed by centrifugation at 35 000 *g* or by filtration. Isolation was finally achieved by discarding most of the lower part of the aqueous phase and drying the remainder, which contained the bulk of the transferred material, in a vacuum desiccator for 10 days over concentrated sulphuric acid. Although the infrared spectra of the dried materials were not identical with those of the alcohols or surfactants, a number of features which could be attributed to the alcohols were identified, e.g., a vestigial peak at 3·55 μm in the spectrum of material from cetostearyl alcohol–cetomacrogol 1000 systems and a prominent peak at 6·85 μm for material from cetyl alcohol–sodium lauryl sulphate systems. Optical microscopy showed the amorphous masses to consist of thin laminae or filaments (Fig. 1a). These were somewhat similar in appearance to the fatty alcohols (Fig. 1b, c) or fatty acids after penetration by a surfactant. There was also a distinct resemblance to the filaments which were seen in coarse emulsions prepared with cetostearyl alcohol and cetomacrogol 1000 or cetrimide which were reported earlier (Talman & Rowan, 1968). When the strength of the surfactant solution in systems containing cetostearyl alcohol was increased from 0·5 to 5·0% w/w, visual examination suggested that the amount of alcohol that migrated to the aqueous phase also increased. The difficulty of isolation, mentioned above, precluded quantitative estimation of the amount of transferred material. Varying the quantities of cetostearyl alcohol to correspond with those used in the emulsion (viz. 0·25, 0·5, 0·75, 1·5, 2·5, 4·0 and

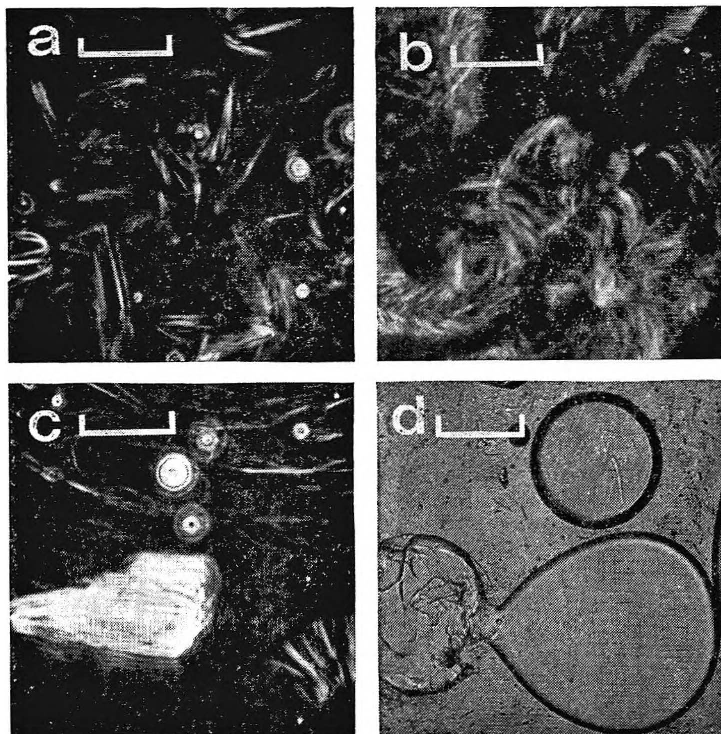

FIG. 1. Photomicrographs of (a) amorphous mass in aqueous phase of cetostearyl alcohol–cetomacrogol 1000 transfer experiment; (b) penetration of cetostearyl alcohol by cetrimide; (c) penetration of cetostearyl a cohol by cetomacrogol 1000; (d) mechanical separation of cetostearyl alcohol from a liquid paraffin droplet. One division = 30 μm.

7·0% w/w), showed that transfer occurred at all levels of concentration, even those at which the alcohol was completely soluble in the liquid paraffin.

Penetration of amphiphile

Penetration of an amphiphile by a surfactant in aqueous solution to form a ternary liquid crystalline phase has been studied by Lawrence (1958, 1961a, b). In 1959 he postulated that penetration took place, not on the flat surface of the amphiphilic crystal, but on the edges, in those positions where the polar heads lie adjacent to one another. He pointed out that this was not due to simple thermal opening of the crystal lattice, although both he and Barry & Shotton (1967) reported that there was a critical temperature below which interaction would not occur. The latter workers quoted 46° as the temperature of penetration for cetyl alcohol by 1% w/w sodium dodecyl sulphate. A small quantity of coarsely powdered cetostearyl alcohol was mixed with 0·5% w/w cetrimide solution at room temperature. After approximately 30 min a weak but definite "set" or structure had developed. This effect was much enhanced by a reduction in particle size; micronized cetostearyl alcohol gave a gel in 10 min when dispersed in the 0·5% w/w cetrimide solution. The occurrence of interaction without the aid of heat strongly indicated that interaction was not due to simple thermal opening in the crystal lattice which is in agreement with Lawrence (1959). If this is so, it would be reasonable to suppose that the size and shape of the surfactant molecule should affect penetration.

Both cetomacrogol 1000 and Sorbester Q 12 penetrated the cetostearyl alcohol lattice much more slowly and less extensively than either sodium lauryl sulphate or cetrimide. Of the two latter surfactants, attack with cetrimide was the more vigorous. The relative ease with which sodium lauryl sulphate and cetrimide penetrated the alcohol may be due to the fact that their molecular size is about one-third that of either cetomacrogol 1000 or Sorbester Q 12. Differences in the vigour of penetration with the ionic surfactants, in spite of their similar molecular size, may be ascribed to the nature of their polar heads (Lawrence, 1958). It is significant that the ease with which emulsions containing surfactants and cetostearyl alcohol could be prepared was paralleled by the ease with which the surfactants penetrated the crystal lattice. Neither Solumin FX170SD nor Texofor FX170 penetrated cetostearyl alcohol even after prolonged heating; emulsions made with these surfactants and high concentrations of the alcohol, contained waxy particles. Solumin FX170SD and Texofor FX170 are cresol derivatives comparable in molecular size to cetomacrogol 1000 and Sorbester Q 12. The lack of penetration may be accounted for by the presence of a substituted aromatic ring. It should be noted that Solumin FX170SD and Texofor FX170 permitted the preparation of stable emulsions with oleyl, lauryl or low concentrations of cetostearyl alcohol. This shows that they are capable of forming a condensed complex film in spite of their molecular size and shape.

Myristic, palmitic and stearic acids were penetrated by 5·0% w/w cetrimide solution but, contrary to Lawrence's (1959) report that penetration occurred with 0·1% surfactant solutions, we were unable to demonstrate the penetration of the foregoing acids by 0·5% w/w cetrimide solution. It is possible that attack by the weaker surfactant solution was so slow that it was not detected by our experimental procedure which involved a period of time similar to that required for the preparation of emulsions. Emulsions containing these acids and 5·0% w/w cetrimide were semi-solid preparations, whereas waxy particles were present in products containing large quantities of the acids and the lower concentration of surface-active agent. Fatty acids were generally less readily penetrated than the alcohols. Both acids and alcohols associate to form dimers by hydrogen bonding. Such association is much stronger in the case of the acids where dimers persist even in the liquid state. This accounts for their unexpectedly high melting and boiling points (Allen & Caldin, 1953). The strong bonding between carboxyl groups, at the point in the crystal lattice of the solid acids where Lawrence (1959) has suggested that penetration occurs, may explain their less ready penetration by surfactants.

Requirements for self-bodying action

Caution must be exercised in using the results of static transfer and penetration experiments to predict the likely behaviour of amphiphiles under the more dynamic conditions which are obtained during the preparation of emulsions. Nevertheless, from the result as a whole it may be concluded that two requirements must be fulfilled before a fatty acid or a fatty alcohol, in conjunction with a surfactant, can produce bodied gels or emulsions. Firstly, a portion of the alcohol or acid must be transferred across the oil–water interface into the aqueous phase. Secondly, the surfactant must penetrate the amphiphile crystal lattice and form a ternary liquid crystal phase. Lauryl alcohol, for example, produced weak gels with cetomacrogol 1000 or Sorbester Q 12 but transfer could not be detected under static conditions. In

emulsions containing these surfactants there would be no opportunity for gel formation in the aqueous phase and this explains why these emulsions were thin fluids. For products which had been prepared with high concentrations of the C_{14}–C_{22} acids and 0·5% w/w surfactant it was clear that the first condition had been fulfilled since the aqueous phase contained oil-soluble component in the form of hard waxy particles. The C_{14}–C_{22} acids were either not penetrated or only slightly penetrated by this concentration of surfactant. It was, therefore, the second condition which had not been met and which was apparently responsible for the lack of bodying action.

With the acid–0·5% w/w surfactant systems, transfer of the amphiphile was not detected *under static conditions* and may be contrasted with the ready migration of cetostearyl alcohol when treated in the same way. Since this alcohol transferred at concentrations lower than its solubility in liquid paraffin, migration was probably taking place in these circumstances by a partitioning process as postulated previously for lauryl alcohol (Talman, Davies & Rowan, 1967). Whilst we consider such a process to be an essential feature of self-bodying action—see also Barry (1969)—it should be noted that we have shown the location of cetostearyl alcohol in an emulsion to be influenced by the method of preparation (Talman & Rowan, 1968). Hand stirring gave poor emulsions with large globules containing crystals of alcohol. Fig. 1d shows the effect of shearing such emulsions between a slide and coverslip and demonstrates that these crystals may be mechanically separated from the oil phase. When preparations were homogenized the alcohol was probably transferred by the combined action of partitioning and mechanical separation. It was not possible to determine the relative contribution of these mechanisms to the overall transfer process in the case of cetostearyl alcohol, but mechanical separation alone would satisfactorily explain the presence of waxy particles of amphiphile in emulsions made with C_{14}–C_{22} acids.

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An *in vitro* model for drug absorption studies

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The transfer of salicylic acid and of three sulphonamides from an aqueous phase of pH 2 or pH 5 through an intervening organic phase to an aqueous phase of pH 7·4 has been studied using a rotating cell. The manner of operation of the cell promotes rapid drug transfer without vortexing or emulsification of the phases. The rates of transfer of the drugs showed the anticipated pH dependance.

The gastrointestinal absorption of drugs is often dependent upon their ability to penetrate a lipid barrier; for some compounds absorption is accomplished by passive diffusion of the unionized moiety (Brodie, 1964).

In vitro experimental techniques intended to mimic *in vivo* absorption behaviour have been described by Doluisio & Swintowsky (1964), and Perrin (1967). In general, in the apparatus used in these techniques the compound being examined is in solution in one of two aqueous phases that are buffered to suitable pH values, e.g. pH 2 or pH 5 to simulate stomach pH, and pH 7·4 to simulate the pH of the plasma. The aqueous phases are separate from each other and are overlaid by an organic phase (simulating a lipid barrier) which is in contact with both aqueous phases.

In the Perrin apparatus, the transfer rate is limited by the fixed interfacial area and by the tendency of the phases to mix by vortexing when stirred too rapidly. In the Doluisio–Swintowsky apparatus the interface alternately expands and contracts in a rather narrow tube; too rapid rocking tends to promote emulsification. The apparatus described here is of the same general design but has continuously generated interfaces allowing rapid interchange of drug between the aqueous and organic phases without vortexing or emulsification of the phases. The kinetics of transfer of salicylic acid and of some sulphonamides have been studied in the apparatus and are reported.

EXPERIMENTAL

Apparatus

The apparatus (Fig. 1a and b) consists of a circular, partitioned cell which is slowly rotated on rollers. The cell body is made of two pieces of copper tubing 6·3 cm long, 12·7 cm in diameter and 3·2 mm wall thickness, threaded at each end, and coated internally with "Vitrenite",* to protect the walls against chemical attack. The half cells are joined together by a partition made from a block of Perspex that has been suitably shaped and threaded on both faces, and having a central hole of 5·6 cm diameter. The end pieces are of Perspex, suitably threaded, with a hole of 1·4 cm diameter drilled 1 cm in from the edge; these holes are plugged with the type of rubber stopper used to seal multidose vials. A small hole drilled through the centre of one of the end pieces allows air to enter the cell, when sampling. The seams between Perspex and metal are sealed externally with a water-resistant glue. The cell is rotated at 32 rev/min on rubber-covered rollers driven by a 110 W constant

* Metal Protectives Co. Pty. Ltd., 1 Hamilton Street, Granville, N.S.W. 2142.

speed motor. (A variable speed motor would give greater flexibility of operating conditions.) The rollers are long enough to accommodate six cells at once.

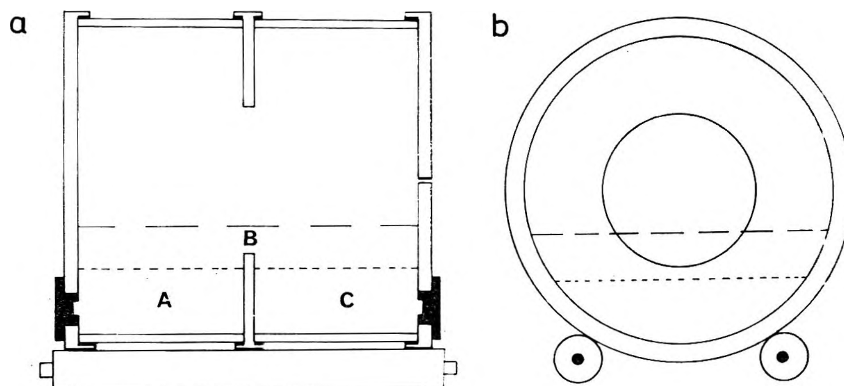

FIG. 1. a. Sectional drawing along length of circular cell. b. Sectional drawing along diameter of cell.

Materials

Salicylic acid, sulphacetamide sodium, sulphadiazine sodium and sulphadimidine sodium were of B.P. standard. Cyclohexane was supplied by Ajax Chemicals Ltd., Sydney, and octan-2-ol by British Drug Houses, U.K. The buffer solutions of pH values 2, 5 and 7·4 were those of the British Pharmacopoeia, p. 1209. Buffer solutions were equilibrated with the organic phase before use.

Salicylic acid was determined spectrophotometrically. The sulphonamides were determined by the Bratton & Marshall technique (Bratton & Marshall, 1939).

Method

100 ml of buffer solution pH 2 or pH 5 containing the drug was placed in compartment A (Fig. 1a), and 100 ml of buffer solution pH 7·4 was placed in compartment C. The aqueous phases were carefully overlaid with 200 ml of organic phase (compartment B). The cell was rotated on the rollers, and samples were removed by syringe via the plugs at suitable time intervals and assayed.

RESULTS

As can be seen from Fig. 2A, salicylic acid (pK_a 2·97) was readily transferred from an aqueous phase of pH 2, through a cyclohexane layer to an aqueous phase of pH 7·4, where it was almost entirely ionized and trapped. Since all of the salicylic acid in the system was found entirely in the aqueous phases, the transfer could be described symbolically as $D_A \rightarrow D_C$ (Doluisio & Swintowsky, 1965), where D_A is the drug concentration in the pH 2 phase, and D_C the concentration in the pH 7·4 phase. A first order plot of the data from Fig. 2A yielded a straight line.

The dependance of amount of drug transferred on pH of the aqueous phase is shown in Fig. 2B; the concentration of unionized drug at a pH of 5 is approximately 1%.

The influence of the polarity of the organic phase is also shown in Fig. 2B; addition of 1% v/v octan-2-ol to the cyclohexane markedly increased the rate of transfer.

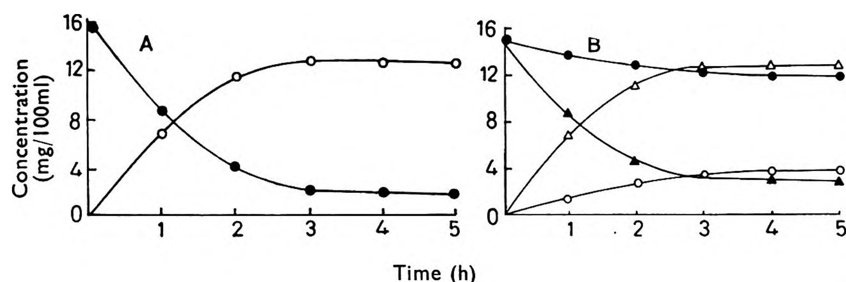

FIG. 2. A. Transfer of salicylic acid from pH 2 phase through a barrier of cyclohexane to a pH 7·4 phase. ● pH 2 phase. ○ pH 7·4 phase.

B. Effect of pH and of polarity of the organic phase on the transfer rate of salicylic acid from pH 5 phase to pH 7·4 phase. ● Compound in pH 5 phase (cyclohexane only). ○ Compound in corresponding pH 7·4 phase. ▲ Compound in pH 5 (cyclohexane plus 1% v/v octan-2-ol). △ Compound in corresponding pH 7·4 phase.

The transfer characteristics of sulphacetamide, sulphadiazine and sulphadimidine were examined; these drugs each have two ionizable groups, the pK_a values of which are: sulphacetamide,* 1·4, 5·38; sulphadimidine,* 2·76, 7·37; sulphadiazine,† 2·24, 6·42.

The percentages of unionized species present between pH 2 and pH 7·4 were calculated from the formula:

$$\% \text{ unionized} = \frac{1}{1 + 10^{pK-pH} + 10^{pH-pK}} \times 100.$$

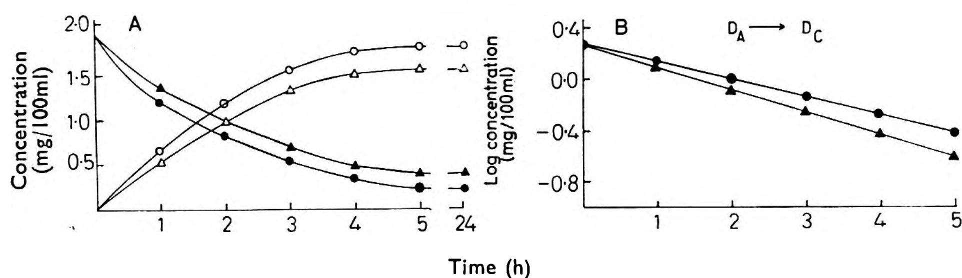

FIG. 3. A. Transfer of sulphacetamide from pH 2 phase and pH 5 phase through a barrier of cyclohexane-octan-2-ol (35:65 v/v) to a pH 7·4 phase. ● pH 2 phase. ○ Corresponding pH 7·4 phase. ▲ pH 5 phase. △ Corresponding pH 7·4 phase.

B. Plot of data in Fig. 3A, showing apparent first order disappearance of sulphacetamide from phases pH 2 and pH 5. ● pH 2 phase. ○ pH 5 phase.

The results of the transfer studies (Figs 3 and 4) showed that sulphacetamide behaved in a manner similar to that of salicylic acid, i.e. in a given time more compound was transferred from a pH 2 phase than from a pH 5 phase. However the difference between the amounts so transferred was much less than with salicylic acid. A first order plot of the data from Fig. 3A yielded straight lines (Fig. 3B).

With both sulphadiazine and sulphadimidine Fig. 4A,B there was a greater amount of drug transported at pH 5 than at pH 2. This behaviour reflects the concentration of unionized species of each drug available for transfer at the chosen pH values.

* Data taken from "The Sulphonamides and Allied Compounds".
† Data supplied by Dr. Brian Rawson of this Department.

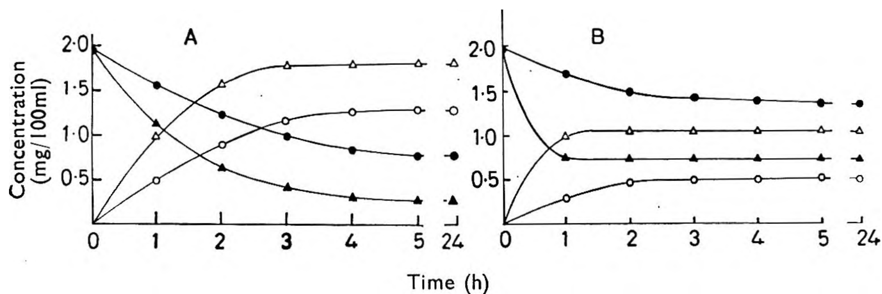

FIG. 4. Transfer of (A) sulphadiazine and (B) sulphadimidine from pH 2 phase and pH 5 phase through a barrier of cyclohexane-octan-2-ol (35 : 65 v/v), to a pH 7·4 phase. ● pH 2 phase. ○ Corresponding pH 7·4 phase. ▲ pH 5 phase. △ Corresponding pH 7·4 phase.

Moreover, first order plots of the transfer data for these two drugs did not yield straight lines, but curves. However, since all of each drug was contained only in the aqueous phases, a plot of $D_A - D_A^{\infty}$ against time should yield straight lines (Doluisio & Swintowsky, 1965). D_A = concentration of drug in compartment A at any time, and D_A^{∞} = equilibrium concentration of drug in compartment A). Straight line graphs were obtained (Fig. 5).

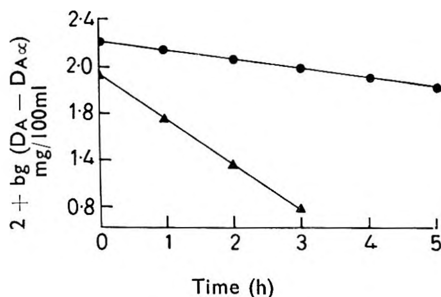

FIG. 5. Plot D_A–D_A^{∞} for data in Fig. 4, pH 5 phase only. ● Sulphadiazine. ▲ Sulphadimidine.

These results indicated that the apparatus described was satisfactory for studying the transport of drug molecules between aqueous phases via a lipid phase.

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Medicament release from fatty suppository bases

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The consistency in terms of viscosity index and the rheology of mixtures of cacao butter with different fats was examined with a Stormer viscometer. Most bases showed shear rate thickening at low stresses and marked fall in consistency between 35°–40°. The drug release from them was related inversely to the consistency of the bases.

Earlier work on the fatty suppository bases has been mainly qualitative, including studies on melting points (Melangeau, 1948), viscosity (Zampfira Csath-Stincel, 1966), softening and liquefaction ranges (Setnikar and Fantelli, 1962, 1963) and rheological changes in mixtures of cacao butter with added water soluble substances (Tufegdzic and Lj Parezanovic-Dordevic, 1961).

Cacao butter possesses non-Newtonian flow characteristics (Sterling & Wuhrman, 1960). Describing the consistency of its mixtures with other fats in terms of a single physical property such as melting point or viscosity, therefore appears inadequate. We have tried to measure the consistency of such mixtures and have examined changes in their consistency with temperature.

Reports on the release of medicaments from fatty suppository bases and their *in vitro* disintegration are few. Plaxo (1967) has suggested dialysis through cellophane membrane and subsequent measurement of extinction. Medicament release has also been estimated microbiologically (Ghafoor & Huych, 1962; Blissitt, Tinker & Husa, 1961). We have estimated medicament release from the bases using an apparatus affording simulated body conditions.

EXPERIMENTAL

Materials. Cacao butter (B.D.H.), beeswax (B.P.), spermaceti (B.P.C.) and kokum butter (I.P.).

Preparation of mixtures of fats as bases. Cacao butter was gently melted on a water-bath below its critical temperature of 36°, since above that temperature the stability of the fat is affected (Sterling & Wuhrmann, 1960). The melted fat was added slowly with constant stirring to other previously melted fats. The mixtures were warmed sufficiently to effect a thorough mixing and then chilled immediately to avoid separation of the constituents. They were stored in a refrigerator for 24 h at 5° and then allowed to attain room temperature (28–30°) slowly. The mixtures did not show any separation of the constituents when kept for 12 to 16 weeks at room temperature.

*Based on the work submitted by T. V. Lohit to Bombay University in partial fulfilment of the requirements for the degree of Master of Science (Faculty of Technology).

The mixtures prepared contained (i) 5, 10, 25, 35 and 50% kokum fat, (ii) 1, 2, 3, 4 and 5% beeswax, and (iii) 2, 4, 6, 8 and 10% spermaceti, in cacao butter.

Rheological study. A Searle type rotational viscometer, commercially available as the Stormer viscometer, involving the use of a cup and rotor was used because of the facility of measuring the rate of shear against varying shear stresses. The cup was filled with the sample and allowed to attain the required temperature with the aid of a heater and a thermostat. Ten min were generally allowed for equilibration. The level of the sample in the cup was maintained just enough to keep the rotor completely immersed and also to prevent the formation of a vortex during rotation. After temperature equilibration, loads from 40 to 400 *g* (shear stress) were applied and the number of revolutions of the rotor were noted to give the shear rate, once while increasing the order of weights and again while decreasing their order, with a view to studying any time dependant flow characteristics like thixotropy. Readings were recorded at 35°, 37°, 40°, 45° and 50°. Except for cacao butter-beeswax mixtures, no appreciable (±0·5 s/100 rev) differences in the readings were observed at different loads. Hence the mean of the two readings was taken as the final reading. The results were plotted to obtain flow curves as shown in Fig. 1.

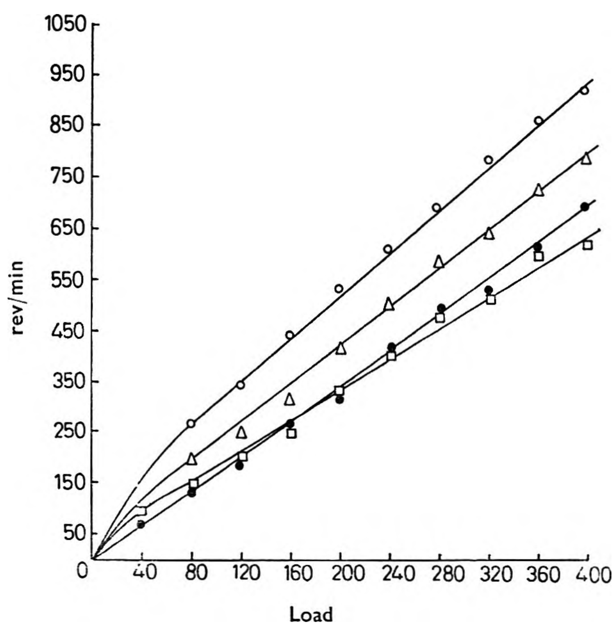

Fig. 1. Rheograms (at 37°). —□—□—, Pure cacao butter; —●—●—, cacao butter + spermaceti 10%; —○—○—, cacao butter + kokum fat 25%; —△—△—, cacao butter + beeswax 1%.

Preparation of suppositories

Salicylic acid (freely soluble in fat), boric acid (less soluble in fat), and copper sulphate (water soluble) were used.

The suppository bases were melted with the minimum amount of heat and part was used to prepare a fine paste of the ground medicament (2 grains/suppository) which was then stirred thoroughly into the bulk of the base. When cloudiness developed the base was poured into chilled moulds. No attempt was made to deter-

mine the particle size or the degree of uniformity of distribution of the medicament. After initial cooling the suppositories were trimmed, kept for 24 h at 5° and then allowed to attain room temperature (28–30°) slowly.

Medicament release from suppositories

The medicament release from the bases was examined using an apparatus in which normal saline at a rate of 100 drops/min was allowed to flow onto the suppository supported on a cotton plug in a glass funnel, mounted on a hot-water jacket. Care was taken to keep the suppository completely immersed in normal saline. Temperature was controlled thermostatically at $37 \pm 0{\cdot}5°$. The amount of medicament released from each suppository was determined by analysing aliquot samples of saline collected at regular intervals over 1 h.

The medicaments were estimated spectrophotometrically using a Beckmann spectrophotometer Model DB. Salicylic acid was estimated by colour development with ferric nitrate and measuring the extinction at 525 nm (Stemler, Cosmides & Miya, 1956). Copper sulphate was estimated by the diethyl dithiocarbamate method measuring the extinctions at 425 nm (Callan & Henderson, 1929; Haddock & Evers, 1932). Estimation or boric acid was by using carmine solution for colour development and measuring the extinction at 585 nm (Hatcher & Wilcox, 1950).

RESULTS

Rheology. Rheograms showing shear rate (rev/min) against shear stress (load g) were plotted for all the samples as shown in Fig. 1. The viscosity indices of the bases were determined using the power law equation originally proposed by Ostwald (1926),

$$T = K\, r^n$$

where T = shear stress; r = shear rate; n = flow index; and K = viscosity index (Rogers & Sabin, 1911). These values as determined from the graphs are recorded

Table 1. *Viscosity indices of bases at different temperatures.* (The main base was cacao butter. The values in parentheses are those obtained by arithmetic interpolation)

Base	Viscosity index at 35°	37°	40°
Pure cacao butter	72·4	51·3 (52·2)	22·4
Added fat			
Cacao + % kokum butter			
5	113·5	70·1 (78·0)	24·0
10	134·9	82·1 (92·8)	18·2
25	—	102·3	18·2
35	—	—	35·3
50	—	—	—
Cacao + % spermaceti			
2	25·7	24·5 (23·9)	20·9
4	35·1	30·1 (28·5)	19·5
6	43·2	34·6 (34·5)	20·9
8	50·7	38·9 (39·0)	20·9
10	57·9	44·6 (44·0)	20·7
Cacao + % beeswax			
1	95·5	59·5 (66·0)	19·5
2	139·6	91·2 (92·4)	20·9
3	171·8	113·2 (114·0)	24·8
4	298·5	160·3 (186·0)	18·2
5	—	—	16·9

in Table 1. The viscosity indices were plotted against temperature to study the changes in consistency of the different bases with temperature (Fig. 2).

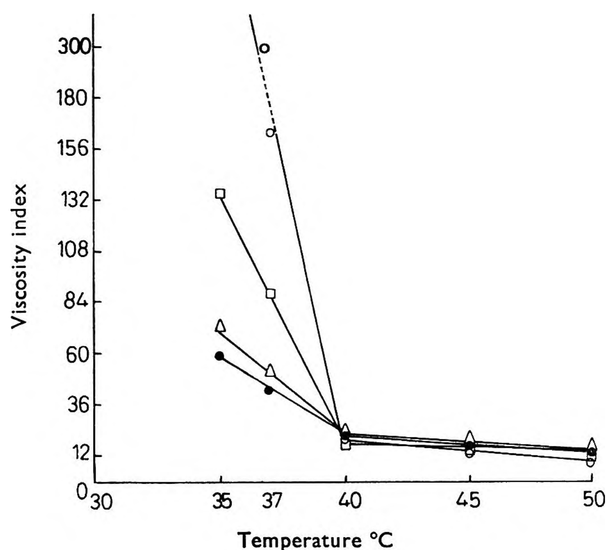

FIG. 2. Effect of temperature on cacao butter and its mixtures. —△—△—, Pure cacao butter; —○—○—, cacao butter + beeswax 4%; —□—□—, Cacao butter + kokum fat 10%; —●—●—, cacao butter + spermaceti 10%.

The rheograms of nearly all bases exhibited slight shear rate thickening. Only mixtures of cacao butter–beeswax showed thixotropy, but this was neither confirmed nor investigated further, because of the operational limitations of the viscometer.

All the bases except those containing 35 and 50% kokum fat in cacao butter and the base with 5% beeswax showed a sharp fall in their consistency, i.e. viscosity index values in the temperature range of 35 to 40°.

Medicament release. To understand the relative drug releasing efficiency of the bases the total amount of medicament released during 1 h was plotted against the viscosity index values at 37° of the bases (Fig. 3). Almost all the graphs were rapidly falling curves.

To examine the pattern of medicament release, the quantity released in an aliquot sample was plotted against the time at which the sample was collected. All the graphs obtained were smooth curves. The rate of drug release was rapid in the first 10 min but fell as the time increased up to 50 min (Fig. 4).

The slopes of the straight lines (fitted for values, between 10–50 min) with the time axis expressed as the tangent of the angle made with the axis have been used to indicate the amount of the drug released per unit time. The slopes were obtained by plotting concentration of medicament against time and were found to fit in accordance with the χ^2 test. In Table 2 the different samples tested and the amount of medicament released is given in terms of the tangent.

When the values for drug release in unit time were plotted against the logarithms of viscosity indices at 37° (calculated as well as verified by experiment, Table 1) of the respective bases, the relation was linear and fitted in accordance with the χ^2 test.

Estimation of medicament release from pure cacao butter suppositories for com-

parative purposes could not be made as it was not possible to obtain suppositories with this base alone at the ambient temperature of 28° to 31°.

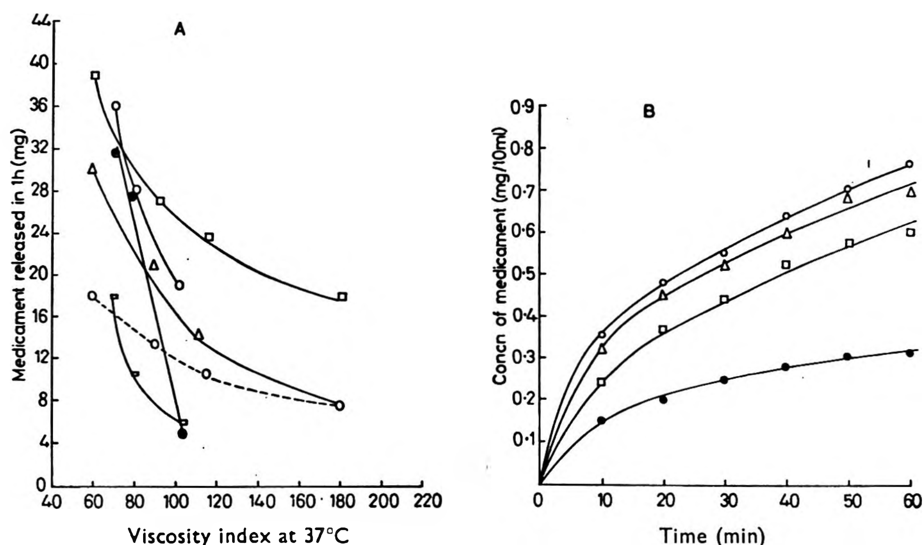

FIG. 3A. Plots showing the total medicament release in 1 h from different bases at 37°. —□—□—, Salicylic acid from cacao butter + beeswax (1, 2, 3 and 4%); —○—○—, salicylic acid from cacao butter + kokum fat (5, 10 and 25%); —△—△—, copper sulphate from cacao butter + beeswax (1, 2, 3 and 4%); —●—●—, copper sulphate from cacao butter + kokum fat (5, 10 and 25%); —·—·—, boric acid from cacao butter + kokum fat (5, 10 and 25%); ..○..○.., Boric acid from cacao butter + beeswax (1, 2, 3 and 4%).
B. Plots showing drug release pattern. —○—○—, Copper sulphate in cacao butter + kokum fat 10%; —△—△—, salicylic acid in cacao butter + kokum fat 10%; —□—□—, salicylic acid in cacao butter + beeswax 3%; —●—●—, copper sulphate in cacao butter + beeswax 3%.

Table 2. *Relative efficiency of medicament release in unit time from various bases, evaluated in terms of the tangent of the angle made by the slope of the straight lines with the* x *axis* (*tan θ*), *higher values indicating greater release.*

Medicament	Base	With % of wax or fat added to cacao butter	Tan θ
Salicylic acid	+ Beeswax	1	0·9325
	,,	2	0·6745
	,,	3	0·6249
	,,	4	0·5317
	+ Kokum fat	5	0·8089
	,,	10	0·5543
	,,	25	0·2679
Boric acid	+ Beeswax	1	0·4663
	,,	2	0·3640
	,,	3	0·1405
	,,	4	0·1051
	+ Kokum fat	5	0·7536
	,,	10	0·6009
	,,	25	0·3640
Copper sulphate	+ Beeswax	1	0·7536
	,,	2	0·4663
	,,	3	0·2126
	,,	4	0·1405
	+ Kokum fat	5	0·6249
	,,	10	0·5319
	,,	25	0·1510

DISCUSSION

The sharp fall in the viscosity index values of the mixtures of fats in the temperature range of 35° to 40° indicated a transition in their consistency and hence these bases could be expected either to melt or soften around body temperature. This was further confirmed by determining the viscosity indices at 37° experimentally.

The viscosity index of the base containing 5% beeswax and 35% kokum fat at 37° did show a comparatively low value at 40°, and it is possible that it may be useful for drugs which lower the m.p. of the base. Further increase in the percentage of kokum fat did not seem to be advantageous. With the preparation containing spermaceti (2–10%) there was a sharp fall in the viscosity index values in the temperature range of 35° to 40°, and below those for pure cacao butter, so such a combination would not offer a suitable preparation.

The extent of change in the viscosity index value is related to the amount of added fat. The mixtures of cacao butter with beeswax up to 5% show comparatively greater effects on the viscosity index values than the other bases under investigation.

The rapidly falling curves in Fig. 3A suggest that the total amount of medicament released varied inversely with the consistency of the base which in turn increased with the amount of fat added.

The release of drug from the bases was linear after an initial period. That the magnitude of release of the medicament depended at least to some extent on the consistency of the base, in which it was incorporated is further substantiated by the linear relation of the plots of logs of viscosity indices of the bases at 37° against values for the release of drug in unit time.

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Pharmacological actions of phosphocholine 2,6-xylyl ether bromide (phospho-TM10; PTM10)

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PPP-Trimethyl-2-(2,6-xylyloxy)ethylphosphonium bromide (PTM10; phospho-TM10), the phosphorus analogue of xylocholine (TM10), has been found to possess local anaesthetic, α-adrenoceptor and ganglion blocking properties. It also shows a blocking action at the neuromuscular junction but has no detectable adrenergic neuron blocking properties. The relation of these results to the 'cholinergic link" hypothesis of Burn and Rand is discussed.

It is well known that substitution of phosphorus for nitrogen in compounds which show agonist activity at acetylcholine receptors results in a reduction in potency (Hunt & Renshaw, 1925; Holton & Ing, 1949). Since Burn & Rand (1959) have postulated that acetylcholine is involved in the release of noradrenaline from adrenergic nerve terminals and that adrenergic neuron blocking agents, such as bretylium and xylocholine, act by preventing this "nicotinic" action of acetylcholine on the noradrenaline stores (Burn & Rand, 1962), it was felt that it might be of interest to investigate the pharmacology of *PPP*-trimethyl-2-(2,6-xylyloxy)ethylphosphonium bromide (phospho-TM10; PTM10) with special reference to any action on the adrenergic nerve or at "nicotinic" sites of action of acetylcholine. The synthesis and an initial pharmacological investigation of phospho-TM10 (PTM10) have already been reported (Clark & Hughes, 1970) and a more comprehensive pharmacological assessment of this compound is now presented.

EXPERIMENTAL

Rat blood pressure

Male rats, 180–250 g, were anaesthetized with pentobarbitone (50 mg/kg) intraperitoneally, or with urethane (15% w/v in 0·9% NaCl; 1·2 g/kg) by the same route. Plastic tracheal, left carotid and right jugular cannulae were inserted and each animal received 100 units of heparin when the operative procedure was complete. All rats were artificially respired using a Palmer Miniature Ideal pump set to deliver 2·5 ml of air at a rate of 100/min. Blood pressure was recorded from the carotid artery on a Devices M2R recorder with a Bell and Howell pressure transducer (Type 4-327-L221) using a Devices preamplifier. Heart rate was determined by counting the number of pulse waves in a 5 s period. All injections were made through the jugular cannula unless otherwise stated and were washed in with 0·1 ml 0·9% NaCl. Rats were pithed under hexobarbitone anaesthesia (250 mg/kg administered intraperitoneally) using the approach through the orbit. These rats received 1·5 mg/kg atropine sulphate intraperitoneally 30 min before the anaesthetic was administered.

Cat blood pressure and nictitating membrane

Anaesthesia was induced in cats of either sex, 1·3–2·5 kg, with ether and was maintained with chloralose (80 mg/kg) injected through a cannula in the right femoral vein. The trachea was cannulated and respiration maintained artificially. The right postganglionic and left preganglionic cervical sympathetic nerves were exposed and stimulated electrically for 15 s as required with rectilinear pulses of 0·5 ms duration and supramaximal voltage delivered at a rate of 20 or 50 shocks/s. The right vagus was exposed and stimulated electrically as required. All stimuli were applied through hook electrodes from a Palmer H44 stimulator and all nerves were sectioned proximal to the stimulating electrodes and covered with cotton wool soaked in saline-equilibrated liquid paraffin. Contractions of the nictitating membranes were recorded with isometric transducers (Devices, Type 2STO2) and blood pressure was measured from the right femoral artery as described for the rat. Heart rate was obtained from the pulse wave recorded by the pressure transducer using a Devices DC3 preamplifier and Model B Instantaneous Ratemeter. All recordings were made on a Devices M4 recorder and drugs were dissolved in saline, injected through the femoral cannula and washed in with 0·5 ml 0·9% NaCl. All animals received 1000 units of heparin when the operative procedure was complete.

Intracutaneous weal test

As described by Bülbring & Wajda (1945).

Isolated cervical vagus nerve

As described by Clark & Hughes (1966) except that stimuli were applied from a Grass S8 stimulator through a Grass isolation unit (Type SIU 4678). Recordings were made differentially through a Tektronix Type 122 preamplifier and were photographed after display on a Tektronix Type 502A oscillograph. The nerve under one of the recording electrodes was destroyed by crushing.

Phrenic nerve-diaphragm

Preparations were mounted on Palmer phrenic nerve electrodes in Krebs solution at 37° and gassed with 5% carbon dioxide in oxygen. Stimuli were applied either directly or indirectly from a Palmer H44 stimulator and recordings were made on a Devices M2R recorder with a Devices isometric transducer.

Frog rectus abdominis

Preparations were removed from freshly killed frogs, set up in Frog Ringer at room temperature and gassed with air. Recordings were made and displayed as for the phrenic nerve-diaphragm.

The drugs used were: acetylcholine bromide, atropine sulphate monohydrate, carbachol chloride, chloralose, heparin injection B.P., hexabarbitone sodium, hexamethonium bromide, (−)noradrenaline bitartrate monohydrate, pentobarbitone sodium, piperoxan hydrochloride, phentolamine methane sulphonate, neostigmine methyl sulphate, tetraethylammonium iodide, (+)tubocurarine chloride, and urethane. *NNN*-trimethyl-2-(2,6-xylyloxy)ethylammonium bromide (xylocholine; TM10), *NNN*-trimethyl-2-(phenoxy)ethylammonium bromide (TM1), and *PPP*-trimethyl-2-(2,6-xylyloxy)ethylphosphonium bromide (phospho-TM10; PTM10) were

synthesized in these laboratories. All doses and concentrations are expressed in terms of these salts.

RESULTS

Action on the cardiovascular system of the rat

Administration of PTM10 (2–15 mg/kg) to rats anaesthetized with urethane or pentobarbitone produced an immediate bradycardia lasting some 5–20 min which was accompanied by a large depressor response lasting 20–60 min. The depressor response was preceded by a shortlived pressor response and the magnitude and duration of the depressor response and bradycardia were related to the dose of PTM10

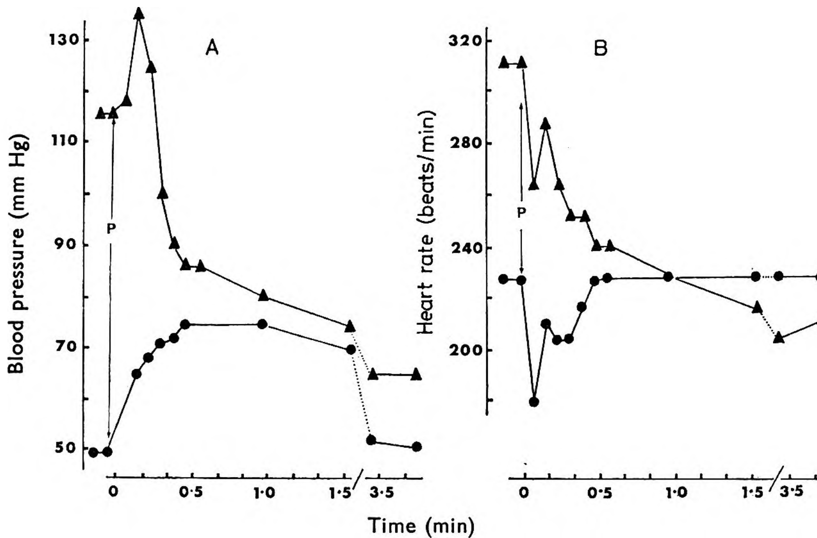

FIG. 1. The effects of PTM10 (1·5 mg, intravenously at P) on the blood pressure (A) and heart rate (B) of a pentobarbitone anaesthetized rat (▲, weight 195 g) and an atropinized pithed rat (●, weight 220 g). Time (min) after the administration of PTM10 is plotted on the abscissa.

administered (Fig. 1). Administration of a second dose of PTM10 during the depressor phase following an initial high dose failed to produce any further reduction in the blood pressure though the initial pressor response was still seen.

In the pithed atropinized rat, similar doses of PTM10 failed to produce a depressor response and the bradycardia was less marked and of much shorter duration than that seen in the anaesthetized animal. The initial pressor response was still seen however and was augmented in both magnitude and duration (Fig. 1). This pressor response was produced on repeated administration of PTM10 and was not reduced by acute bilateral adrenalectomy or by treatment of the rats with α-blocking agents (phentolamine, 0·5 mg/kg; piperoxan, 4 mg/kg) administered intravenously 5 min before the dose of PTM10. Hexamethonium (1·5 mg/kg) also failed to reduce the pressor response to PTM10.

In pithed atropinized rats, the pressor responses evoked by administration of noradrenaline and by the ganglion stimulant TM1 were antagonized by similar doses of PTM10, the action against noradrenaline being shorter in duration than the action against TM1 (Fig. 2).

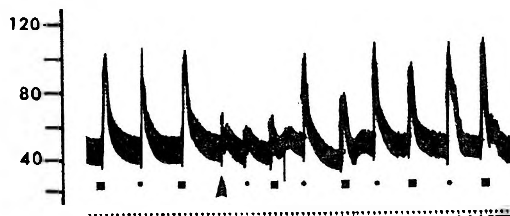

FIG. 2. Pithed atropinized rat (weight 240 g). The effects of noradrenaline (0·05 μg intravenously at ●) and TM1 (20 μg intravenously at ■) before and after 0·8 mg PTM10 intravenously at ▲ on the blood pressure in mm Hg. Time marker, 60 s.

Action on cat blood pressure and nictitating membranes

In doses of 1–8 mg/kg, PTM10 caused a fall in blood pressure and heart rate in the anaesthetized cat. The bradycardia was less marked in this species than in the rat and the initial pressor response which was always seen in the rat was observed in only one out of 10 experiments (Fig. 3).

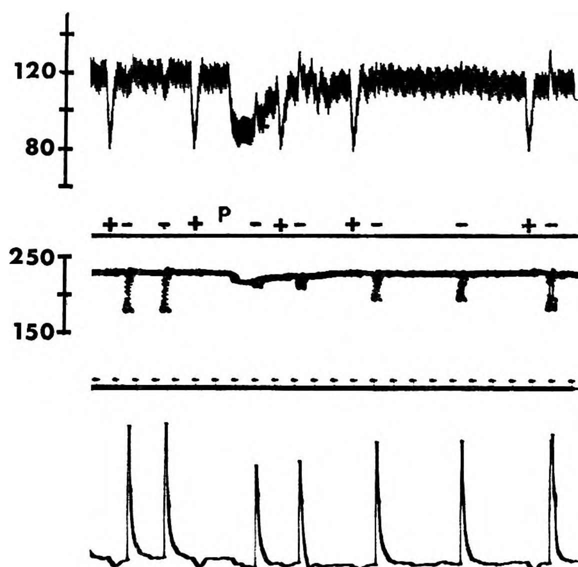

FIG. 3. Nictitating membrane preparation of the cat (1·4 kg). Upper record: Blood pressure in mm Hg. Middle record: Heart rate in beats/min. Lower record: contraction of the nictitating membrane. At –, electrical stimulation was applied to the vagus (10/s, 0·2 ms duration, 10 V for 7 s) and preganglionic cervical sympathetic (20/s, 0·5 ms duration, 10 V for 15 s) nerves simultaneously. At P, 4·5 mg PTM10 and at +, 0·5 μg acetylcholine were injected intravenously. PTM10 reduced the response of the nictitating membrane and the fall in heart rate produced by electrical stimulation with a similar time course but left the response to injected acetylcholine unchanged. Time marker, 60 s.

The fall in heart rate produced by electrical stimulation of the distal portion of the sectioned vagus was reduced or abolished by these doses of PTM10, though the depressor response to injected acetylcholine remained unchanged. Responses of the nictitating membrane to stimulation of the cervical sympathetic nerve preganglionically were also reduced, the time course of this reduction being similar to that of the effect on vagal stimulation (Fig. 3). Responses of the nictitating membrane to postganglionic stimulation were transiently reduced, this effect being smaller and of

much shorter duration than the effect against preganglionic stimulation. In all cases the blocking action of PTM10 was fast in onset and of only moderate duration, the responses returning to control levels within 40 min of the administration of the dose of PTM10. Although larger doses killed the animals (producing a precipitous fall in heart rate, pulse pressure and blood pressure which was quickly followed by the death of the animal), the responses of the nictitating membranes to pre- and post ganglionic stimulation of the cervical sympathetic nerves returned to control levels within 45 min after the administration of 50 mg/kg of PTM10 in divided doses over a period of 1–2 h in three experiments, and after administration of 100 mg/kg over 3 h in one further experiment (Fig. 4).

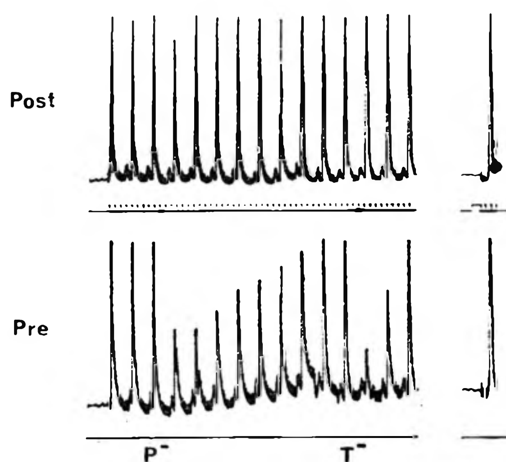

FIG. 4. Nictitating membrane preparation of cat (1·9 kg). Responses are to stimulation (0·5 ms duration, 50/s, 14 V: applied for 15 s every 3 min 45 s for the whole experiment) of the pre-(lower) and post-(upper) ganglionic cervical sympathetic nerves. At P, 9 mg PTM10 and at T, 17 mg tetraethylammonium were injected intravenously. Time interval between parts 1 and 2 of the record was 160 min and during the first 120 min of this period 50 mg/kg PTM10 was administered in divided doses and had no prolonged effect on the response to electrical stimulation. Time marker, 60 s.

Assessment of local anaesthetic activity

Intracutaneous injections of solutions of PTM10 in 0·9% NaCl into guinea-pigs according to the method of Bülbring & Wajda (1945) produced a loss of sensation in the area injected. A delay in the onset of the anaesthesia, which is characteristic of xylocholine, was also apparent with PTM10, though to a lesser extent. Since differences in rates of onset of anaesthesia complicate estimations of potency by this method, no accurate comparisons of potency were made. However, solutions of xylocholine and PTM10 of equal molarity produced approximately similar degrees of anaesthesia indicating that there was no great difference in the local anaesthetic potency of these two compounds.

In the isolated cervical vagus preparation of the rabbit, PTM10 in concentrations of 0·5–1 mg/ml produced a reduction in the size of the action potential which was usually in excess of 90% after 15 min exposure to the drug. In similar concentrations xylocholine also reduced the size of the action potential though the effect was not as great as that seen with PTM10. With both drugs complete abolition of the action potential could be achieved on exposure to higher concentrations and complete

reversal of this blockade was usually produced after washing the preparation for 2–4 h (Fig. 5).

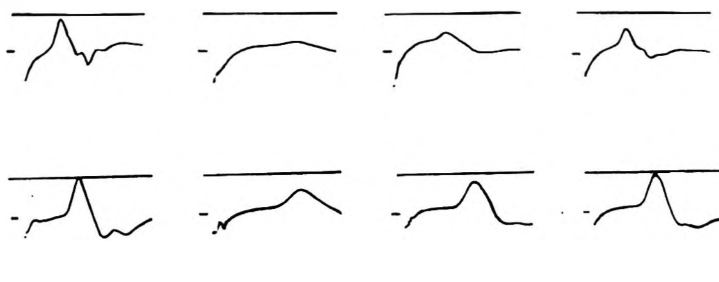

FIG. 5. Action of PTM10 (0·6 mg/ml—upper record) and xylocholine (0·6 mg/ml—lower record) in reducing the size of the action potential recorded on stimulation (0·5 ms duration, 32 V) of the isolated cervical vagus from the rabbit. From left to right: control: after 15 min exposure to the drug: after washing for 1 and 3 h respectively. Calibration at bottom right is 3 mV vertical and 20 ms horizontal.

Action on the isolated phrenic nerve-diaphragm

In concentrations of 10 μg/ml and above, PTM10 produced a reduction in the twitch tension developed in the isolated diaphragm in response to electrical stimulation of the phrenic nerve. At low concentrations development of the blockade was slow and equilibrium was not attained in 90 min when the experiments were terminated. At higher concentrations the blockade was fast in onset and responses to direct stimulation of the muscle were unaffected by concentrations of PTM10 which completely abolished the response to indirect stimulation. The blockade could be reversed easily on washing the tissue and was partially reversed by KCl (0·4 mg/ml) but not by tetraethylammonium (50 μg/ml) or by neostigmine (5 μg/ml), though these concentrations were effective in reversing the blockade due to tubocurarine. Partial blocking concentrations of tubocurarine and PTM10 were additive and in no case was any antagonism observed. Estimations of blocking potency after exposure of the tissue to the drug for 15 min (i.e. under non-equilibrium conditions) indicated that PTM10 was a more potent blocking agent than xylocholine on this preparation, concentrations needed to produce 50% blockade being 35 and 45 μg/ml for PTM10 and 52 and 60 μg/ml for xylocholine in two experiments respectively (Fig. 6).

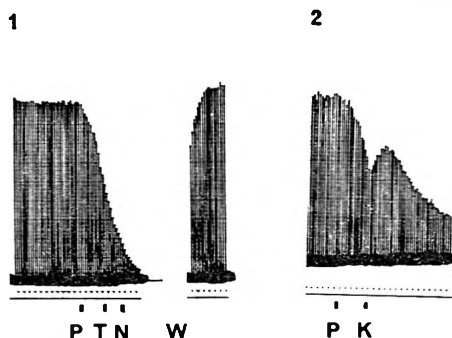

FIG. 6. Rat phrenic nerve-diaphragm. Maximal twitches in response to stimulation of the phrenic nerve (0·03 ms duration, 11 V, 3/min). Time marker, 60 s. Part 1. Effect of PTM10 (80 μg/ml at P) and of tetraethylammonium (50 μg/ml at T) and neostigmine (5 μg/ml at N). Time interval between the two sections of part 1 was 45 s during which the tissue was washed once by drainage. Part 2. Effect of KCl (0·4 mg/ml at K) on the blockade produced by PTM10 (75 μg/ml at P).

Action on the rectus abdominis

On this preparation responses to both acetylcholine and carbachol were antagonized by PTM10 in concentrations above 2 μg/ml. Dose-response curves constructed in the presence of various concentrations of PTM10 showed that not only were the dose-response curves to acetylcholine and carbachol shifted to higher concentrations along the dose axis, but that the maximal response which could be elicited from the tissue was also reduced. (Fig. 7).

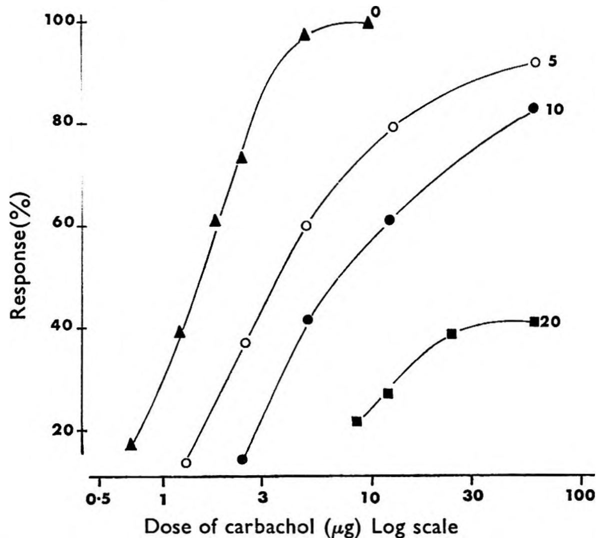

FIG. 7. Frog rectus abdominis. Plot of $\log_{10}$ (in μg) against response (% of maximal) for carbachol in the presence of 0, 5, 10 and 20 μg/ml of PTM10.

DISCUSSION

Clark & Hughes, (1970) indicated that replacement of the nitrogen atom in xylocholine by phosphorus produced a compound (PTM10) which was devoid of adrenergic neuron blocking properties. The results here presented substantiate this finding.

Since PTM10 reduced the effect of noradrenaline on the blood pressure of the pithed rat and also had a more prolonged blocking action against the ganglion stimulant TM1, it is apparent that it possesses α-blocking properties and also interferes with transmission in adrenergic nerves. As the response of the nictitating membranes to preganglionic stimulation was much reduced while the response to postganglionic stimulation remained relatively unchanged it is probable that this interference takes place at the level of the ganglion.

The effect of PTM10 on vagal stimulation is unlikely to be due to any antimuscarinic action since the response to injected acetylcholine was unchanged. It is likely therefore that this action is also due to ganglion blockade and this is supported by the observation that the time course of the effect is similar to that of the action of PTM10 on the response of the nictitating membrane to electrical stimulation of the cervical sympathetic nerve preganglionically.

The possession of ganglion blocking properties by PTM10 could also explain the effect of this compound on the blood pressure and heart rate of the cat and rat, the differences in size of the reduction in heart rate being due to differences in the degree of normal sympathetic tone in the two species. The initial pressor response to

PTM10, seen mainly in the rat, was probably not due to an action at ganglia as it was unaffected by hexamethonium. Release of catecholamines (which is responsible for the pressor action of xylocholine) also seems unlikely in the case of PTM10 as bilateral adrenalectomy and α-blocking agents did not reduce the response. The mechanism of this initial pressor response has yet to be established but may be due to a direct action.

The blocking actions of PTM10 were fast in onset and of only moderate duration. In no case, even at high dose levels, was any blocking action observed which had the characteristic long duration of the adrenergic neuron blocking agents.

The actions of PTM10 in the guinea-pig intracutaneous weal test and on the isolated vagus can be accounted for in terms of local anaesthetic activity and in this respect PTM10 appears to be equipotent or slightly more potent than xylocholine. The local anaesthetic properties demonstrated in these preparations probably account for the blocking action of PTM10 on the Finkleman preparation which was demonstrated earlier (Clark & Hughes, 1970).

The actions of PTM10 on the phrenic nerve diaphragm preparation appear to be similar to those of bretylium (Dixit, Gulati & Gokhale, 1961). Blockade by PTM10 was additive to a partial tubocurarine blockade, was easily reversed by washing and by KCl but was unaffected by neostigmine and tetraethylammonium. This blocking action was at least as potent as that possessed by xylocholine. In contrast to bretylium however, PTM10 had a non-competitive blocking action against acetylcholine and carbachol on the frog rectus abdominis muscle.

The multiple actions of PTM10 make it difficult to establish that this compound has no adrenergic neuron blocking properties. However, the fact that all the actions of PTM10 that I observed can be accounted for by mechanisms other than adrenergic neuron blockade, suggests that any adrenergic neuron blocking properties possessed by PTM10 must be very weak. In spite of this, the actions of PTM10 at the neuromuscular junction are at least as strong as those of xylocholine and are of a similar type to those possessed by other adrenergic neuron blocking agents. It may be therefore that support for the Burn & Rand "cholinergic link" hypothesis drawn from the actions of drugs at the neuromuscular junction should be treated with caution.

The lack of adrenergic neuron blocking activity in PTM10 indicates that the requirements for adrenergic neuron blocking activity are very closely defined in terms of molecular structure. The loss of adrenergic neuron blocking properties on substitution of phosphorus for nitrogen in xylocholine is not dissimilar to the reduced potency observed in phosphonium analogues of compounds which are active at acetylcholine receptors and might therefore be interpreted as support for the Burn & Rand "cholinergic link" theory of transmission at adrenergic nerve terminals.

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The influence of angiotensin on the uptake of noradrenaline by the isolated heart of the rabbit

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The influence of angiotensin on the removal of noradrenaline (10 ng/ml) from the perfusion medium and on the net-uptake of noradrenaline (5 and 20 μg/ml) was examined in the rabbit isolated heart. Concentrations of angiotensin, known to augment the output of noradrenaline from rabbit heart during sympathetic nerve stimulation, did not inhibit the removal of infused noradrenaline from the perfusion fluid. Only very high concentrations of angiotensin (13 μg/ml) significantly diminished the loss of noradrenaline. The positive inotropic effect of noradrenaline was not potentiated by angiotensin. On the other hand, cocaine, in doses which enhance the output of noradrenaline during sympathetic stimulation greatly reduced the loss of noradrenaline from the perfusion fluid. Neither angiotensin (130 ng/ml) nor metanephrine (5 and 20 μg/ml) significantly influenced the net-uptake of noradrenaline from high concentrations. The observations made support the assumption that an increase of transmitter liberation rather than inhibition of transmitter inactivation is responsible for the increase caused by angiotensin in the output of noradrenaline during stimulation of sympathetic nerves.

Noradrenaline is released into the perfusion medium during stimulation of the postganglionic sympathetic nerves of rabbit isolated hearts (Huković & Muscholl, 1962). This outflow of noradrenaline is augmented if angiotensin is infused into the aortic cannula (Starke, Werner & Schümann, 1969). A similar increase of amine output has been observed in the dog paw and kidney (Zimmerman & Whitmore, 1967; Zimmerman & Gisslen, 1968) but at higher concentrations of angiotensin.

Noradrenaline released from the sympathetic nerve terminals is largely inactivated by re-uptake through the neuronal membrane (Iversen, 1967). The same mechanism is mainly responsible for the removal of noradrenaline from fluid perfusing an isolated organ. Cocaine, which increases the output of noradrenaline during sympathetic stimulation by interference with re-uptake, also prevents removal of noradrenaline from the perfusion fluid (Huković & Muscholl, 1962; Lindmar & Muscholl, 1964). High concentrations of angiotensin diminish the uptake of [^{3}H]noradrenaline into several organs (e.g. Palaić & Khairallah, 1967a). If the peptide augments the output of noradrenaline induced by sympathetic nerve stimulation by inhibition of re-uptake, it should also reduce the amount of noradrenaline removed from the perfusion fluid.

We tested this possibility and examined additionally the influence of angiotensin on the net-uptake of noradrenaline into rabbit isolated hearts at high concentrations of noradrenaline.

EXPERIMENTAL

Rabbits of either sex (89), 1·5-2·0 kg, were killed by a blow on the head. The hearts were immediately removed and perfused at 34° or 37° and at a constant flow rate of 10 or 25 ml/min (roller pump Desaga, Heidelberg). By means of a four-way stop-cock, several physiological salt solutions (PSS) could be perfused successively. Modified Tyrode solution (Starke & others, 1969) or Krebs solution (Peach, Bumpus & Khairallah, 1969) were used. Ascorbic acid and disodium-EDTA 10 mg/litre were always added, and the solution was saturated with a mixture of 5% carbon dioxide in oxygen. Contractions were monitored by means of a strain gauge connected to a Hellige multiscriptor, diastolic tension being adjusted to 2 g. Experiments were started after 45 or 120 min perfusion with normal PSS.

To estimate the removal of noradrenaline from the perfusion medium, the hearts were perfused with PSS containing 10 ng/ml (—)-noradrenaline. Four 4 min samples (if the perfusion rate was 10 ml/min) or 2 min samples (if the perfusion rate was 25 ml/min) of the venous effluent were successively collected, starting 10 s or 2 min after the onset of noradrenaline perfusion, respectively. The samples were acidified and analysed for noradrenaline.

The uptake of noradrenaline at high concentrations was measured in hearts perfused with Tyrode solution at 25 ml/min 125 or 500 μg/min (—)-noradrenaline were infused into the aortic cannula (infusion apparatus Unita, Braun, Melsungen), giving final concentrations of 5 or 20 μg/ml, respectively. Perfusion with 20 μg/ml was carried out for 10 min, followed by a 2 min washout, perfusion at 5 μg/ml for 5 min, without a final wash-out. The hearts were then removed from the apparatus, rinsed with PSS, blotted, weighed, and homogenized by means of an Ultra-Turrax (Janke + Kunkel, Staufen) in 20 ml 0·4N $HClO_4$ containing 0·1% EDTA and ascorbic acid. After centrifugation, the residue was once more extracted and the final volume made up to 50 ml. 10 ml aliquots were analysed for noradrenaline.

The catecholamines were adsorbed on Al_2O_3 (Aluminiumoxid basisch, Woelm, Eschwege) by stirring at pH 8·5. After elution with 0·1N HCl, noradrenaline was determined fluorimetrically as described by Euler & Floding (1956) and Palmer (1964). The recovery from alumina was tested for each series of samples adsorbed on one occasion by adding appropriate amounts of noradrenaline to perfusates or extracts. Values are corrected for the corresponding recovery (mean: 69·4 ± 0·8%; N = 54). Recovery of 40 μg noradrenaline added before homogenization of hearts was 85·0 ± 6·3%; N = 3).

Drugs. Val^5-angiotensin II-Asp^1-β-amide (Hypertensin, Ciba AG, Basel); (—)-noradrenaline base (Farbwerke Hoechst AG, Frankfurt/M.); cocaine hydrochloride (Merck AG, Darmstadt); (±)-metanephrine hydrochloride (Calbiochem AG, Luzern). Doses refer to the bases.

Mean values ± s.e. are given throughout. Student's *t*-test was used to calculate significance. n = number of experiments.

RESULTS

Noradrenaline elimination

Nearly equal amounts of noradrenaline were recovered from the four venous effluent samples collected in each experiment. The four values were averaged to calculate the percentage of noradrenaline removed during passage through the coron-

ary vessels and, further, the rate of elimination, expressed as ng noradrenaline/heart min^{-1} and ng noradrenaline/g heart min^{-1}.

Data are presented in Table 1. At a perfusion rate of 25 ml/min, the control hearts removed 41·1% of the perfused noradrenaline; 66·1% was removed at the lower

Table 1. *Effect of angiotensin and cocaine on the removal of noradrenaline* (10 *ng/ml*) *from the perfusion medium by isolated rabbit hearts*

Drug concentration ng/ml	Noradrenaline removal % ± s.e.	Rate of noradrenaline removal ng/heart min^{-1} ± s.e.	Rate of noradrenaline removal ng/g heart min^{-1} ± s.e.	n
	Perfusion rate 25 ml/min, Tyrode, 34°			
Controls	41·1 ± 1·3	103·0 ± 3·2	23·4 ± 1·2	12
Angiotensin				
0·013	44·8 ± 2·2	112·1 ± 5·4	24·1 ± 1·4	5
0·13	41·5 ± 0·9	104·0 ± 2·3	23·3 ± 1·7	6
1·3	38·0 ± 2·2	95·1 ± 5·4	23·4 ± 1·0	5
13·0	39·5 ± 2·3	99·0 ± 5·7	20·7 ± 1·8	6
130·0	39·9 ± 2·2	100·0 ± 5·5	22·3 ± 2·1	6
13 000·0	33·2 ± 3·0†	83·1 ± 7·6†	18·5 ± 1·4†	5
Cocaine				
10 000·0	12·3 ± 3·6‡	30·8 ± 9·0‡	5·5 ± 1·3‡	3
	Perfusion rate 10 ml/min, Tyrode, 34°			
Controls	66·1 ± 1·1	66·1 ± 1·1	17·1 ± 1·9	3
Angiotensin				
2·0	66·1 ± 2·3	66·1 ± 2·3	15·2 ± 1·3	4
	Perfusion rate 10 ml/min, Krebs, 37°			
Controls	69·2 ± 1·7	69·2 ± 1·7	14·6 ± 1·1	6
Angiotensin				
2·0	69·4 ± 3·6	69·4 ± 3·6	13·4 ± 1·0	3
Cocaine				
10 000·0	9·0 ± 4·0‡	9·0 ± 4·0‡	2·1 ± 1·1‡	3

Experiments with Tyrode solution: 45 min perfusion with PSS; 8 min perfusion with angiotensin or cocaine; thereafter with angiotensin or cocaine + noradrenaline. Experiments with Krebs solution: 120 min perfusion with PSS; thereafter simultaneous perfusion with angiotensin or cocaine + noradrenaline.

* The amount eliminated expressed as per cent of the amount infused.

† Significantly different from corresponding controls ($P<0·05$).

‡ Significantly different from corresponding controls ($P<0·001$).

perfusion rate of 10 ml/min (Tyrode solution, 34°). The two values are significantly different (P <0·001). The total amount removed per g heart per min was, however, greater at the higher coronary flow (P <0·001). There was no significant difference caused by substition of Tyrode solution at 34° by Krebs solutions at 37°, at equivalent flow rates. Cocaine (10 μg/ml) reduced the loss of noradrenaline from the perfusate by 70% (perfusion rate 25 ml/min) or 87% (perfusion rate 10 ml/min). Angiotensin caused a much smaller but significant inhibition (19%) only at a concentration of 13 μg/ml.

Angiotensin and cocaine were also compared for their influence on the positive inotropic effect of noradrenaline. In hearts perfused at a rate of 25 ml/min, the peak systolic tension developed was 7·1 ± 0·3 g (n = 43). The increase in the strength of contraction caused by 10 ng/ml noradrenaline was 2·4 ± 0·5 g (n = 10). In the presence of cocaine, the effect of noradrenaline was increased (11·0 ± 0·1 g; P <0·001, n = 3). Angiotensin did not influence the inotropic action of noradrenaline (2·3 ± 0·3 g; n = 5) even in the very high concentrations necessary to reduce the removal of noradrenaline from the perfusion fluid.

Noradrenaline uptake

The mean noradrenaline content of 12 hearts perfused with Tyrode solution for 45 min was 1123 ± 99 (range, 606 to 1918) ng/g. The mean endogenous content was subtracted from the total amount found in hearts perfused with noradrenaline at high concentrations. In the experiments with 5 μg/ml, moreover, extracellular noradrenaline was not washed out at the end of the infusion. Extracellular space of rat hearts is 325 μl/g (Iversen, 1965); we used this value for correction.

The results are presented in Table 2. Neither metanephrine (5 μg/ml) nor angiotensin (130 ng/ml) significantly influenced the uptake at 5 μg noradrenaline/ml. After 10 min perfusion with 20 μg/ml noradrenaline and 2 min wash-out, the amount taken up and retained did not significantly differ in control hearts and those infused with metanephrine (20 μg/ml) or angiotensin (130 ng/ml).

Table 2. *Effect of metanephrine and angiotensin on the uptake of noradrenaline by the rabbit isolated heart*

Noradrenaline concentration	Drug	Noradrenaline uptake (ng/g ± s.e.)	n
	—	1708 ± 222	6
5 μg/ml*	Metanephrine 5 μg/ml	1318 ± 307	3
	Angiotensin 130 ng/ml	2166 ± 680	3
	—	3738 ± 133	8
20 μg/ml†	Metanephrine 20 μg/ml	3327 ± 588	3
	Angiotensin 130 ng/ml	3409 ± 249	6

Infusion of the drugs tested for inhibition of uptake started 8 min before noradrenaline infusion.

* Noradrenaline infused for 5 min. Values corrected for endogenous noradrenaline content and that of extracellular fluid.

† Noradrenaline infused for 10 min, followed by a 2 min wash-out. Values corrected for endogenous noradrenaline content.

DISCUSSION

The output of noradrenaline from isolated rabbit hearts during sympathetic nerve stimulation is enhanced by angiotensin (Starke & others, 1969). If the coronary flow is kept constant, significant augmentation is obtained by 130 pg/ml, and maximal effects by 1·3 ng/ml. In these concentrations, angiotensin is without any effect of the removal of infused noradrenaline from the perfusion medium. The ratio of the dose producing a small inhibition of removal of noradrenaline from the perfusion fluid (13 μg/ml) and that augmenting the output of noradrenaline on sympathetic stimulation is 10^5. On the other hand, cocaine, a typical blocker of noradrenaline uptake across the neuronal membrane, greatly reduced the removal of noradrenaline from the perfusion fluid in concentrations which enhance the output of noradrenaline during sympathetic nerve stimulation (10 μg/ml; Huković & Muscholl, 1962). It seems unlikely, therefore, that the cocaine-like action observed with excessive concentrations of angiotensin is related to the increased output of noradrenaline during sympathetic stimulation which is caused by low concentrations of angiotensin.

Recently, Peach & others (1969) demonstrated that the uptake of (±)-[^{3}H]noradrenaline into isolated perfused rabbit hearts is reduced by angiotensin; 0·05 ng/ml

caused a diminution by 50%, 2·0 ng/ml by 80%. As the elimination of infused noradrenaline from the perfusion medium is mainly the result of net-uptake into the heart (Lindmar & Muscholl, 1964), inhibition of net-uptake should be accompanied by the appearance of a greater amount of noradrenaline in the venous effluent. In hearts perfused with Tyrode solution at a rate of 25 ml/min, we did not find an effect on the removal of perfused noradrenaline by angiotensin even with doses of angiotensin much higher than those of Peach & others (1969). Moreover, the experimental conditions used were similar to those of these authors (Krebs solution, 37°, 10 ml/min, 2 h perfusion with PSS before the administration of drugs, simultaneous addition of 2 ng/ml angiotensin and 10 ng/ml noradrenaline). Cocaine, on the other hand, always caused a clearcut reduction of noradrenaline elimination.

The accumulation of labelled noradrenaline may be diminished, without a simultaneous increase of the amount of fluorimetrically determined noradrenaline in the perfusate, by several mechanisms. In the experiments of Peach & others (1969), (±)-[^{3}H]noradrenaline was diluted with unlabelled (—)-noradrenaline. Angiotensin might specifically inhibit the uptake of the (+)-isomer. This would result in a great decrease of the accumulation of labelled amine in the heart, but only a comparatively small increase of fluorimetrically determined noradrenaline in the venous effluent. It is not very likely, however, that the above-mentioned reduction of [^{3}H]noradrenaline uptake by 80% can be explained in this way (cf. Draskóczy & Trendelenburg, 1968).

Secondly, angiotensin might accelerate the metabolism of noradrenaline. The percentage of total myocardial radioactivity present as metabolites was not changed by the peptide (Peach & others, 1969). Apparently, metabolic products were not determined in the perfusate. That portion of noradrenaline which, in the presence of angiotensin, is neither retained in the heart nor recovered from the perfusate, may be contained in the effluent as metabolites.

Finally, the accumulation of [^{3}H]noradrenaline in the heart is the result not only of net-uptake, but also of an exchange with the endogenous stores. Exchange with endogenous noradrenaline is slow in rat hearts (Iversen, 1963), but it may be more rapid in rabbit hearts. Angiotensin might inhibit the exchange process without appreciably diminishing net-uptake. This would reduce the accumulation of [^{3}H]noradrenaline in the heart, leaving the amount of chemically determined noradrenaline in the venous effluent unaffected. Single and combined, these mechanisms may help to explain the results of Peach & others (1969) as well as our own.

In earlier reports, large doses of angiotensin were needed to demonstrate an inhibition of the uptake of [^{3}H]noradrenaline (Panisset & Bourdois, 1968; Palaić & Khairallah, 1967a,b). Angiotensin (1 ng/ml) did not inhibit the elimination of (—)-noradrenaline from the perfusion medium by the cat spleen (Thoenen, Huerlimann & Haefely, 1965); intravenous infusion into pithed rats at a rate of 30 ng/kg min^{-1} did not influence the uptake of (±)-[^{3}H]noradrenaline by several organs (Pals & Masucci, 1968).

The drug sensitivity of the uptake of noradrenaline at very high concentrations ($Uptake_2$) differs from that of $Uptake_1$ (Iversen, 1965). Therefore, we examined the influence of angiotensin and metanephrine on $Uptake_2$. Metanephrine is a potent blocker of $Uptake_2$ in the rat heart (Iversen, 1965). In our experiments, the accumulation of noradrenaline was only insignificantly reduced. It is known that metanephrine does not uniformly decrease the uptake of noradrenaline; it increases for

instance the uptake into the submaxillary gland of rats *in vivo* (Iversen, Fischer & Axelrod, 1966). The uptake of noradrenaline was unaffected by 130 ng/ml of angiotensin, i.e. 10^3 of the dose increasing the output during sympathetic nerve stimulation. Inhibition of an Uptake$_2$-like mechanism, therefore, does not seem to be responsible for the potentiating effect of angiotensin on noradrenaline output.

Zimmerman & Gisslen (1968), comparing the effects of angiotensin and cocaine, concluded that the peptide did not increase noradrenaline outflow by a cocaine-like mechanism. They assumed that the amount of noradrenaline liberated per nerve impulse might be augmented. Our results support their conclusion. At high concentrations of noradrenaline, net-uptake is unaffected by angiotensin. At low concentrations of noradrenaline, a cocaine-like effect can be obtained by very high doses of angiotensin, but it is absent at the low doses which increase the output of transmitter during sympathetic nerve stimulation.

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The inhibitory effect of sulphonylurea derivatives on liver glycogenolysis increased by catecholamines

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Hypoglycaemic sulphonylureas inhibit the increased glycogenolysis and glucose release of the rat perfused liver produced by β-adrenergic agonists. The β-receptor antagonist propranolol exerted a similar effect. Dichloroisoprenaline increases the hypoglycaemic effect of chlorpropamide given in doses ineffective in the intact rat. Hypoglycaemic sulphonylureas have no effect on the catecholamine sensitivity of the nictitating membrane and heart auricle of the cat, indicating that their ability to inhibit glycogenolysis induced by β-receptor agonists is not due to a β-receptor antagonist activity as is the action of propranolol.

Previously reported experiments from our laboratory have demonstrated that the rate of glucose release from the isolated perfused liver of rats is stimulated by adrenergic β-receptor agonists and that this effect is abolished by specific β-receptor antagonists (Vizi & Pogátsa, 1965; Vizi, Pogátsa & Káldor, 1965). Similarly perfusion of the liver with a glucose-free Tyrode solution augmented both glucose release and glycogenolysis and these actions were also inhibited by β-receptor blocking drugs. It is well known that one of the principal characteristics of the hypoglycaemic sulphonylureas is to decrease hepatic glucose release and glycogenolysis (Purnell, Arai & others, 1956; Ashmore, Cahill & others, 1958; Pogátsa & Káldor, 1965). Chrusciel, Janiec & Brus (1964) found the sulphonylurea derivative, chlorpropamide, to antagonize isoprenaline's action on the blood pressure of cat and in relaxing the cat uterus. On the other hand, according to de Divitiis, Giordano & others (1968) propranolol decreases the hypoglycaemic effect of tolbutamide in healthy subjects. Propranolol also increases the toxicity of insulin in the rat (Byers & Friedman, 1966) and delays the compensatory response to hypoglycaemia in man (Abramson, Arky & Woeber,1966) presumably due to antagonism of increased amounts of circulating adrenaline (Goldfien, Zileli & others, 1958). However, Calvey & Summerill (1968) observed in rabbits, that propranol decreased resting plasma glucose, augmented the effects of insulin, and delayed the compensatory response to hypoglycaemia. It seemed worthwhile to examine whether the sulphonylureas have any influence on the effect of isoprenaline or adrenaline on the carbohydrate metabolism of the liver, and whether the β-adrenergic blocking effect of the sulphonylureas can be unequivocally established or not.

EXPERIMENTAL

Methods

Isolated liver perfusion was by a modification (Vizi & others, 1965) of the method of Issekutz (1924). To examine the possible effect of β-adrenergic blockade on the

hypoglycaemic effect of sulphonylurea compounds, albino rats of either sex, 150–200 g, fasting for 8 h were given dichloroisoprenaline (7 mg/kg) and chlorpropamide (20 mg/kg) intraperitoneally. Blood samples for glucose determination (Hagedorn & Jensen, 1923) were taken from the tail vein of the animals before the administration of the compounds and after 1, 2 and 3 h.

The possible β-adrenergic blocking effect of the sulphonylureas was determined by the method of Smith (1963) and György, Molnár & Dóda (1965) on the nictitating membrane of the cat and on cat auricle suspended in Tyrode solution. The auricles were placed in 10 ml of Tyrode solution at 31°. The left auricle preparations were stimulated with a frequency of 120/min using square waves at twice the threshold voltage. For right auricle preparations the increases in heart rate caused by isoprenaline were recorded as well as the isotonic myocardium contractions. We wanted to know whether or not the sulphonylureas had any influence on the positive inotropic and chronotropic effect of $2{\cdot}85 \times 10^{-8}$ M isoprenaline. The compounds in 0·2 ml volume were placed in the organ bath to give a bath concentration of $3{\cdot}69 \times 10^{-3}$ M 3–10 min before the administration of isoprenaline which was administered at 20–25 min intervals. In these experiments cats of either sex, 2·5–3 kg were used.

Statistical significance was analysed by Student's *t*-test.

Drugs. Adrenaline tartrate (Burroughs Wellcome). (−)-Isoprenaline bitartrate (Isolevin, Cilag-Chemie). Propranolol hydrochloride (Inderal, ICI). Dichloroisoprenaline hydrochloride (DCI, Boehringer). Chlorpropamide (Diabinese, Pfizer). Carbutamide (Nadisan, Boehringer). N_1-Sulphanilyl-N_2-methoxypropyl-carbamide (AH 6848, Boehringer). Insulin (Gedeon Richter). The concentrations are expressed in molar terms.

RESULTS

The rate of glucose release from perfused rat liver was significantly potentiated by removing glucose from the perfusing Tyrode solution. Similarly, there was a greater fall in hepatic glycogen content. When carbutamide or chlorpropamide, 1·85 ×

Table 1. *Perfusion of the isolated liver of the rat. Alterations of glucose release, glycogen concentration into normal Tyrode solution or into glucose-free Tyrode solution, after the administration of various concentrations of carbutamide, chlorpropamide, AH 6848 compound or insulin in glucose-free solution*

		mg glucose/g wet liver h^{-1} (mean ± s.e.) at times (min)		Change of glycogen content
		30	120	%
Tyrode solution	(10)	10·2 ± 1·5	4·2 ± 0·9	−49 ± 9
Glucose-free Tyrode solution	(10)	18·4 ± 2·3[1]	6·7 ± 1·4	−76 ± 4[1]
Carbutamide, $1{\cdot}86 \times 10^{-3}$ M	(10)	16·1 ± 2·2	7·6 ± 0·9	−66 ± 5
Carbutamide, $3{\cdot}72 \times 10^{-3}$ M	(10)	11·1 ± 1·1[3]	3·1 ± 0·9[2]	−58 ± 4[3]
Chlorpropamide, $1{\cdot}86 \times 10^{-3}$ M	(10)	14·8 ± 1·9	6·9 ± 1·0	−64 ± 6
Chlorpropamide, $3{\cdot}72 \times 10^{-3}$ M	(10)	9·3 ± 1·6[3]	4·7 ± 0·9[2]	−61 ± 6[2]
AH 6848 comp., $3{\cdot}48 \times 10^{-3}$ M	(6)	17·3 ± 1·5	8·5 ± 1·3	−79 ± 5
Insulin 0·02 U/100 ml	(10)	16·1 ± 3·0	7·8 ± 1·8	
Insulin 0·4 U/100 ml	(7)	6·2 ± 2·8[3]	1·4 ± 1·6[2]	

Carbutamide, chlorpropamide, AH 6848 compound and insulin were each given at the beginning of perfusion. Number in brackets indicate the number of perfusions. The significance of the corresponding solutions with respect to the normal Tyrode $^1P < 0{\cdot}02$. The significance of the corresponding solutions with respect to the glucose-free Tyrode $^2P < 0{\cdot}05$; $^3P < 0{\cdot}01$.

10^{-3} M, were added to glucose-free Tyrode there was no significant change in glucose loss although at twice this concentration both measurements were significantly reduced (Table 1). The chemically related AH 6848 which is devoid of hypoglycaemic activity, was ineffective. Insulin markedly inhibited the loss of glucose.

In the presence of glucose (in Tyrode solution), both carbutamide and chlorpropamide significantly antagonized the glucose release caused by isoprenaline. Propranolol and insulin were also effective in preventing glucose release, so much so that there appeared to be a net uptake of glucose from the perfusate into the liver. AH 6848 was again inactive. Qualitatively similar results were obtained when adrenaline was substituted for isoprenaline (Table 2).

Table 2. *Perfusion of the isolated liver of the rat. Alterations of glucose release and glycogen concentration after the administration of isoprenaline, adrenaline and various concentrations of carbutamide, chlorpropamide, AH 6848 compound, insulin and propranolol in normal Tyrode solution*

		mg glucose/g wet liver h^{-1} (mean ± s.e.) at times (min)		Change of glycogen content
		30	120	%
Tyrode solution	(10)	10·2 ± 1·5	4·2 ± 0·9	—49 ± 9
(—)-Isoprenaline, 5·7 × 10^{-8} M	(10)	11·9 ± 1·8	10·9 ± 2·3[1]	—89 ± 3[3]
Carbutamide, 0·37 × 10^{-3} M and (—)-isoprenaline	(10)	10·2 ± 0·9	4·2 ± 0·5[5]	—62 ± 11[4]
Carbutamide, 3·72 × 10^{-3} M and (—)-isoprenaline	(10)	3·6 ± 4·4[4]	1·1 ± 1·7[6]	—48 ± 7[7]
Chlorpropamide, 0·37 × 10^{-3} M and (—)-isoprenaline	(10)	9·5 ± 1·3	5·0 ± 0·8[4]	—66 ± 6[6]
Chlorpropamide, 3·72 × 10^{-3} M and (—)-isoprenaline	(10)	8·6 ± 1·4	2·8 ± 1·2[5]	—60 ± 9[6]
AH 6848 comp., 3·48 × 10^{-3} M and (—)-isoprenaline	(10)	11·5 ± 1·5	11·1 ± 1·5	—80 ± 3
Insulin, 0·02 U/100 ml and (—)-isoprenaline	(10)	13·2 ± 2·9	15·6 ± 2·3	—59 ± 5[6]
Insulin, 0·4 U/100 ml and (—)-isoprenaline	(11)	9·2 ± 2·6	—2·7 ± 1·7[7]	—10 ± 24[6]
Propranolol, 2·38 × 10^{-5} M and (—)-isoprenaline	(10)	8·0 ± 3·4	—1·9 ± 1·2[7]	—54 ± 35[6]
Adrenaline, 6·6 × 10^{-7} M	(10)	11·1 ± 2·5	9·9 ± 1·4[1]	—84 ± 5[2]
Carbutamide, 0·37 × 10^{-3} M and adrenaline	(10)	13·3 ± 0·9	0·9 ± 0·7[9]	—37 ± 4[9]
Carbutamide, 3·72 × 10^{-3} M and adrenaline	(10)	3·7 ± 0·7[8]	1·3 ± 0·3[9]	—49 ± 6[9]
AH 6848, 3·48 × 10^{-3} M and adrenaline	(6)	10·9 ± 0·7	10·0 ± 0·7	—82 ± 5
Insulin, 0·02 U/100 ml and adrenaline	(6)	16·3 ± 2·3	14·3 ± 2·9	—57 ± 9
Insulin, 0·4 U/100 ml and adrenaline	(6)	9·6 ± 5·2	0·5 ± 0·6[9]	—23 ± 18[8]

(—)-Isoprenaline, 5·7 × 10^{-8} M and adrenaline, 6·6 × 10^{-7} M were each given 30 min after the beginning of the perfusion and carbutamide, chlorpropamide AH 6848, insulin and propranolol were each given at the beginning of the perfusion. Numbers in brackets indicate the number of perfusions. The significance of the corresponding solutions with respect to normal Tyrode [1]$P < 0·02$; [2]$P < 0·01$; [3]$P < 0·001$. Significance of the corresponding solutions with respect to normal Tyrode containing isoprenaline is: [4]$P < 0·05$; [5]$P < 0·02$; [6]$P < 0·01$; [7]$P < 0·001$. The significance of the corresponding solution with respect to normal Tyrode containing adrenaline is: [8]$P < 0·02$; [9]$P < 0·001$.

The dilator effect of isoprenaline on the nictitating membrane, contracted by amphetamine, was not antagonized by chlorpropamide or AH 6848 (Table 3).

Stimulation of cardiac frequency and contractile force was blocked by propranolol and dichloroisoprenaline but not by chlorpropamide or carbutamide (Table 4).

Chlorpropamide (20 mg/kg) and dichloroisoprenaline (7 mg/kg) have themselves no effect on the blood glucose level. On simultaneous administration, however, there was definite decrease of the blood sugar in the first and second hours (Fig. 1).

Table 3. *The effect of chlorpropamide, AH 6848, dichloroisoprenaline and propranolol on the (—)-isoprenaline induced relaxation of the nictitating membrane of the cat contracted by amphetamine* (5 mg/kg., i.v.)

		% inhibition of relaxation induced by (—)-isoprenaline
(—)-Isoprenaline, 30 μg/kg		0
Chlorpropamide, 50 mg/kg	(4)	5 *$P < 0{\cdot}05$
AH 6848, 50 mg/kg	(3)	7 *$P < 0{\cdot}02$
Dichloroisoprenaline, 4 mg/kg	(8)	93 *$P < 0{\cdot}01$
Propranolol, 0·7 mg/kg	(5)	94 *$P < 0{\cdot}001$

Numbers in brackets indicate the number of experiments.
*Significance of the corresponding compound with respect to (—)-isoprenaline.

Table 4. *The effect of hypoglycaemic sulphonylureas on the (—)-isoprenaline induced changes of frequency and increase of isotonic muscular contraction of the heart*

		Changes in frequency (beats/min)	Increase of muscular contraction (mm)
(—)-Isoprenaline, $2{\cdot}85 \times 10^{-8}$ M	(12)	49 ± 12	19 ± 4
Carbutamide, $3{\cdot}72 \times 10^{-3}$ M and (—)-isoprenaline	(12)	45 ± 11	21 ± 5
(—)-Isoprenaline, $2{\cdot}85 \times 10^{-8}$ M	(12)	42 ± 3	19 ± 2
Chlorpropamide, $3{\cdot}72 \times 10^{-3}$ M and (—)-isoprenaline	(12)	38 ± 3	17 ± 1

Numbers in brackets indicate numbers of experiments. Recording of alterations of frequency was made only in half of these cases. There is no significant difference among the groups.

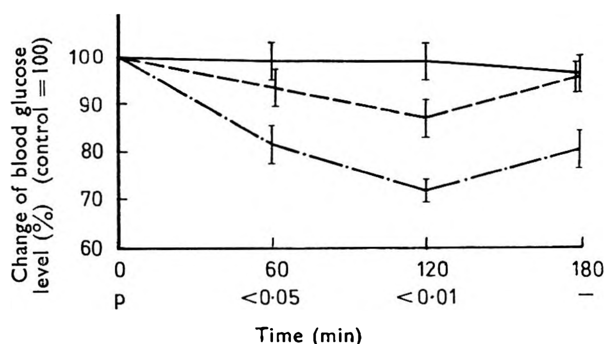

FIG. 1. The effect of dichloroisoprenaline and chlorpropamide on the blood glucose level of the intact rat. Each curve represents the mean values of ten rats. Vertical bars represent the standard error of mean. *P* represents the significance between chlorpropamide and chlorpropamide + dichloroisoprenaline. Dichloroisoprenaline (7 mg/kg, i.p.) ——. Chlorpropamide (20 mg/kg, i.p.) – – –. Dichloroisoprenaline (7 mg/kg) + chlopropamide (20 mg/kg) —·—.

DISCUSSION

The results of the present studies demonstrate that two sulphonylureas, with hypoglycaemic activity, exert an antagonistic action against liver glycogenolysis and glucose release stimulated by β-adrenergic agonists. This property was also exhibited by insulin and propranolol. Another sulphonylurea without hypoglycaemic activity was not effective in this context. In our experiments, we were unable to demonstrate any β-blocking properties of the hypoglycaemic sulphonylureas either by actions on the nictitating membrane or hearts of cats. The effectiveness of propranolol confirms the results we obtained earlier with dichloroisoprenaline and pronethalol (Vizi & others, 1965) in suggesting that the response to isoprenaline is mediated by β-receptors. It may be argued that the mode of action of these β-receptor antagonists is specific β-blockade, although they also possess direct actions on cell membranes at concentrations similar to those used in this study (Lucchesi & Whitsitt, 1969). It also may be assumed that the local anaesthetic effect of the β-receptor blocking agents have a role in the phenomenon observed. Therefore we studied the effect of practalol [4-(2-hydroxy-3-isopropylaminopropoxy) acetanilide; ICI 50 172) which is devoid of local anaesthetic effect (Fitzgerald, 1969). Practalol, $4{\cdot}04 \times 10^{-4}$ M, failed to influence the glucose release augmented by glucose-free Tyrode solution or by isoprenaline ($5{\cdot}7 \times 10^{-8}$) neither was the decrease of glycogen level affected. But these observations did not throw any further light on the mechanism of the β-blocking agents studied because practalol is a very weak antagonist of isoprenaline (Dunlop & Schanks, 1968).

Failure to demonstrate peripheral β-blocking actions with sulphonylureas suggests that their mode of action in the liver differs from that of the β-blockers, in preventing catecholamine induced glycogenolysis.

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Acid gastric secretory responses to histamine, crude porcine gastrin and pentagastrin in rats

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The log dose-response curves for graded doses of the secretagogues porcine gastrin (a partially purified sample; the crude and its gastrin II equivalent), histamine, and a gastrin pentapeptide (pentagastrin) on the perfused stomachs of urethanized rats are parallel. On weight basis, pentagastrin is 60 times and histamine four times more active than the crude porcine gastrin preparation. The partially purified porcine gastrin sample is six times more potent than histamine but half as potent as pentagastrin. On molar basis gastrin (as the pure porcine gastrin II) has 3000 times the activity of histamine dihydrochloride and 5000 times that of the histamine base. Gastrin is 50 times more potent than pentagastrin. Gastrin and pentagastrin are more potent and have less undesirable side-effects than histamine.

The two most important endogenous substances that control acid gastric secretion are histamine and gastrin (Amure & Ginsburg, 1964) Secretory activity in human stomach and in experimental animals is routinely tested with histamine, preceded by an antihistamine (Kay, 1953) or its pyrazole isomer (histalog) with or without antihistamine drug (Rosiere & Grossman, 1951). In spite of this, the use of histamine has its limitations and is not without dangers. The aim of the present work therefore is to compare the efficacy of the three substances, namely, histamine, crude and standardized gastrin and pentagastrin (ICI 50 123) on acid gastric secretion in rats.

EXPERIMENTAL

Materials. Histamine dihydrochloride (L. Light & Co.); pentagastrin (ICI 50 123) ICI Ltd; Crude gastrin (hog gastrin). Standardized gastrin (gift from Prof. M. Ginsburg, Chelsea College, London).

Methods. Male albino Wistar rats, 190 to 240 g, were given sugar lumps (cane sugar) instead of their normal food pellets, 24 h before the start of the experiments. This made the cleaning of the stomachs easy for the perfusion experiments. Water was given freely. Anaesthesia was induced with urethane (0·6 ml/100 g weight of a 25% w/v solution) given intramuscularly. This dose maintained a satisfactory and uniform anaesthesia for up to 13 h. Stomachs for perfusion and the continuous recording of acid secretion were prepared according to Ghosh & Schild (1958),using 0·00025–0·001N NaOH as the perfusion fluid. This fluid acted as an approximate linear buffer from pH 8·5–4·0 and pH 7·0–3·9 in 2 rats. The response metameter was the maximum fall in the pH of the gastric effluent fluid after injection. All injections were intravenous through a cannulated femoral vein in volumes of 0·05 to 0·2 ml and the cannula washed with 0·1 ml of 0·9% saline, after each injection.

Histamine doses refer to its salt in μg. Doses of pentagastrin and crude gastrin are also expressed in μg. The crude gastrin was the water soluble extract from hog antral mucosa (Blair, Harper & others, 1961). The standardized gastrin was a

partially purified porcine gastrin previously assayed by Prof. M. Ginsburg against the pure gastrin II. A laboratory unit of it is equivalent to 2 μg by weight and on assay was found to be equivalent to 50 ng of the pure gastrin II.

To facilitate comparison of the various secretagogues a standard procedure consisting of high and low doses of the standard, and high and low doses of the test drugs in randomized order was adopted. The response metameters are plotted against log dose of each secretagogue for graded doses in 4 rats as shown in Fig. 1. All the doses used were from the linear portion of the log dose-response curves. The responses for potency comparison were required to operate closely both in intensity and duration. The crude porcine gastrin extracts were assayed against the standardized gastrin.

The histamine content of the crude gastrin extracts assayed on guinea-pig ileum (Adam, Hardwick & Spencer, 1954) was less than 0·05 μg/mg of crude extract, an amount that does not interfere with acid secretory responses elicited by crude gastrin.

RESULTS

The acid secretory responses to successive injections of the same dose of a secretagogue increased up to the third or fourth dose before a steady response was reached when little or no alteration in sensitivity of the rats to the secretagogues was detected in experiments lasting over 9 h. Assays were begun when the steady state was reached. Responses to graded doses of the secretagogues are shown in Table 1.

Table 1. *Responses to graded doses of standardized porcine gastrin, crude porcine gastrin, pentagastrin and histamine dihydrochloride*

Stimulant	Dose (μg)	pH change (mean and standard deviation)	No. of rats used
Standardized porcine gastrin	10	2·40 ± 0·1	(10)
	20	2·75 ± 0·15	(12)
	30	2·90 ± 0·1	(8)
Crude porcine gastrin	200	2·0 ± 0·25	(12)
	400	2·2 ± 0·15	(10)
	800	2·4 ± 0·40	(14)
Histamine dihydrochloride	100	2·60 ± 0·25	(16)
	200	2·80 ± 0·10	(12)
	400	2·97 ± 0·20	(10)
	800	3·20 ± 0·05	(14)
Pentagastrin	6·25	2·10 ± 0·25	(13)
	12·5	2·30 ± 0·30	(15)
	25	2·60 ± 0·32	(12)

Responses to histamine and crude gastrin

Acid secretory responses to gastrin and histamine were typical and followed the course described by Ghosh & Schild (1958) and Amure & Ginsburg (1964). In most experiments, the maximum fall in pH of the gastric effluent was within 15 min of injection and the responses lasted about 30 min. Animals tolerated small doses of histamine (100 to 800 μg); but larger doses caused respiratory disturbances.

Responses to pentagastrin and crude gastrin

Acid secretory responses to pentagastrin started within 2 to 3½ min after the completion of the injection (2·72 ± 0·75 min, mean and standard deviation for 20 observa-

tions in 13 rats). The maximum fall was reached within 20 min and recovery was complete within 1 h. The time-course responses for histamine, crude gastrin and pentagastrin showed no marked differences and the pattern is comparable in all three secretagogues with low doses of histamine (100 to 200 μg); pentagastrin (6·25 to 12·5 μg) and high doses of crude gastrin (400 to 800 μg). The results are shown in Fig. 2.

The log dose-response curves for histamine, crude gastrin, and pentagastrin are judged paralleled as shown in Fig. 1 (slopes 0·60; 0·66 and 0·7). This indicates a common mode of action and thus allowed the comparison of these secretagogues.

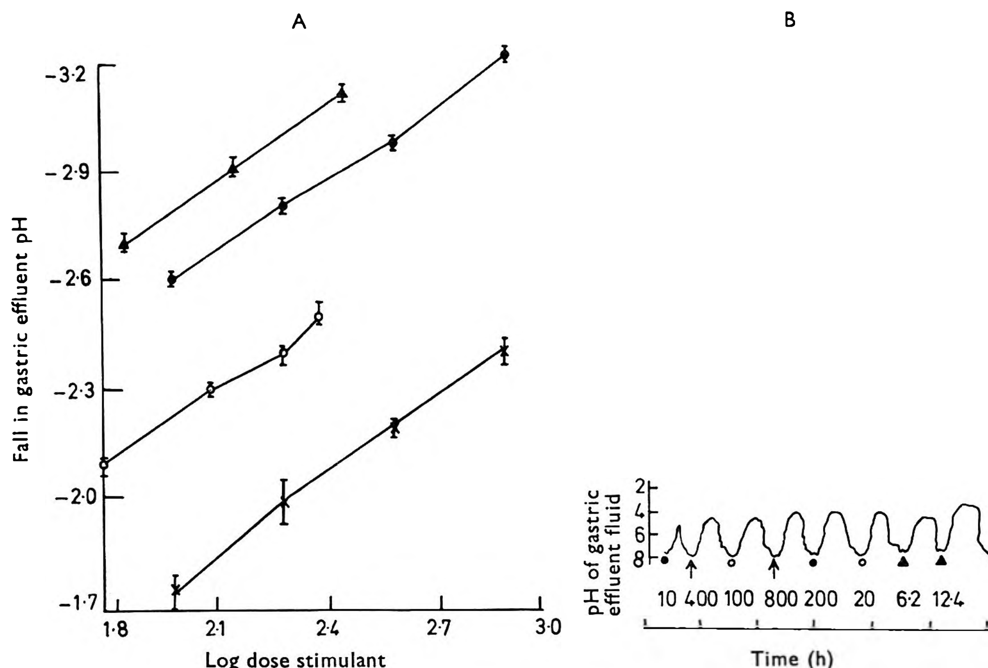

FIG. 1A. Log dose response curves for stimulants. Each point is the mean for 4 rats of maximum fall in gastric effluent pH. Vertical bars represent standard error of the mean. ▲—▲, Standardized gastrin (×10 on dose scale); ●—●, histamine dihydrochloride; ○—○, pentagastrin; (×10 on dose scale); ×—× crude porcine gastrin.

B. Effects of intravenous injection of standardized porcine gastrin (●), crude porcine gastrin (↑), histamine dihydrochloride (○) and pentagastrin (▲). The signs indicate the points of injection. Doses (μg) are shown below the signs.

On weight to weight basis, pentagastrin was found to possess about 60 times the activity of the crude porcine gastrin, while histamine (expressed as the dihydrochloride) has four times the potency of crude porcine gastrin. The standardized gastrin (partially-purified porcine gastrin) was six times more potent than histamine (95% confidence limit 5·1–6·9) but it is half as potent as pentagastrin (1·5–2·5). After conversion of the crude porcine gastrin to its pure porcine gastrin II equivalent, gastrin was found on a weight basis to be 270 times more potent than histamine (95% confidence limit 231–309) and 18 times more potent than pentagastrin (16·6–19·6). On molar basis the differences became more pronounced, when pure gastrin was found to be 3000 times more potent than histamine (95% confidence limit 2600–3500) and 50 times more potent than pentagastrin (45·1–53·5). Pentagastrin was, on weight basis, 15 times more potent than histamine (95% confidence limit 14·2–15·7 and 60 times more potent on molar basis (59–66). The index of precision of assays

calculated from two log dose–response curves for graded doses was 0·18 and 0·25. Thus the assays may be regarded as reliable.

In the doses used, pentagastrin had a slight but consistent pressor effect on rat blood pressure (4·37 ± 1·75 mm Hg rise, mean and standard deviation for 8 rats). Histamine in doses used in the assays exhibited pronounced and consistent depressor effects on blood pressure (37·7 ± 8·3 mm Hg fall in 10 rats). In rats in which simultaneous recordings of blood pressure and gastric secretion were made, the hypotensive effect of histamine started before the gastric secretory effect and the recovery of blood pressure occurred long before the recovery of the gastric secretion. Crude porcine gastrin had no consistent effect on blood pressure except occasionally when slight pressor effects occurred with large doses and these were significantly lower when compared with the depressor effects of histamine ($P < 0{\cdot}001$).

DISCUSSION

The increase in the sensitivity of the rat stomach to the injected secretagogues with the first three or more doses of an assay confirms the observation of Rosenoer & Schild (1962) for carbachol in rats. The necessity for stabilizing the sensitivity of the rat stomach before beginning an assay is thus seen. Also with histamine there was a difference in the time for the responses to develop in the 'stabilized' and 'non-stabilized' stomachs. The differences were not apparent with crude gastrin, standardized gastrin or pentagastrin. In stabilized stomachs the onset of responses to histamine is as short as those of gastrin and pentagastrin. The short latency of response to extracts containing gastrin confirms the finding of Amure & Ginsburg (1964) in rats and Makhlouf, McManus & Card (1964) in man. The over-all time course of responses to gastrin confirms in its entirety the findings of Amure & Ginsburg (1964) but only part of the confirmation of their findings and those of Ghosh & Schild (1958) were obtained for histamine.

The crude porcine gastrin was less pure than the purified gastrin, thus explaining the lower potency of the crude material compared with histamine and pentagastrin.

Barrett, Raventos & Siddal (1966) using a modification of the method of Ghosh & Schild (1958) found that gastrin possessed on molar basis 6000 times the activity of histamine base and about ten times the activity of pentagastrin. On a weight for weight basis, Hansky & Eu (1967), using subcutaneous injection in pyloric and oesophageal ligated rats, found pentagastrin seven times less potent than gastrin. We found histamine (as the dihydrochloride) to be about 3000 times less potent than gastrin on a molar basis and 5000 times less active as the histamine base. Gastrin is 50 times more potent than pentagastrin on molar basis.

The findings confirm that gastrin is the most potent of the three secretagogues.

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LETTERS TO THE EDITOR

Investigation of the mechanism of gelatin-induced *in vitro* red cell aggregation

We reported (Görög & Kovács, 1970) that *in vitro* non-steroid anti-inflammatory compounds were highly specific inhibitors of gelatin-induced aggregation of red cells of the rat. The present report is concerned with the mechanism responsible for the inhibitory effect on aggregation and its investigation using compounds with known biochemical mechanisms of action.

The applied method corresponds to that described earlier (Görög & Kovács, 1970). The buffered saline solution of the compounds to be tested was added to the saline suspension of the red cells of the rat and after incubation for 5 min at 20° (or 16 h at 4°) gelatin, 0·6% final concentration, was added to the cell suspension. Aggregation was estimated by the sedimentation rate of the column of cells (ESR).

The potency of the inhibitory effect is compared with phenylbutazone in Table 1. This shows that cell-aggregation was inhibited by fluoride at a higher concentration than that inhibiting red cell glycolysis (2–8 mM). When the red cells were left standing for 16 h in a medium containing fluoride before the addition of gelatin, the aggregation-inhibitory effect of the compound was significantly increased. It is known that after such a time lapse the adenosine triphosphate (ATP) content of the cells is reduced because of inhibited glycolysis. On the other hand, in high concentration, fluoride strongly inhibits the ATPase activity of red cells (Straub, 1952). Our results have therefore been explained by the assumption that the direct effect of fluoride is produced by the ATPase inhibitory action, while responsibility for the enhanced inhibition of cell-aggregation seen after prolonged incubation is ascribed to the reduced energy level of the cells. The red cell aggregating effect of gelatin was strongly inhibited by the two sulphydryl binding compounds, *p*-chloromercuribenzoate (PCMB) and Salyrgan. After incubation of the cells for 16 h the effect of PCMB was

Table 1. *Effect of various metabolic inhibitors on gelatin-induced* in vitro *aggregation of red cells of the rat*

Compound	IC50 (mM)* after incubation for 5 min	16 h	Relative effectiveness† (Phenylbutazone = 1·0) after incubation for 5 min	16 h
Fluoride	90	3·0	9782	319
PCMB	1	0·08	108	8·5
Salyrgan	0·49	0·12	53	12·7
DNP	0·24	0·19	26	20·2
PCP	0·045	0·042	4·8	4·4
Na-Arsenate	>30			
Na-Azide	>20			
KCN	>10			
Na-EDTA‡	35			
Na-EGTA§	16	8·0		
Phenylbutazone	0·0092	0·0094	1·0	1·0

* Concentrations producing 50% inhibition on red cell aggregation.
† The smaller the value the greater the inhibitory effect.
‡ 2 mM EDTA or EGTA produced 20 and 23% inhibition respectively.
§ In the presence of 20 mM Mg ion ($MgCl_2$).

strongly increased, while that of Salyrgan showed a moderate increase. According to Weber & Portzehl (1952), these mercuric agents inhibit the energy transfer from ATP to the actomyosin system, which explains their potent inhibitory effect on actomyosin ATPase. Of the compounds investigated, the strongest inhibitor of red cell aggregation was the pentachlorophenol (PCP), a potent uncoupling and actomyosin ATPase inhibitory compound. The inhibitory effect of 2,4-dinitrophenol (DNP) on cell aggregation was also significant. Aggregation of red cells was not inhibited by arsenate, cyanide and azide.

Our results suggest that gelatin-induced aggregation of red cells is a Ca ion dependent process. Aggregation was inhibited slightly by EDTA, but ethylene glycol bis(2-amino-ethyl) tetra-acetic acid (EGTA; Chel-De, Geigy) produced inhibition of aggregation in the presence of excess Mg ion. EGTA is a chelate-forming agent which has practically no binding to Mg ion at neutral pH and forms a specific Ca-chelate in the presence of excess Mg ion (Ebashi, Ebashi & Fujite, 1960), hence its effectiveness emphasizes the significance of the Ca ion in the process. Presumably the relatively high concentration was needed because the gelatin preparation contained fairly large amounts of Ca ion; moreover, in the red cell membrane the Ca ion is bonded at a site which is hardly accessible to the chelate-forming agents.

The results obtained point to a close similarity in the mechanism between the gelatin-induced aggregation of red cells and the aggregation of blood platelets under various influences (Mason & Saba, 1969). On the evidence of our findings the aggregation of red cells—like that of the platelets—is strongly inhibited by compounds which bind sulphydryl groups, inhibit actomyosin ATPase or have an uncoupling effect on oxidative phosphorylation. In the case of platelets, a decisive role in bringing about aggregation is ascribed to the contractile protein with ecto-ATPase activity, situated on the outer surface of the cell-membrane. Much evidence indicates that the physical and enzymatic properties of this superficial contractile protein are altered by the compounds inhibiting the aggregation of platelets (Mason & Saba, 1969). Our results indicate that gelatin-induced aggregation of red cells is an energy-requiring process in which a decisive role is played by a platelet-like Ca sensitive ecto-ATPase (Schatzmann, 1966), most probably the site of action of inhibitory compounds. Our results partly explain the resemblance to be noted between the relative inhibitory effect of non-steroid anti-inflammatory agents on red cell and platelet aggregation.

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Free fatty acid inhibition of α-glycerophosphate dehydrogenase activity in rat brain

α-Glycerophosphate dehydrogenase is known to play an important rate-limiting role in phospholipid synthesis and intracellular hydrogen ion transport (Kornberg & Pricer, 1953; Kennedy, 1953). A recent report from this laboratory demonstrated that the free fatty acid, octanoate, inhibited competitively the activity of α-glycerophosphate dehydrogenase in both adipose tissue and heart (Vijayvargiya & Singhal, 1970). We now report that octanoate produces competitive inhibition of α-glycerophosphate dehydrogenase in rat cerebral cortex and cerebellum.

Female Sprague-Dawley rats, approximately 250 g, were killed by decapitation and bled. The cerebral cortex and cerebellum were rapidly excised and supernatant fluids were obtained as described previously (Singhal, Valadares & Ling, 1967; Singhal, Valadares & Schwark, 1969). The activity of α-glycerophosphate dehydrogenase was determined in the supernatant fluid under strictly linear kinetic conditions by measuring the formation of NAD^+ from NADH in an assay system coupled with aldolase (Vijayvargiya & Singhal, 1970). Changes in extinction were recorded for a period of 5 min and enzyme activity was calculated as μmol of substrate metabolized per g of tissue per h at 37°.

The effects of various concentrations of sodium octanoate on the activity of α-glycerophosphate dehydrogenase in the cerebral cortex and cerebellum are shown in Table 1. A definite inhibition of the enzyme activity was observed with 10 mM concentration of the free fatty acid in both regions of the brain. α-Glycerophosphate dehydrogenase in cerebellum and cerebral cortex declined further when the concentration of sodium octanoate was increased and was almost completely inhibited in the presence of 80·0 mM octanoate.

Table 1. *Effect of sodium octanoate on α-glycerophosphate dehydrogenase activity in rat cerebral cortex and cerebellum.* Various concentrations of sodium octanoate (pH 7·4) were added directly to the reaction mixture just before the addition of the substrate, fructose 1,6-diphosphate. Each value is the mean ± s.e. of three determinations of enzyme activity. Values in parentheses indicate the percentages of control values which are taken as 100%.

Sodium octanoate (mM)	α-Glycerophosphate dehydrogenase activity (μmol/g h^{-1})	
	Cerebral cortex	Cerebellum
None (control)	284 ± 20 (100)	365 ± 2 (100)
2·5	245 ± 22 (86)	340 ± 3 (93)
5·0	207 ± 26 (73)	301 ± 3 (82)*
10·0	178 ± 12 (63)*	255 ± 8 (70)*
20·0	133 ± 11 (47)*	196 ± 9 (54)*
40·0	84 ± 14 (30)*	108 ± 4 (30)*
80·0	12 ± 0 (4)*	11 ± 2 (3)*

* Statistically significant difference compared with the control values ($P = <0·05$).

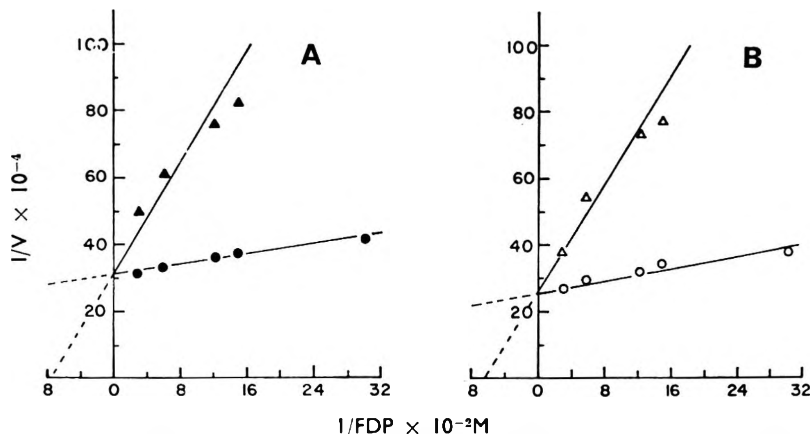

FIG. 1. Lineweaver-Burk plots showing competitive inhibition of α-glycerophosphate dehydrogenase by sodium octanoate in rat cerebral cortex (A) and cerebellum (B). The final concentration of sodium octanoate in the reaction mixture was 10·0 mM. ▲—▲, $K_m = 1{\cdot}35 \times 10^{-2}$M; ●—●, $K_m = 1{\cdot}19 \times 10^{-3}$M; △—△, $K_m = 1{\cdot}56 \times 10^{-2}$M; ○—○, $K_m = 1{\cdot}67 \times 10^{-3}$M.

The nature of octanoate-induced inhibition of α-glycerophosphate dehydrogenase was examined by determining the enzyme activity in presence of varying amounts of the substrate, fructose 1,6-diphosphate, with or without the addition of octanoate (10·0 mM). Lineweaver-Burk plots of the results obtained are shown in Fig. 1. These plots, which extrapolate to the same point on the ordinate, indicate that octanoate produces a competitive inhibition of α-glycerophosphate dehydrogenase in cerebral cortex and cerebellum. The Ki values calculated from these data were found to be $9{\cdot}7 \times 10^{-4}$M for the cerebro-cortical and $1{\cdot}2 \times 10^{-3}$M for the cerebellar enzyme.

It has been demonstrated that preincubation of supernatant fluids with free fatty acids produces marked inhibition of hepatic and cardiac pyruvate kinase as well as that of α-glycerophosphate dehydrogenase in heart and adipose tissue (Weber, Convery & others, 1966; Tsutsumi & Takenaka, 1969; Vijayvargiya & Singhal, 1970). Fig. 2 shows the effects of preincubation with octanoate on the activity of

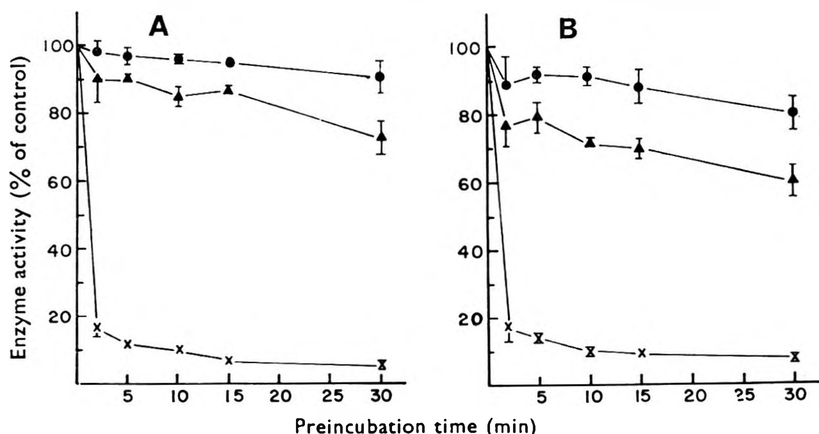

FIG. 2. Effect of preincubation of supernatant fluids with octanoate on α-glycerophosphate dehydrogenase activity in cerebral cortex (A) and cerebellum (B). 0·9 ml of the supernatant fluid was incubated at 37° for various periods of time with either 0·3 ml distilled water or sodium octanoate (1·0 or 5·0 mM). At the end of incubation, a 0·4 ml aliquot from this mixture was added to the assay system for determining enzyme activity. Each point represents the mean ± s.e. of 2 or 3 enzyme determination. ●—●, control; ▲—▲, 1 mM octanoa; ×—×, 5 mM octonoate.

α-glycerophosphate dehydrogenase in the rat cerebral cortex and cerebellum. Incubation at 37° without octanoate for 30 min decreased enzyme activity to 80% of the control values in cerebral cortex and to 91% in the cerebellum. When 1·0 mM sodium octanoate was included in the incubation mixture, the enzyme activity was inhibited to 60% in the cerebral cortex and to 73% of the control values in the cerebellum following 30 min incubation. However, in the presence of 5·0 mM octanoate, a more rapid and pronounced inhibition of the enzyme activity was observed. In this case, α-glycerophosphate dehydrogenase was inhibited by 80% in both cerebral cortex and cerebellum with 2 min preincubation and by 90% when the supernatant fluids were incubated with octanoate for a period of 5 min.

α-Glycerophosphate dehydrogenase plays an important role in nervous tissue since it provides glycerol phosphate for the synthesis of myelin lipids (DeVellis, Schjeide & Clemente, 1967). Laatsch (1962) has shown that the period of most active myelination in the brain is accompanied by a marked rise in the activity of α-glycerophosphate dehydrogenase. DeVellis & others (1967) reported that myelin deficiency observed in the brains of neonatal rats subjected to head X-irradiation was accompanied by a marked decrease in the activity of α-glycerophosphate dehydrogenase. It is generally believed that a reciprocal relation exists between the consumption of carbohydrates and free fatty acids since glycolysis is inhibited while free fatty acids are being utilized (Shipp, Opie & Challoner, 1961; Opie, Evans & Shipp, 1963). Demonstration of the inhibitory effects of free fatty acids on hepatic and cardiac glycolytic enzymes (Weber & others, 1966; Tsutsumi & Takenaka, 1969) led to the suggestion that the free fatty acids may function physiologically in acute adaptation as a "metabolic directional switch" to restrict the flow of glycolysis. The present study demonstrates that octanoate inhibits the activity of α-glycerophosphate dehydrogenase in cerebral cortex and cerebellum of the rat. While the precise significance of these findings must await the demonstration that physiological concentrations of other free fatty acids can produce inhibition of this brain enzyme, it is conceivable that an increase in the intracellular level of free fatty acids may affect the process of myelin formation in nervous tissue.

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Utilization of acetylsalicylic acid as sole carbon source and the induction of its enzymatic hydrolysis by an isolated strain of *Acinetobacter lwoffii*

From glass distilled water that had been allowed to stand at room temperature for several days in a glass bottle open to the atmosphere, a bacterium was isolated which was capable of consuming acetylsalicylic acid (aspirin). The organism grew readily with aeration at 20–30° but not at 37° or above in the following medium (g/litre): aspirin 0·5, $(NH_4)_2SO_4$ 1·54, KH_2PO_4 1·83, Na_2HPO_4 3·91, $MgSO_4.7H_2O$ 0·0617, $FeSO_4.7H_2O$ 0·00016; pH 7·12. The bacterium was a Gram-negative, non-motile rod which grew in air; it was catalase positive, oxidase negative and urease negative; it was unable to produce acids from glucose; it gave a negative Hugh & Leifson (1953) test; it grew on nutrient and MacConkey agars; the ability to hydrolyse gelatin was uncertain; it was unable to utilize citrate, reduce nitrate, oxidize gluconate or produce H_2S; in nutrient broth it grew at 37° but not at 5°; it did not turn blood agar green; with litmus milk it reduced the indicator but produced no pH change. These results suggest that the organism is *Acinetobacter* (*Moraxella*) *lwoffii* (Cowan & Steel, 1965; Thornley, 1967). The bacterium is similar to NCIB 8250 (Vibrio O1; Fewson, 1967) but unlike that organism did not utilize citrate, L- or DL-mandelate or 4-hydroxymandelate as sole source of carbon and energy, but did utilize 2,3-dihydroxybenzoate.

In the following experiments salicylate and aspirin were determined at pH 7·12 using a Unicam SP 800 ultraviolet recording spectrophotometer after removing the bacteria by centrifugation. Complete spectral curves of suitably diluted samples were plotted from 350–200 nm for all samples and the concentrations of salicylate and aspirin were calculated from the extinction at the peaks and shoulders (Gore, Naik & others, 1968). At 296 nm salicylate absorbs maximally and obeys Beer's Law, but aspirin does not absorb. The rate of hydrolysis of aspirin was, therefore, determined by measuring the appearance of salicylate at 296 nm. At 262 nm aspirin gives a shoulder and obeys Beer's Law but salicylate, if present, interferes. The rate of disappearance of aspirin was determined at 262 nm only when the very sensitive method showed that salicylate was absent.

During initial growth of the organism in aspirin medium no salicylate was detected in the culture fluid at 296 nm and the specific rate of aspirin disappearance (at 262 nm) was equal to the first order specific rate of *spontaneous* hydrolysis of aspirin (at 296 nm). Since the ratio of increase in dry weight to the accompanying decrease in concentration of aspirin is constant (Table 1), the growth rate is equal to the rate of disappearance of, and spontaneous hydrolysis of, aspirin and does not increase exponentially with time as is usual. The growth rate is therefore governed by the the release of acetate and salicylate in the spontaneous, non-enzymatic hydrolysis of aspirin. Salicylate, and probably acetate as well, are consumed as soon as they are formed.

During the 5th and subsequent subcultures of the organism in aspirin medium, salicylate accumulated faster than in an uninoculated sterile medium and then disappeared (Table 2). The organism grew at 20–30° without a lag in the above medium containing instead of aspirin, sodium acetate (0·5 g/litre) or salicylic acid (0·5 g/litre). Bacteria repeatedly subcultured in aspirin medium therefore catalyse the hydrolysis of aspirin to acetate and salicylate which are then consumed but not necessarily immediately.

The following experiment with a cell-free extract indicates that the hydrolysis of aspirin is catalysed by a soluble intracellular esterase. After growth in aspirin medium

Table 1. *Disappearance of aspirin from the culture fluid during the initial growth of the aspirin dissimilating bacterium at 22° in a chemically defined medium containing aspirin as sole source of carbon and energy*

Time after inoculation (h)	Dry weight of bacteria (μg/ml)	Aspirin remaining (mM)	Increase in dry weight / Decrease in aspirin (μg ml^{-1} mM^{-1})
0	6·0	2·78	
24	43	2·20	64
48	74	1·72	65
72	109	1·22	70
96	148	0·61	64

Table 2. *Concentration of salicylate in the culture fluid during the 5th and 7th subcultures of the aspirin dissimilating bacterium at 22° in chemically defined medium containing aspirin as sole source of carbon and energy*

Time after inoculation (h)	Dry weight of bacteria (μg/ml)	Salicylate in medium (mM)	Salicylate in uninoculated control (mM)
	5th subculture		
0	3·4	0	0
16·3	20	0·83	0·42
41·5	118	0·81	0·81
65·3	159	0	1·30
	7th subculture		
0	1·8	0	0
15·5	16	0·76	0·39
41·5	127	0·51	0·81
66·8	155	0	1·37

at 22° the bacteria were harvested by centrifugation, resuspended in phosphate buffer (Na_2HPO_4 6·34 g/litre, KH_2PO_4 2·96 g/litre; pH 7·12), centrifuged, resuspended in distilled water, centrifuged and stored at −20° until required. Bacteria equivalent to a dry weight of 4 mg were suspended in 5 ml of phosphate buffer and disintegrated for 30 min at 0° using a MSE ultrasonic disintegrator with a stainless steel probe of diameter 19 mm. The preparation was centrifuged at 9000 *g* for 30 min at 4°. The cell-free supernatant, containing 1·0 mg of protein, was shaken at 30° with 90 μmol of aspirin in 30 ml of phosphate buffer under an atmosphere of N_2. Anaerobic conditions were maintained to prevent further metabolism (oxidation) of the hydrolysis products. From spectrophotometric determinations of salicylate at various times the first order specific rate of hydrolysis of aspirin was found to be 0·78 h^{-1} with the above cell-free extract and 0·022 h^{-1} with a boiled cell-free extract. The specific activity of acetylsalicylate hydrolase in the cell-free extract was 0·97 μmol/min mg^{-1} of protein in the phosphate buffer at 30°.

The above results indicate that the organism undergoes processes of adaptation during repeated subculture in aspirin medium such that the growth rate increases (cf. Tables 1 and 2) and the synthesis of acetylsalicylate hydrolase is induced. The rate-limiting step before adaptation is the non-enzymatic, spontaneous hydrolysis of aspirin but after adaptation is the dissimilation of salicylate, since it accumulates in the medium.

The deterioration of preparations containing salicylic acid, its salts and derivatives, especially aspirin, has long been studied. Sodium salicylate disappears slowly from

its solutions in the presence of air or oxygen with the formation of brown or black colourations or precipitates. The darkening is not very sensitive to light. The decomposition occurs in concentrated or in dilute solutions containing sodium bicarbonate or alkali, is accompanied by the absorption of molecular oxygen and is ascribed to the auto-oxidation of salicylate (Greenish & Beesley, 1915; Hilton & Bailey, 1938; Brecht & Rogers, 1940; Tomski, 1942). The discolouration is catalysed by traces of metal ions (Zwikker & Weber, 1940; Laszlovzky & Barcza, 1963) and is retarded by some substances.

Sodium salicylate preparations may also deteriorate through microbial attack. The growth of fungi in sodium salicylate solutions and its inhibition by chloroform was observed by Hanzlik & Wetzel (1920). That salicylic acid and its anion can act as a source of carbon and energy for the growth of micro-organisms (Evans, 1963) is amply demonstrated by the following recent examples: *Mycobacterium fortuitum* (Tsukamura, 1965); *Pseudomonas* species (Yamamoto, Katigiri & others, 1965; Stanier, Palleroni & Douderoff, 1966); *Trichoderma lignorum* (Vidal, Robert-Gero & others, 1967); *Aspergillus niger* (Krupka, Racle & Marderosian, 1969). Fewson (1967) reported that not only salicylate, but also aspirin, can act as sole source of carbon and energy for *Acinetobacter lwoffii* NCIB 8250 (Vibrio O1).

The spontaneous hydrolysis of aspirin in aqueous solution at physiological pH values to acetate and salicylate anions (reviewed by Stempel, 1961) might be expected to precede the growth of micro-organisms at the expense of aspirin. The experiments here described show that this indeed occurred immediately after the isolation of an aspirin-degrading bacterium from a laboratory environment. After a few subcultures in aspirin medium however, this organism produced an enzyme which hydrolysed aspirin very rapidly thus enabling it to grow and consume this substrate at a still greater rate.

The strains described here and by Fewson (1967) are variations of the widespread species *Acinetobacter lwoffii* which sometimes contaminates water and no doubt aqueous solutions. It seems likely that these organisms or related bacteria may be partially responsible for the deterioration of solutions of aspirin, salicylic acid and salicylates.

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Sympathomimetic action of tetanus toxin

Clinical reports by Kerr, Corbett & others (1968) revealed that in tetanus, there may be labile hypertension, tachycardia and irregularity of cardiac rhythm. We have looked for evidence of action of the toxin on the sympathetic nervous system. Tetanus toxin (Haffkein's Institute), 20 000 MLD/ml, given in amounts of 0·01 to 1·0 ml, produced 15–25% reduction of flow in the hind limb of the white rat perfused with oxygenated Ringer-Locke solution at 37°. The reduction was approximately doubled after pretreatment with cocaine (1 mg), but there was no reduction of flow in animals pretreated with reserpine (1 mg), 24 h before, or with phenoxybenzamine (1 mg) 45 min before the toxin.

A triphasic response was seen in the systemic blood pressure of the dog treated with (1·0 ml, of toxin per kg). There was an immediate sharp rise of 10 mm of mercury, then a similar fall below the pre-injection level and later a more sustained pressor effect of 30–40 mm of mercury for 15–20 min. There was no tachyphylaxis. Pretreatment with phenoxybenzamine (10 mg/kg) abolished the delayed pressor effect without affecting the earlier two phases. Similar observations were made on the rat. In about one quarter of the dogs, administration of toxin produced only a depressor response; when these animals were pretreated with mepyramine, the injection of toxin produced a sharp transient pressor effect; this was followed by a more sustained pressor effect which was abolished by pretreatment with phenoxybenzamine.

Injection of low doses of toxin (0·01–0·5 ml) in the perfused frog or rabbit heart produced positive inotropic and chronotropic effects with higher doses of toxin (0·5 ml) there was a subsequent depression. These effects were blocked by suitable doses of β-adrenergic blocking agents like dichloroisopropyl noradrenaline or pronethalol.

The toxin also produced contractions of the smooth muscles of the dog spleen and the guinea-pig vas deferens which were antagonized by phenoxybenzamine.

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Toxicity of ethanol barbiturate mixtures

We feel that Smith & Herxheimer (1969) may have misunderstood the objectives of our initial study on the ethanol-barbiturate interaction (Wiberg, Coldwell & Trenholm, 1969). Our experiments were not designed to establish whether the interaction was additive or synergistic. If this had been our purpose we would have used an experimental design similar to that recently described by Gebhart, Plaa & Mitchell (1969).

The information available from newer techniques should be used for a better comprehension of the mechanisms of drug interaction. Our studies of the ethanol-barbiturate interaction have now progressed far beyond the report referred to above (Coldwell, Wiberg & Trenholm, 1970; Trenholm, Maxwell & others, 1970; Wiberg, Trenholm & Coldwell, 1970). Thus, (1) ethanol (3 g/kg, i.p.) with barbiturates markedly reduces blood pressure with concomitant reduction in urine formation and renal clearance of barbiturates; (2) ethanol produces a dose-related decrease in body temperature (as much as 2–3°), an effect which would be expected to decrease the hepatic metabolism of barbiturate and ethanol; (3) ethanol depresses the respiration rate and lowers the blood pO_2 levels, an effect which would be expected to reduce ethanol and barbiturate metabolism; (4) pentobarbitone enhanced the activity of purified rat liver alcohol dehydrogenase but retarded the metabolism by liver slices; (5) ethanol is more toxic in older rats (12–14 months) and there is an increased sensitivity to ethanol-barbiturate mixtures; (6) ethanol alters the distribution of barbiturates in body compartments. The distribution pattern for barbiturates was studied in the presence and absence of ethanol for some seventeen different organs and tissues and several changes were noted.

Magnussen (1968) has noted that in oral dosing, with ethanol-barbiturate mixtures, there is an optimal ethanol concentration of between 5–10% which facilitates barbiturate absorption. Decreased barbiturate absorption was found when ethanol concentrations exceeded 10%.

Such information has convinced us that the use of the terms "additive" or "synergistic" do not help to clarify our understanding of the ethanol-barbiturate toxicity. Obviously, there are inherent dangers associated with the combined use of ethanol and barbiturates which must be repeatedly brought to the attention of the public.

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Pharmacological observations on phyllomedusin

Phyllomedusin is a natural decapeptide recently isolated from methanol extracts of the skin of *Phyllomedusa bicolor*, an hylid frog from the Amazonian region (Anastasi & Erspamer Falconieri, 1970).

The formulae reported below show the strict chemical resemblance existing between phyllomedusin, physalaemin from the skin of the South American amphibian *Physalaemus fuscumaculatus* (Anastasi, Erspamer & Cei, 1964), and eledoisin from the posterior salivary glands of the Mediterranean octopod *Eledone moschata* (Anastasi & Erspamer, 1963).

Pyr–Asn–Pro–Asn–Arg–Phe–Ile–Gly–Leu–Met–NH_2	Phyllomedusin
Pyr–Ala–Asp–Pro–Asn–Lys–Phe–Tyr–Gly–Leu–Met–NH_2	Physalaemin
Pyr–Pro–Ser–Lys–Asp–Ala–Phe–Ile–Gly–Leu–Met–NH_2	Eledoisin

Pure natural phyllomedusin was assayed biologically, in parallel with physalaemin, on a number of test preparations. The results are shown in Table 1.

Like that of the other tachykinins (physalaemin, eledoisin, substance P), the activity of phyllomedusin was destroyed by incubation with both chymotrypsin and trypsin (Bertaccini, Cei & Erspamer, 1965).

It may be seen from Table 1 that the activity ratio physalaemin : phyllomedusin varied not only for the different test preparations but also within the preparations; not infrequently it varied even for the same preparation during the course of an experiment.

Data reported in Table 1 reflect only the intensity of the actions displayed by the two polypeptides. Other distinctive features were represented by the duration of the fall of blood pressure, by the rapidity of relaxation of the smooth muscle after washing, and finally by the shape of the smooth muscle contraction curve.

The fresh skins which served for the preparation of pure phyllomedusin contained approximately 1100 μg of the pure peptide per g fresh tissue. The dry skin of a single large specimen of *Phyllomedusa bicolor* captured at Leticia (Colombian Amazonas) contained as much as 3500–4500 μg of phyllomedusin per g tissue.

In addition to phyllomedusin, *Phyllomedusa bicolor* contains in its skin conspicuous amounts of caerulein (probably phyllocaerulein), of phyllokinin, and of other active peptides.

It has been repeatedly pointed out that physalaemin-like peptides occur in the skin of several other *Phyllomedusa* species (Bertaccini & others, 1965). Whether these peptides are identical or not with phyllomedusin is a problem which must be solved separately in the individual species.

Table 1. *The result of parallel bioassay of physalaemin and phyllomedusin on nine test preparations.* The activity of physalaemin was always considered equal to 100, that of phyllomedusin was expressed in per cent.

Test preparation	Phyllomedusin activity (in %) (relative to physalaemin = 100%)
Dog blood pressure	40–70
Rabbit large intestine	60–150
Rabbit duodenum	30–80
Rat duodenum	70–150
Rat colon	100–150
Guinea-pig ileum	30–80
Rat oestrous uterus	150–200
Rabbit uterus	100–350
Rat salivary secretion	100–200

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A simple method for chronic measurement of the electrocardiogram and blood pressure in the conscious rat

The cardiovascular responses to drugs and toxic chemicals may be markedly different in conscious animals and anaesthetized animals. Electrocardiograms (ECG) are difficult to obtain from the conscious rat without training (Farmer & Levy, 1968) or restraint (Fujita & Tedeschi, 1968). The procedure described permits implantation of electrodes which can be used for several months to monitor ECG. With slight modifications, arterial and venous cannulas have also been used for up to 2 weeks.

The mounting device (Fig. 1) consisted of a small curved polyethylene "saddle" approximately 3 × 2 cm which was cut from the side of a standard 16 oz polyethylene reagent bottle. Two electrode leads, about 10 cm in length and consisting of No 30 Teflon coated 7 strand stainless steel wire, were woven through 6 holes in the saddle leaving about 8 cm on one side for attachment to the sternum of the rat and 2 cm on the other side for connection to the electrocardiograph. Approximately 4 mm of the short lead was stripped of Teflon insulation and soldered so as to leave a small solder blob. If required, silicone rubber cannulas were also passed through additional holes in the same "saddle".

Under ether anaesthesia, two 1 cm long skin incisions were made over the anterior and posterior ends of the sternum. A 4 cm dorsal midline incision was made beginning at about the 4th vertebrae and proceeding posteriorly. The skin adjacent to these incisions was dissected free from subcutaneous tissue. The electrode leads were passed under the skin from the dorsal incision to the incisions over the sternum. The end of the electrode wires were then passed through the cartilage at each end of the sternum, using a small curved needle, and secured by twisting around the main electrode lead in the same way as standard electrical connections are made. The saddle was gently manipulated beneath the skin through the dorsal incision and the skin sutured over the saddle leaving the short connecting parts of the electrodes protruding. About 20 min was required to complete this procedure. When required, silicone rubber cannulas filled with heparinized saline, were passed under the skin and implanted in the carotid artery and jugular vein in a similar manner.

After surgery the animals were housed in individual cages to prevent other rats from damaging the electrode leads or cannulas. When an ECG was required the rat was placed in a small open-top stainless steel cage which was grounded to the electrocardiograph. Two wires (made of the same material as the implanted leads) about 60 cm in length and with small aligator clips attached were used to connect the implanted leads to the electrocardiograph. These light-weight leads allowed free movement of the animal within the cage and permitted the measurement of the ECG with only rare artifacts occurring as the result of the aligator clips shorting together.

Where vascular cannulas were used about 2 cm of cannula was permitted to protrude from the "saddle". The cannulas were filled with heparinized saline and ligated about 0·5 cm from the tip. To maintain patency of the cannulas the ligature was untied and about 0·5 ml of heparinized saline (10 units/ml) infused every 12 h. In our experience it was possible to maintain cannula patency for about 2 weeks. To measure blood pressure or give drugs intravenously, extension cannulas about 20 cm in length were attached to the indwelling cannulas by means of appropriate size needle tubing and connected to a pressure transducer or syringe.

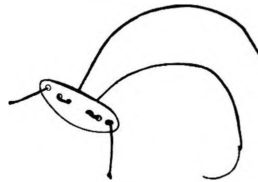

FIG. 1. Device for exteriorizing electrocardiograph leads. If required, cannulas may be exteriorized via the same device.

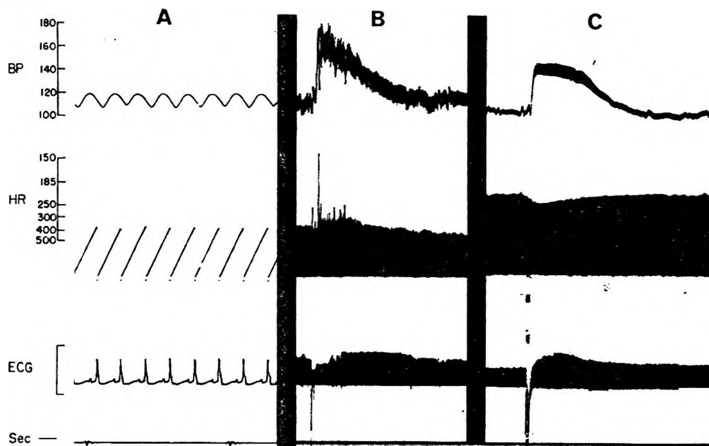

FIG. 2. Blood pressure (BP in mm Hg), heart rate (HR beats/min) and electrocardiogram (ECG) in conscious unrestrained rat. A-control tracing; B-response to 2 μg/kg adrenaline; C-response to 2 μg/kg adrenaline 30 min after administration of 1 mg/kg atropine; H.R. in C must be multiplied by 2 because of the change in attenuation of the cardiotachometer.

The epidermis overlying the plastic plate showed a moderate degree of acanthasis and papillomatosis. The plastic saddle was embedded in a moderately dense connective tissue sheath the inside of which was lined by a smooth shining mesothelial like membrane. The electrodes were enveloped by a thin connective tissue sheath the inside of which was lined by a smooth shining membrane similar to that seen around the plastic plate. The smooth mesothelial like membrane and the connective tissue sheath enveloped the electrodes throughout their entire length in the subcutaneous tissue so that the electrodes were permitted to move freely with the movement of the animal, but were not displaced from their general position in the subcutis.

The distal ends of some of the electrodes were surrounded by a small 3 × 3 mm whitish-grey tissue nodule. On sectioning, these nodules were found to be composed of granulation tissue with a small central cavity surrounded by a variable degree of fibrous tissue. The reaction was characterized by central polymorphonuclear infiltrates, epithelial cells, fibroblasts and histiocytes.

Figure 2 shows a blood pressure and ECG tracing obtained in this way and the response to 2 μg/kg adrenaline before and after administration of atropine. The reflex bradycardia produced by adrenaline before atropine and the tachycardia after atropine emphasizes the differences in cardiovascular response in the conscious animal compared with the anaesthetized animal where tachycardia predominates. This type of phenomenon has been described previously in other species (Whitty & Shepard, 1967; van Miert, 1969).

In a series of 6 animals prepared to determine how long satisfactory ECG recordings could be obtained, excellent recordings showing all components of the ECG were obtained 5 months after surgery in 4 animals. Electrode failure occurred in 2 animals after 3 weeks. Thus, this method has been found useful for the long-term assessment of cardiotoxic effects of chemicals (Grice, Heggtveit & others, 1970) and for the study of the cardiac effects of drugs in the conscious rat.

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February 12, 1970

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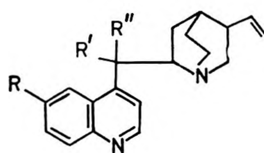
The isolation and identification of quininone from *Cinchona ledgeriana*

We have isolated and identified an alkaloid from the bark of a variation of *C. ledgeriana* collected in Guatamala. To the mother liquors remaining after the industrial isolation of quinine (I, R=OMe, R′=H, R″=OH) from this bark (these were supplied, as the residual bases in the form of their thiocyanate salts in aqueous solution, by Lake & Cruickshank Ltd.) (1 litre) was added excess sodium carbonate. The liberated bases were extracted with ether, the total ethereal extract was reduced to small volume under reduced pressure and the residue was subjected to column chromatography on alumina. Elution with ether–chloroform (2:1 v/v) afforded quinamine (II) (0·21 g) (Henry, 1949; Turner & Woodward, 1953), and subsequently with ether–chloroform (1:1 v/v) a white crystalline solid (0·04 g) (initial eluate) and cinchonine (I, R=H, R′=H, R″=OH) (1·24 g) (Henry, 1949; Turner & Woodward, 1953) (latter eluate). Both quinamine and cinchonine, along with quinine, have been isolated previously (Henry, Kirby & Shaw, 1945) from *C. ledgeriana*. The above white crystalline solid was recrystallized from ether–light petroleum (b.p. 40°–60°) to afford prisms, m.p. 98–101°. Elemental analysis gave an empirical formula $C_{20}H_{22}N_2O_2$ which was confirmed and shown to be the molecular formula by mass spectrometry. The ultraviolet spectrum in absolute ethanol showed λ_{max} 361–364 nm



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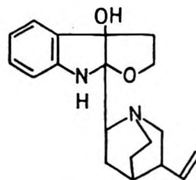
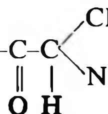


II

(log ϵ = 3·77), λ_{infl} 242–245 nm (log ϵ = 4·18) and λ_{infl} 252–257 nm (log ϵ = 3·99) and in ethanolic hydrochloric acid showed λ_{max} 342–344 nm (log ϵ = 3·79) and λ_{sh} 250–261 nm (log ϵ = 3·96) and the infrared spectrum in Nujol showed a strong band at 1685 ± 3 cm^{-1} (C=O) but was devoid of absorption between 3100–4000 cm^{-1} (N–H and O–H groups absent). The proton magnetic resonance spectrum in $CDCl_3$ included a 3-proton singlet at 6·14 τ (OMe), a 5-proton signal between 1·20–2·65 τ (5 aromatic protons), a 1-proton multiplet between 3·85–4·29 τ and a 2-proton multiplet between 4·81–5·18 τ (CH_2=CH–) and a 1-proton triplet centred at 5·88 τ (J = 9Hz) 

). The mass spectrum was similar to that of quinine (Budzikiewicz, Djerrasi & Williams, 1964) and indicated a molecular ion at m/e 322, a base peak at m/e 136 and other significant peaks at m/e 307, 292, 186, 172, 159, 158, 137 and 81.

These above data suggest the alkaloid is quininone (I, R=OMe, R'+R''=O). This was verified by its synthesis by oxidation of quinine (I, R=OMe, R'=H, R''=OH) using potassium t-butoxide–fluorenone mixture (Warnhoff & Reynolds-Warnhoff, 1963) (see also Doering, Cortes & Knox, 1947; Turner & Woodward, 1953), the natural and synthetic compounds having identical melting points and mixed melting point and infrared, ultraviolet, proton magnetic resonance and mass spectra.

Quininone has previously been detected by thin-layer and paper chromatography and by ultraviolet spectroscopy in several *Cinchona* species although it was only isolated in an amorphous state (Vácha, Čuba & others, 1964). The above studies further establish quininone as a natural product and represent its first isolation in a crystalline form from a natural source.

We are grateful to Dr. Walker of Lake & Cruickshank Ltd., Berkhamstead, for supplying the mixed base thiocyanate residues.

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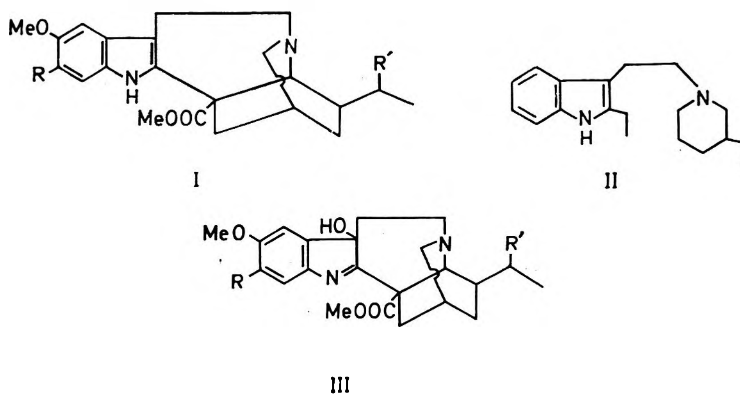
The isolation and identification of jollyanine from *Tabernamontana cumminsii*

As well as conopharyngine (I,R=OCH_3, R'=H), the major alkaloidal component (Thomas & Starmer, 1963; see also Renner, Prins & Stoll, 1959), 2-ethyl-3-[2-(3-ethylpiperidino)ethyl]indole (II), an alkaloid of particular biogenetic significance, has also been isolated in very small amounts (Crooks, Robinson & Smith, 1968) from the ether-soluble bases obtained from the leaves of *T. cumminsii*. We now report the isolation and identification of a third alkaloid from this source.



The total ether-soluble basic extract (14·2 g) was subjected to column chromatography on alumina (grade H) using ether as eluant. The initial eluates afforded a base which, after further purification by rechromatography under the conditions already described, gave an oil (1·9 mg) which was identified as (II) (Crooks, Robinson & Smith, 1968). The subsequent eluates from the initial column consisted mainly of conopharyngine together with two minor basic components. Recrystallization of this mixture from ethanol-ether gave conopharyngine (9·74 g), and further smaller amounts of slightly impure conopharygine were obtained by three further successive crystallizations from the mother liquors. The final mother liquor was subjected to preparative thin-layer chromatography on alumina using ether-light petroleum (b.p. 30–40°) (5:1 v/v) as solvent and iodine vapour followed by exposure to ultraviolet light as developer. The zone with Rf=0·80 was extracted with ether and, on crystallization from ether, afforded light-tan coloured prisms (6·8 mg), m.p. 184–191° which upon vacuum sublimation (200°/0·04 mm) afforded white prisms, m.p. 188–191° (with sublimation to needles at 145°), which are presently being investigated. The zone with Rf = 0·65 was similarly extracted to give a white crystalline solid, which after recrystallization from ether afforded white prisms (11·7 mg), m.p. 154–156°. Elemental analysis gave an empirical formula $C_{23}H_{30}N_2O_5$ which was confirmed and shown to be the molecular formula by mass spectrometry. The ultraviolet spectrum in absolute ethanol had λ_{max} 239·5 nm (log ϵ = 4·23), λ_{max} 312 nm (log ϵ = 3·54), λ_{infl} 302–305 nm (log ϵ = 3·50) and λ_{min} 276–277 nm (log ϵ = 3·21) and in ethanolic hydrochloric acid had λ_{max} 244 nm (log ϵ = 4·23), λ_{max} 332·5 nm (log ϵ = 3·56), λ_{infl} 303–305 nm (log ϵ = 3·40) and λ_{min} 272–274 nm (log ϵ = 3·10), data which suggest the presence of an indolenine-type chromophore in the alkaloid. The infrared spectrum in chloroform showed bands at 3585 $\pm$ 10 and 3480 $\pm$ 10 cm^{-1} (O–H stretchings), but no other absorption bands between this and 3100 cm^{-1}, showing N–H groups to be absent, and also had strong absorption bands at 1740 and 1692

cm^{-1}, characteristic of the free and hydrogen-bonded stretchings respectively of an ester carbonyl group intramolecularly hydrogen bonded to the hydroxyl function [cf. voacangine hydroxyindolenine (III, R=R'=H) (Thomas & Biemann, 1968)]. The proton magnetic resonance spectrum showed 3-proton singlets at 6·14, 6·15 τ (2 aromatic O–CH_3) and 6·34 τ ($COOCH_3$), 1-proton singlets at 2·95 and 3·14 τ (2 aromatic protons in a 1:4 substitution relation), a 3-proton triplet centred at 9·16 τ (J = 6Hz) (methyl group protons of an ethyl group) and a singlet at 6·53 τ which disappeared upon addition of D_2O (O–H). The mass spectrum indicated a molecular ion at m/e = 414, which was also the base peak, and other significant peaks at m/e = 399 (M–CH_3), 397 (M–OH), 385 (M–C_2H_5), 367 [M-(C_2H_5 + HO)], 355 (M–$COOCH_3$) 290, 248, 231, 220 and 122.

The above data are in good agreement with those reported for the alkaloid jollyanine (conopharyngine hydroxyindolenine) (III, R = OCH_3, R' = H) whose isolation from *Conopharyngia jollyana* and structural elucidation has been reported (Hootele, Levy & others, 1967). The structure of the alkaloid at present under investigation was further confirmed as III (R = OCH_3, R' = H) by its synthesis by continuous oxygenation for 7 h of a solution of conopharyngine (I, R = OCH_3, R' = H) in benzene in the presence of ultraviolet light under the conditions already described for the conversion of voacristine (I, R = H, R' = OH) into its hydroxyindolenine (III, R=H, R'=OH) (Schnoes, Thomas & others, 1968).

Three hydroxyindolenines, III (R=R'=H) (Thomas & Biemann, 1968), III (R=H, R'=OH) (Schnoes, Thomas & others, 1968) and jollyanine III (R=OCH_3, R'=H) have been isolated from plant sources. Owing to the facile preparation of these compounds by oxidation of the corresponding indoles (I, R=R'=H; R=H, R'=OH and R=OCH_3, R'=H, respectively) it is possible that they are artifacts formed by similar oxidations of their corresponding indoles during extract "work-up" (Hootele, Levy & others, 1967; Thomas & Biemann, 1968).

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