Journal of Pharmacy and Pharmacology

Published by
The Pharmaceutical Society
of Great Britain

Volume 16 No. 9
September 1964

Journal of Pharmacy and Pharmacology

Published by THE PHARMACEUTICAL SOCIETY OF GREAT BRITAIN

17 Bloomsbury Square, London, W.C.1. HOLborn 8967

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J. Pharm. Pharmacol., 1964, **16**, 569–595

Review Article

Biological activity in steroids possessing nitrogen atoms: recent advances

M. MARTIN-SMITH, M.Sc., Ph.D., AND M. F. SUGRUE, M.Sc.

WHEN the biological activities of steroids possessing nitrogen atoms were reviewed two years ago (Alauddin & Martin-Smith, 1962 a, b) it was predicted that a great deal of attention would be focussed on compounds of this class in the future. As anticipated, nitrogenous steroids possessing pharmacological properties not previously found within the group have been discovered—the most notable of these new properties being anti-inflammatory activity, anti-hypercholesterolaemic activity, digitalis-like activity, coronary dilatatory activity and central nervous system depressant activity. At the same time new nitrogenous steroids have been prepared which show weak androgenic, oestrogenic, progestational and anti-tumour activity (suggesting perhaps, that more potent agents exhibiting these types of activities await discovery within the nitrogen-containing steroid group) whilst the numbers of nitrogenous steroids showing high anabolic activity or antibacterial properties have also grown. It thus seemed desirable to extend the original reviews to these recent developments. The present survey covers the literature appearing since the publication of the two preceding reviews was completed, up to the end of 1963, although several 1964 references have been included and the opportunity has been taken to make reference to earlier contributions then unavailable to the authors.

Advances in the synthetic nitrogenous steroid field

Perhaps the most spectacular advances in the realm of synthetic nitrogenous steroids are connected with the preparation and biological testing of further steroids possessing heterocyclic rings incorporating nitrogen atoms, although many of the compounds so obtained still await full pharmacological evaluation and some of the claims concerning activity, which are based solely on routine assay procedures designed to detect compounds of potential interest, may need modification in the light of further studies. Differences in the activity of steroids as evidenced in screening assays and under conditions more closely akin to those of potential therapeutic application have been stressed by Bush (1962). Apart from new aza-steroids and new homo aza-steroids in which a nitrogen function has been substituted for a ring methylene group (for example, Engel & Rakhit, 1962; Huisman, Speckamp & Pandit, 1963; Kutney, Johnson & Vlattas, 1963; Kutney, Vlattas & Rao, 1963; Mazur,

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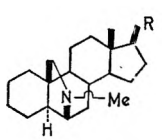
1963; Morgan, 1963; Morisawa, Kishida & Tanabe, 1963; Shoppee, Lack & Roy, 1963; Shroff, 1963; Uskokovic, Toome & Gut, 1962) prepared since the field was last reviewed (Rosseels, 1961; Tökés, 1963), several diaza-steroids have been prepared. These embrace both the heteroannular type (Doorenbos & Singh, 1961; 1962), giving an extension of the earlier work leading to a diazaequilenin-like molecule (Bhide, Tikotkar & Tilak, 1960), and the homoannular type in which a carbocyclic ring of the steroid nucleus can be considered to have been replaced by a pyrazole ring (Vida & Gut, 1963a), a pyrimidine ring (Caspi & Piatak, 1963a) or a pyridazine ring (Caspi, Grover & Piatak, 1963), thus extending earlier work in which ring A of the steroid nucleus was replaced by substituted pyridazine rings (Weisenborn, Remy & Jacobs, 1954). A further extension of this type of replacement of ring A of the steroid molecule is represented by the preparation of compounds in which an isoxazole ring is the replacing entity (Vida & Gut, 1963b). The biological properties shown by these compounds, when all have been tested, should be of theoretical interest, since the only modified steroid hormone so far discovered, in which a methylene carbon atom of the nucleus has been replaced by an atom of another element, and which shows enhancement of the activity characteristic of the parent compound, would appear to be 17α-methyl-2-oxa-5α-androstan-17β-ol-3-one (oxandrolone) (Fox, Minot & Liddle, 1962; Pappo & Jung, 1962). Certainly the pyrazole derivative prepared by Vida & Gut (1963a) was inactive as an androgen, anabolic agent, oestrogen or anti-oestrogen just as the steroidal 4-aza-5-en-3-ones prepared earlier by Dorfman, Uskokovic & Gut (1960) showed only weak anabolic or androgenic activities.

The first examples of aza-steroids in which a nitrogen atom replaces a carbon atom common to two rings of the steroid nucleus have been prepared (Meltzer & others, 1963a,b; Meyers, Ralhan & Munoz, 1963). The compounds so far reported include 8-aza-19-nortestosterone, 8-aza-19-norprogesterone and 8-azaoestrone and it will be interesting to learn of their biological activities particularly of the derived quaternary salts in view of the anti-shock properties of a structurally related compound reported by Osborne, Winbury & Govier (1963). It remains to be seen whether bisaza steroidal quaternary salts involving replacement of C-10 and C-13 by nitrogen will be prepared. These compounds, which will exhibit optical isomerism at the 10-aza- and 13-aza- quaternary nitrogen atoms, should be of great interest with respect to the relationship between chemical structure and neuromuscular-or ganglion-blocking activity. A further new type of aza-steroid is furnished by 17-azaprogesterone in which the carbon atom bearing the steroid side chain has been replaced by a nitrogen atom (Rakhit & Gut, 1964).

Many new nitrogenous steroids in which the perhydrocyclopentenophenanthrene nucleus is fused to a heterocyclic ring system have also been prepared, mainly as potential new anabolic agents. In addition to further derivatives of steroidal [3,2-*c*]-pyrazoles (for example, de Ruggieri, Gandolfi & Chiaramonti, 1962a; de Ruggieri, Gandolfi & Guzzi, 1963c; Fried & others, 1963a; Hirschmann & Patchett, 1963; Hirschmann &

others, 1963; Palmer, 1963; Patchett, Arth & Schwam, 1963; Schaub & Weiss, 1963), steroidal [2,3-*d*]-isoxazoles (for example, Marchetti & Donini, 1961; de Ruggieri & others, 1962a; Caspi & Piatak, 1963b; Manson & others, 1963) and steroidal [3,2-*d*]-thiazoles (for example, Doorenbos & Dorn, 1962; Holton & Necoechea, 1962; Clinton, 1963; Zderic & others, 1963), earlier representative members of which were discussed by Alauddin & Martin-Smith (1962a), a number of new fused steroidal heterocyclic compounds have been reported. The examples involving a 5-membered heterocyclic ring embrace steroidal [3,2-*b*]-pyrroles (Orr & Bowers, 1962), [16,17-*b*]-pyrroles (Mueller & Jiu, 1961), [3,2-*b*]- and [3,4-*b*]-indoles (Doorenbos & Wu, 1962), [3,4-*c*]-pyrazoles (Clinton & others, 1961, 1962), [17,16-*c*] -pyrazoles (for example, Moore, Holton & Wittle, 1962; de Ruggieri, Gandolfi & Chiaramonti, 1963a,b; Robinson, Bruce & Oliveto, 1963), [16,17-*c*]-pyrazoles (Dodson, 1960; Morita, 1963; Sciaky & Facciano, 1963), [3,2-*c*]-isoxazoles (de Ruggieri & others, 1962a), [6,7-*d*]-isoxazoles (Caspi & Piatak, 1962), [2,3-*d*]-thiazoles (Bowers & Edwards, 1963; Kraemer & others, 1963), [11,9-*d*]-thiazoles (Kitagawa & others, 1963), [12,11-*d*]-thiazoles (Takeda & Komeno, 1960), [17,16-*d*]-isothiazoles and [17,16-*d*]-thiazoles (Takeda & Komeno, 1962),



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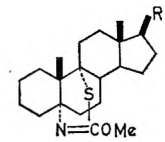

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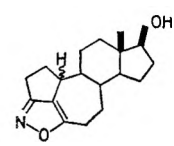


III

[2,3-*d*]-triazoles (Nathansohn, Testa & Di Mola, 1962; Fried, Buchschacher & Mrozik, 1963), [16,17]-1,2-diazabicyclo [3,2,0]-heptenes (Moore & others, 1962) and a D-homo-17-aza-[17,16-*a*]-indole (Hassner & Haddadin, 1962a). Those involving a six-membered ring include [17,16-*b*]-pyridines (Ketcheson & Taurins, 1960), [3,2-*d*]-pyrimidines (de Ruggieri & others, 1962a; de Ruggieri & others, 1963c; Smith, Teller & Foell, 1963; Zderic & others, 1963), [17,16-*d*]-pyrimidines (Ketcheson & Taurins, 1960; Smith & others, 1963), [2,3-*b*]-pyrazines (Jellinck & Irwin, 1963), [2,3-*e*]- and [4,3-*e*]-dihydro-*m*-oxazines (Kuehne, Konopka & Lambert, 1962), [2,3-*g*]- and [4,3-*g*]-pteridines (Bardos & others, 1963; Raman, Chmielewicz & Bardos, 1963), and finally [17,16-*b*]-quinolines (Hassner & Haddadin, 1962b), the preparation of this last group as potential anti-tumour agents being inspired by earlier reports (Buu-Hoi & Cagniant, 1944) of haemolytic properties in quinolino-steroids. An example involving a seven-membered ring is afforded by an androsteno [17,16-*e*]-1,2-diazepin-4-one (Moore & Pandya, 1964). In addition, several novel bridged heterocyclic nitrogenous steroidal systems have been prepared, including the types shown in I, II and III (Ledger & McKenna, 1963; Kitagawa & others, 1963; Roussel-UCLAF, 1963a; Kitagawa & Sato, 1964).

Compound III is claimed to possess anabolic properties.

The preparation of nitrogenous steroids in which the nitrogen atom forms part of an appended rather than a fused heterocyclic system has also continued. Among such compounds of biological interest, where the appended heterocycle forms a spiro system, are certain 16-spiro-dihydropyrazole derivatives (Werder & Brueckner, 1962), several 3-spiro-tetrahydrothiazole derivatives (Djerassi, Crossley & Kielezewski, 1962) and a number of lactams of 17β-amino-17α-(2-carboxyethyl)-androstane derivatives (Nysted & Burtner, 1962; Patchett, Arth & Hoffman, 1963; Patchett, Arth & Schwam, 1963; Patchett & others, 1962) which are nitrogen isosteres of the anti-aldosterone spirolactones and some of which (Burtner & Nysted, 1960) exhibit similar properties to the spirolactones.

In addition to the anti-accelerator activity shown by steroids substituted at C-16 by a piperidino ring (Swaine & Waud, 1960) steroids possessing appended piperidine rings at various positions have proved active as agents exerting a digitalis-like action (Nysted, 1958) or potentiating the contraction of striated muscle (Loomis, 1963), as central nervous system depressants (Babcock, 1959), as antihypertensive agents (Hershberg, 1960; Sterling, 1963), as antimicrobial agents (Counsell, 1963), as weak mineralotrophic (Szporny & Meszaros, 1962) and thymolytic agents (Dorfman & others, 1961; Stephenson, 1963), as antihypercholesterolaemic agents (Counsell & Klimstra, 1962; Tiernan, 1962) and as weakly active progestational (Kincl & Dorfman, 1963a,b) or oestrogenic (Takabatake & Ariyoshi, 1962) agents. It will also be interesting to learn of the biological properties of a number of 16α-substituted amino-glucocorticoid hormones which include 16α-piperidino-derivatives (Hoffman, Kissman & Weiss, 1962).

Steroids possessing appended pyrrolidine or morpholine rings, in several instances, appear to exhibit activities analogous to those just listed for the piperidine derivatives (for example, Camerino, Sciaky & Sala, 1962; Clinton, 1962; Gailliot & Robert, 1960; Hull, 1963; Marshall, 1961; Nysted, 1960; Panouse, Schmitt & Brunaud, 1961a,b; Gedeon Richter, 1963; Sciaky, 1962; Nakagawa, Mori & Tanaka, 1963), whilst a number of piperazine derivatives possess glucocorticoid activities (Brown & Sarett, 1963; Dömök & Szporny, 1963) and digitalis-like action is characteristic of several steroids possessing a substituted thiazole ring in the 17β-position (Ralls & Bergstrom, 1957; Takamura & others, 1963a,b).

The preparation of cyano- and thiocyanato-steroids has continued (for example, Cantrall, Littell & Bernstein, 1964a,b; Christiansen & Johnson, 1963; Crabbé & others, 1963; Julia, Linarès & Simon, 1963; Kissman, Hoffman & Weiss, 1961; Lednicer & Babcock, 1962; Nagata & others, 1961; Ueda & Mosettig, 1963; Valcavi, 1963). It would seem that in general the introduction of a cyano-group into a steroid hormone molecule greatly reduces the activity of the parent compound (Jen & Wolff, 1962; Kissman, Hoffman & Weiss, 1962) and some members of the group appear to actually exhibit antihormonal properties (Cella, 1960; Lincoln & Hogg, 1957; Mazur, 1957) although yet others are claimed to

be anabolic/androgenic agents (Bowers, Edwards & Orr, 1963), progestational or oestrogenic agents (de Ruggieri, 1962) or glucocorticoid agents (Fried, 1958). Certain 2α-cyano-compounds are stated to be useful as central nervous system depressants (Kissman & others, 1962). Interestingly, anti-inflammatory activity has been demonstrated in various 6β-nitro-steroids (Abildgaard, 1961) and antihypertensive activity is stated to be present in certain 16-nitromethylpregnenes (Dodson, 1959).

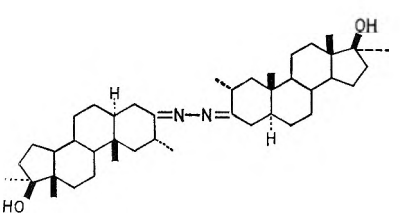
Several 21-azido analogues of glucocorticoids (Boland, 1961; Brown & others, 1961) have been shown to retain glucocorticoid activity, thus demonstrating extension of such properties to this group from steroidal diazoketones (Christensen, Steinberg & Hirschmann, 1958) and arylazo compounds (Dodson, 1957) known earlier. Perhaps the most dramatic discovery in this area, however, has been the demonstration that certain steroidal azines based on the androstane skeleton possess potent anabolic activity (de Ruggieri, Gandolfi & Chiaramonti, 1962b).

Further new nitrogenous steroids of great biological interest are afforded by several diaza-steroids in which carbon atoms of the steroidal side chain have been isosterically replaced by nitrogen atoms and which have proved to be inhibitors of cholesterol biosynthesis (Counsell & others, 1962b; Counsell, Klimstra & Ranney, 1962a; Ranney & Counsell, 1962a,b; Velluz & others, 1962).

A variety of nitrogenous steroids possessing various biological properties which have mainly been reported in the patent literature and which were not covered in the two previous reviews (Alauddin & Martin-Smith, 1962a,b) have been listed in a recently published compendium of steroidal drugs (Applezweig, 1962a).

ANABOLIC AGENTS

A number of new nitrogenous steroids possessing what is generally described as anabolic activity—despite objections which have been raised to the use of this term on account of its misleading connotations (Bush, 1962)—have been reported within the past two years. In addition to further representatives belonging to the group in which the steroid nucleus



IV

is fused to a heterocyclic ring, a new class of nitrogenous steroidal anabolic agents has been discovered in the androstane 3,3′-azines (de Ruggieri, & others 1963d; de Ruggieri & others, 1962b), the most potent of which is dimethazine, 2α,17α-dimethyl-17β-hydroxy-5α-androstane-3,3′-azine (IV). This compound, which, on oral administration, is stated to induce

an appreciable weight gain in adult rats, to show greater myotrophic activity than methyltestosterone, oxymetholone, stanozolol or testosterone propionate in castrated rats and to induce greater nitrogen retention in adult male rats than methyltestosterone (Matscher, Lupo & de Ruggieri, 1962) while exhibiting no oestrogenic, progestational or corticoid activity (Lupo, Matscher & de Ruggieri, 1962), can perhaps be regarded as an example of drug latentiation, especially in view of the obvious parallel to the relationship between prontosil red and sulphanilamide. On injection dimethazine is, however, less potent than testosterone and the nitrogenous steroids, 2-cyano-17β-hydroxy-5α-androst-2-ene caproate, stanozolol, 2-(*NN*-diethylaminomethylene)-17β-hydroxy-17α-methyl-5α-androstan-3-one and dihydrotestosterone-3-isonicotinyl hydrazone in the levator ani, ventral prostate and seminal vesicle weight gain tests in the rat (Dorfman & Kincl, 1963a). Dimethazine has been further shown to be without effect on the pituitary gland of castrated female rats (Beghelli & Mavrulis, 1962), to have a beneficial effect on bone regeneration in rats (Mavrulis & Pezzoli, 1962) and to have a higher activity in the levator ani test than methyltestosterone (Bianco & others, 1962).

In the heterocyclic field, clinical studies have shown that stanozolol (17β-hydroxy-17α-methylandrostano-[3,2-*c*]-pyrazole) may be a useful anabolic agent in man (Howard & Furman, 1962; Tainter & others, 1963), and, from nitrogen balance studies in the rat, 17β-hydroxy-17α-methylandrostano-[2,3-*d*]-isoxazole appears to be an even more potent agent than stanozolol (Arnold, Potts & Beyler, 1963a), although the initial claims of high potency in stanozolol have been questioned (Edgren, 1963).

Structure-action studies with some androstano- and androsteno-[2,3-*d*]-isoxazoles (Manson & others, 1963) have shown that the presence of a 4-ene or 4,6-diene system reduces anabolic activity where C-19 is present (Arnold, Potts & Beyler, 1963b) as is also true for the corresponding acetates (Caspi & Piatak, 1962), but that in the 19-nor series, where anabolic activity is present, it is more pronounced in the 4-ene than in the corresponding fully saturated compound. Unlike the situation with the corresponding 4-enes or 4,6-dienes of the [3,2-*c*]-pyrazole series, no oestrogenic activity is observed with the 4-enes or 4,6-dienes of the [2,3-*d*]-isoxazole series possessing a C-19 methyl group. The presence of a 17β-hydroxyl group seems crucial for high potency. Interestingly, 17β-hydroxyandrostano-[2,3-*d*]-isoxazole is inactive by the oral route but shows comparable activity to the corresponding 17α-methyl compound when given parenterally, thus providing a parallel to the situation pertaining between testosterone and 17α-methyltestosterone. Esterification of the hydroxyl group of 17β-hydroxyandrostano-[2,3-*d*]-isoxazole with 3-cyclohexylpropionic acid affords a potent anabolic agent with long duration of action and minimal androgenicity. A good myotrophic to androgenic ratio appears also to be present in 17β-hydroxy-17α-methylandrostano-[3,2-*c*]-isoxazole (Arnold & others, 1963b; Dorini & Montezemolo, 1961) where the side of the isoxazole ring involved in the ring fusion has been changed. This compound would seem to show negligible

progestational activity and to have a low inhibitory effect on pituitary gonadotrophin production. Insertion of methyl groups at C-4 to give 17β-hydroxy-4,4,17α-trimethylandrost-5-eno-[2,3-*d*]-isoxazole results in a compound which, like 2α-cyano-17β-hydroxy-4,4,17α-trimethylandrost-5-en-3-one, produces marked adrenocortical hypertrophy in mature female rats and blocks ACTH-induced thymolysis (Potts, Burnham & Beyler, 1963).

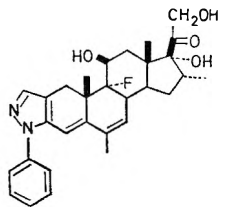
In addition to the anabolic properties of certain androstano-[3,2-*c*]-pyrazoles (Junkmann & Suchowsky, 1962) and [2,3-*d*]-triazoles (Nathansohn & others, 1962) a good anabolic to androgenic ratio is present in various androstano-[3,2-*d*]-thiazoles (Holton & Necoechea, 1962; Zderic & others, 1963). However, the androstano-[17,16-*c*]-pyrazoles so far prepared have nearly all proved devoid of hormonal activity (de Ruggieri, & others, 1963b), although weak anti-ovulatory properties are present in 3β-hydroxyandrostano-[17,16-*c*]-pyrazole and 3β-acetoxy-20-oxo-5-pregneno-[17,16-*c*]-pyrazole (Kincl & Dorfman, 1963b) and 3-methoxy-1,3,5(10)-oestratrieno-[17,16-*c*]-pyrazole, while without oestrogenic activity has been shown to possess antihypercholesterolaemic properties (Robinson & others, 1963). Similar lack of hormonal properties were found in the case of the androstano-[3,2-*d*]- and [17,16-*d*]-pyrimidines prepared by Smith & others (1963) although in this case antibacterial activity was present. The low degree of hormonal activity in the androstano-[17,16-*c*]-pyrazoles is perhaps of some significance in the light of the interesting suggestion (Bush, 1962) that the receptor site responsible for androgenic activity might be capable of accepting androgenic steroids in either of two positions in which the C-3 to C-17 axis is rotated through 180° in the plane of the steroid nucleus.

Two other nitrogenous steroids showing good anabolic to androgenic ratios are 2-(aminomethylene)-17α-methyl-5α-androstan-17β-ol-3-one and 2-(diethylaminoethylene)-17α-methyl-5α-androstan-17β-ol-3-one which on oral administration to rats had anabolic to androgenic ratios respectively 8 and 6 times that of testosterone (Zderic & others, 1963), whilst anabolic properties have been reported for 3-pyrrolidino-17α-methyl-5α-androst-2-en-17β-ol-4-one (Sciaky, 1962).

NITROGENOUS STEROIDS SHOWING ANTI-INFLAMMATORY ACTIVITY

Apart from further examples of latentiation or attempted latentiation of various anti-inflammatory steroids through esterification with nitrogen-containing acids (for example, Brunner & Finkelstein, 1960; Dorfman & others, 1961; Engelhardt, 1963; Thomae, 1963; Upjohn, 1960), oxime formation (Poos & Sarett, 1963) or carbamate formation (Brown & others, 1962; Lange & Amundson, 1962), anti-inflammatory activity has been demonstrated in some 20-alkylamino-steroids (Georgian, Kerwin & Wolff, 1961), in certain androsteno and pregneno [3,2-*c*]-pyrazoles (for example, Fried & others, 1963a; Harnik, 1963; Hirschmann & others, 1963; Steelman & others, 1963; Tishler, Steinberg & Hirschmann, 1962) and in several pregneno [3,2-*d*]-triazoles (Fried, Buchschacher & Mrozik, 1963). Although the [3,2-*c*]-pyrazole of hydrocortisone exhibited a

decreased anti-inflammatory activity as compared to hydrocortisone itself, potent activity is present in the [3,2-*c*]-pyrazoles of a number of glucocorticoids bearing substituents in the 6 or 16 positions. As well as their high potency these compounds are characterised by their lack of sodium retention activity (Fried & others, 1963a, Hirschmann & others, 1963). The [3,2-*c*]-2′-phenylpyrazole of 9α-fluoro-6,16α-dimethyl-Δ^6-hydrocortisone (V) has been claimed to be the most potent anti-inflammatory steroid yet known, being some 2000 times as potent as



V

hydrocortisone in the rat systemic granuloma assay (Steelman & others, 1963). Other compounds of the series include the [3,2-*c*]-pyrazole of 16α-methylcortisone which has four times the activity of the parent steroid (Steelman & others, 1963) and the [3,2-*c*]-pyrazole of 9α-fluoro-16α-methylhydrocortisone which has 10 to 20 times the activity of hydrocortisone (Hirschmann & others, 1963). That a high degree of structural specificity would seem to be associated with anti-inflammatory activity is shown by the complete absence of activity in 6,16α-dimethyl-2′-phenyl-4,6-pregnadiene-11β,17α,21-triol-3,20-dione-[3,2-*d*]-3′-H-1′,2′,3′-triazole whereas the corresponding 3′-phenyltriazole exhibits an activity some 190 times that of hydrocortisone in the rat systemic granuloma assay (Fried, Buchschacher & Mrozik 1963).

The hydroxamic analogue of cortisone, 17α-hydroxy-3,11-dioxo-aetiochol-4-enohydroxamic acid, like its *O*-methyl derivative, proved devoid of anti-inflammatory activity (Kierstead, Faraone & Goldberg, 1963) as also appears to be the case with the *N*-acetyl derivatives of the 11-amino isostere of hydrocortisone and closely related compounds (Oliveto & Rausser, 1961; Scherico, 1962). Low or negligible anti-inflammatory activity was found to be present in 2α-cyano-9α-fluoro-16α-hydroxy-hydrocortisone-16,17-acetonide (Kissman & others, 1962), in 21-piperidino-21-deoxyprednisolone (Dorfman & others, 1961; Stephenson, 1963) and in the 20-oxime or 3-oxime of cortisone-21-acetate (Sarrett, Patchett & Steelman, 1963). Although highly active in the local granuloma inhibition assay, a series of 21-carbamate derivatives of hydrocortisone showed little systemic activity on subcutaneous injection (Brown & others, 1962).

ANTI-HYPERCHOLESTEROLAEMIC AGENTS

Apart from one or two exceptions (Nysted, 1960) anti-hypercholesterolaemic activity has been found to be characteristic of nitrogenous steroids

belonging to two main groups. The first group consists of β-dialkylaminoethyl ethers of 3-hydroxy steroids (for example, Birkenmeyer & others, 1961, 1962; Cantrall & others, 1963; Gordon & others, 1961; Phillips & Avigan, 1963; Velluz, 1963) and the second group consists of the steroids in which carbon atoms of the side chain have suffered isosteric replacement by nitrogen. In addition, 3-methoxy-1,3,5(10)-oestratrieno-[17,16-*c*]-pyrazole has been found to retain the antihypocholesterolaemic properties of oestradiol while being virtually devoid of oestrogenic activity as measured in the mouse uterotrophic assay (Robinson & others, 1963), thus affording an excellent example of a synthetic steroid in which two biological properties characteristic of a natural hormone have been dissociated.

The preparation of the side chain diaza-steroids which include 22,25-diazacholestanol (Counsell & others, 1962b), 22,25-diazacholesterol Counsell & others, 1962b), 20,25-diazacholesterol (Counsell, Klimstra & Ranney, 1962a), 22,25-diaza-19-norcholesta-1,3,5(10)-trien-3-ol (Velluz & others, 1962) and some closely related compounds, was inspired by the known inhibitory action of cholesterol upon its own biosynthesis—feedback control (Siperstein, 1960)—and it was reasoned that the nitrogen isosteres might well be more firmly bound to the surface of the feedback-inhibited enzyme (Counsell, Klimstra & Ranney, 1962a). It would now appear that these compounds exert their hypocholesterolaemic effects primarily via a mechanism involving blockade of the *in vivo* conversion of desmosterol into cholesterol (Dvornik & Kraml, 1963; Ranney & others, 1963) and not, as was claimed earlier, via inhibition of hydroxymethylglutarylcoenzyme A reductase (Ranney & Counsell, 1962a,b; Sachs & Wolfman, 1963; Thompson, Du Pont & Robbins, 1963). Thus their primary site of action may well be the same as that of the 3-(β-dialkylaminoethoxy)-steroids which also block the *in vivo* conversion of desmosterol into cholesterol (Gordon & others, 1961; Phillips & Avigan, 1963).

Daily oral administration of 22,25-diazacholestanol in the form of its dihydrochloride to rats at a level of 5 mg/kg for 10 days reduced the plasma cholesterol levels by 24% (Ranney & Counsell, 1962a,b) while in man, it was found to lower the serum cholesterol levels and the β-lipoprotein cholesterol content in hypercholesterolaemic patients (Sachs & Wolfman, 1963), but at the expense of progressive accumulation of desmosterol. The 20,25-diaza isostere of cholesterol was found to be the most active member of the series and on oral administration to rats rendered hypercholesterolaemic with 6-propylthiouracil it exhibited 15 times the hypocholesterolaemic potency of triparanol (Counsell, Klimstra & Ranney, 1962). No oestrogenic activity was present in 22,25-diaza-19-norcholesta-1,3,5(10)-trien-3-ol as evidenced by experiments on castrated female rats (Velluz & others, 1962).

In the 3-(β-dialkylaminoethoxy)-group three compounds, namely 3β-(β-dimethylaminoethoxy)-androst-5-en-17-one oxime, 3β-(β-diethylaminoethoxy)-androst-5-en-17-one methoxime hydrochloride and 3β-(β-dimethylaminoethoxy)-pregn-5-en-3-one, are stated to show at least 20

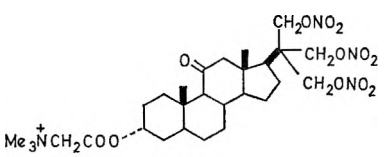
times the hypocholesterolaemic activity of triparanol in rats (Cantrall & others, 1963).

CENTRAL NERVOUS SYSTEM DEPRESSANTS

Anticonvulsant and sedative properties appear to be present in several groups of nitrogenous steroids. These include various steroidal monoximes (Babcock, 1958; Wechter, Schroeter & Buhler, 1961) and dioximes (Babcock & Wechter, 1962; Wechter, 1962; Upjohn, 1962a,b), a number of 17β-acetamido-androstane derivatives (de Ruggieri, Ferrari & Gandolfi, 1963), certain sterol aminoalkyl carbonates (Bergstrom, 1959), and aminoalkanoates (Marshall, 1963), and 2-cyano-1,4-pregnadiene-3,20-dione (Kissman & others, 1962) as well as various 3-aminopregnane derivatives (Schmitt, Brunaud & Panouse, 1961a,b; Schmitt & others, 1962) related chemically to the alkaloid funtumidine, the tranquillising properties of which were mentioned in the two earlier reviews. Similarly, central nervous system depressant activity as evidenced by induction of loss of the righting reflex in the mouse has been shown for several 2β-,6β- and 16β-morpholino-steroids, one of which, 2β-morpholino-3α-hydroxy-5α-pregnan-20-one, protected mice from leptazol-induced seizures at a dose one third of that necessary to produce loss of the righting reflex (Sugrue, 1963). In no case, however, was a degree of anaesthesia sufficient for surgery produced, nor was anti-Parkinson activity present.

VASODILATORY AGENTS

A striking example of the application of the supporting moiety theory is afforded by the preparation of several bis and tris nitratomethyl steroids and related nitrate esters such as VI which can be regarded as analogues



VI

of glyceryl trinitrate and which have proved to be potent coronary vasodilatory agents (for example, Bertin, 1962; Bertin, Nedelec & Locatelli, 1962; Bertin & others, 1962a; Roussel-UCLAF, 1962a, 1963b,c). Similar activity has been demonstrated in nitrogenous analogues such as 3α-acetoxy-11-oxo-20-dimethylaminoethyl-21-dimethylaminopregnane (Roussel-UCLAF, 1962b) whilst papaverine-dehydroepiandrosterone-3-monosulphate, which can be regarded as a steroid-latentiated presentation of papaverine, exhibited coronary vasodilator activity and inhibited histamine-induced bronchospasm and histamine-induced contraction of the guinea-pig small intestine (Setnikar, 1963). The synthesis of compounds of these types suggests many further applications of steroids as supporting moieties and latentiating agents, and it will be interesting

to discover what new drugs will be inspired by the success achieved with the compounds just discussed.

SEX HORMONAL ACTIVITY

The attempts so far recorded to synthesise nitrogenous steroids exhibiting potent androgenic, oestrogenic or progestational activity have not met with any marked success, although weak activity has been encountered in several instances. The preparation of amino isosteres of the steroidal oestrogens and related nitrogenous steroids has continued (for example, Bernstein, Cantrall & Littell, 1962; Schwenk & Gold, 1962; Suzui, Sawai & Chuma, 1962; Tsuda & others, 1963) and application of the α-aminonitrile synthesis to the preparation of a number 17-alkyl-17-dimethylamino-steroids resulted in a new oestrogenic compound, 17β-*NN*-dimethylamino-17α-methyl-3-methoxyoestra-1,3,5(10)-triene (Lednicer & Babcock, 1962). Nitrogenous oestrogen derivatives have played an important part in support of arguments attempting to more closely delineate the molecular requirements for oestrogenic activity (Patton & Dmochowski, 1963). Since replacement of a hydrogen atom of the C-2 methyl group in 2-methyloestrone by a dialkylamino-group leads to a marked decrease in oestrogenic potency as is also observed in 2-nitro-oestradiol and 4-nitro-oestradiol, it is conceivable that the fall in oestrogenic potency is a reflection of the lessened ability of the phenolic hydroxyl group on C-3 to enter intermolecular hydrogen bonding with the receptor, having its origin in steric hindrance, in intramolecular hydrogen bonding or in a combination of the two (cf. Brown, Eglinton & Martin-Smith, 1962).

Oestrone dimethylhydrazone proved only one thirtieth as active as oestrone in increasing the uterine weight of immature rats while the dimethylhydrazone derivatives of pregnenolone, progesterone and ethisterone proved to be without progestational activity (McKinney & Payne, 1961). The dimethylhydrazones of several androgenic steroids also showed a marked decrease in activity compared to the parent compound, and in the case of the 3-dimethylhydrazone of methyltestosterone the anabolic activity was depressed to a greater extent than the androgenic activity (McKinney & Payne, 1961; Wiley & Chang, 1963). On the other hand, the benziloyl hydrazones of testosterone-17-heptanoate and oestrone-3-heptanoate (Gleason & Parker, 1959), like various other steroidal ester hydrazones (Frosst, 1960) showed a marked prolongation of action as compared to the esters from which they are derived. The benziloyl hydrazone of 17α-hydroxyprogesterone-17-heptanoate, however, showed little difference in activity from 17α-hydroxyprogesterone-17-heptanoate (Gleason & Parker, 1959). The epimeric mixture of 17-cyanohydrins derived from 3,17-dioxo-oestr-4-ene has been reported to exhibit oestrogenic activity (de Ruggieri, 1962), while oestrogenic as well as progestational activity is stated to be present in various 4,9-androstadieno-[3,2-*c*]-pyrazoles (Hirschmann, 1963). Anti-ovulatory activity of one fifth and of one hundredth of that observed with norethisterone was found

in 3β-hydroxyandrostano-[17,16-*c*]-pyrazole and 3β-acetoxy-20-oxo-5-pregneno-[17,16-*c*]-pyrazole respectively (Kincl & Dorfman, 1963b) when administered orally to rats. On subcutaneous injection to rats, the anti-ovulatory activities of 3β-hydroxyallopregnane-20-*N*-acetylhydrazone, 3β-hydroxyallopregnane-20-nicotinylhydrazone, 16-dimethylaminoethylene-3β-hydroxyandrostan-17-one and 3,17-dioxo-16-piperidinomethylandrost-4-ene were respectively one fourteenth, one hundredth, one hundredth and one hundredth times that of norethisterone (Kincl & Dorfman, 1963a). Weak progestational activity was also found for 5α-thiocyanato-17α-ethynyloestrenolone and 5α-thiocyanatopregnan-3,20-dione as evidenced by the rabbit endometrial carbonic anhydrase test (Miyake, 1962).

Attention has also been given to the preparation of nitrogenous steroids capable of functioning as "antihormones," i.e. of antagonising endogenously formed hormones. Not only should such antihormones be of value in treating conditions such as prostate hypertrophy but it is conceivable that anti-oestrogens and antiprogestogens might prove of value in controlling fertility. Moreover, antihormones might prove of value in the elucidation of the intimate mechanisms of action of the natural hormones (Applezweig, 1962b). In some instances attempts have been made to secure steroids capable of directly antagonising the natural hormone and in other instances attention has been directed towards the production of antibodies capable of neutralising the endogenously formed hormones.

Examples of nitrogenous steroids resulting from the first approach are 3,11-dioxo-17-oximinoandrost-4-ene which is claimed to exhibit anti-oestrogenic and antimineralocorticoid properties (Nagata, Narisada & Sugasawa, 1962), certain 4-alkylamino-derivatives of testosterone and nortestosterone which are claimed to possess anti-androgenic activity (Suzuki, 1963), a number of steroidal [12,11-*d*]-thiazoles which are stated to exhibit antiprogestational properties (Yeneno, 1963) and various 17-iminosteroids which possess the ability to inhibit the response to testosterone propionate of the seminal vesicles and ventral prostate glands of castrated male rats, although without effect on endogenous androgens in intact male rats (Saunders & others, 1963). Anti-oestrogenic activity, as measured by the ability to inhibit oestrone-induced uterine growth in rats, was observed with the isonicotinylhydrazone of 17β-hydroxy-3-oxo-5α-androstane, with 3β-acetoxy-16β-carbamoyl-17α-isopregn-5-en-20-one, with 17β-hydroxy-17α-methyl-2-methylaminomethylene-5α-androstan-3-one and with 2-aminomethylene-17β-hydroxy-17α-methyl-5α-androstan-3-one (Dorfman & Kincl, 1963b), but only at massive doses. The piperidino-, pyrrolidino- and morpholino-ethers of oestrone and oestradiol also inhibit oestrone-induced uterine growth in rats but exhibit certain oestrogenic effects as well (Takabatake & Ariyoshi, 1962).

As a means of achieving antihormonal antibody production Erlanger & others (1957) prepared protein derivatives of testosterone and cortisone in which the steroid molecule was covalently bound to bovine serum albumin, and found that the products did indeed exhibit antigenic proper-

ties in rabbits. The encouraging results of this earlier work led to the synthesis of conjugates of progesterone, deoxycorticosterone and oestrone with bovine serum albumin (Beiser & others, 1959; Erlanger & others, 1959) but the results were by no means clear-cut (Lieberman & others, 1959). Nevertheless, oestrone conjugates of human serum albumin, formed by reaction of the 17-isocyanate derived from oestrone with the albumin to give the corresponding 17-carbamido-protein conjugate, were prepared (Goodfriend & Sehon, 1960, 1961a,b) and it was found that specific antibodies were produced to both the oestrone residue and the protein carrier. Thus it was established that the oestrone residue could indeed act as a haptenic group in inducing the formation of a steroid-specific antibody, but the antibody site complementary to the oestrone residue was found to be capable of accommodating other steroid molecules as well. The oestrone protein complex was itself devoid of oestrogenic activity and its antiserum proved capable of neutralising the 6-hr utero-trophic activity of exogenous oestrone in immature female rats. Further work involving the passive immunisation of sheep with oestrone bovine serum albumin, testosterone bovine serum albumin, hydrocortisone bovine serum albumin and aldosterone bovine serum albumin resulted in the production of anti-sera which were then found on administration to mice to be capable of blocking the effects of the appropriate steroid (Neri & Tolksdorf, 1962). At present insufficient data are available to permit of firm conclusions, but, in the light of the encouraging results so far, it would seem that complex nitrogenous steroids where the nitrogenous moiety is a protein may well have an important role to play in providing sera for the therapy of disorders which involve overproduction of steroid hormones.

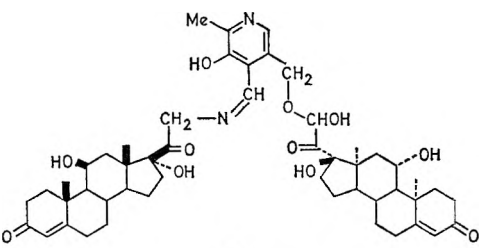
OTHER ACTIVITIES

Further compounds in which the steroidal nucleus can be regarded as acting as a supporting moiety bearing a hydrazide, hydrazone or iso-nicotinylhydrazone radical have been prepared (Volovel'skiĭ, 1961) and like the earlier compounds of the series (Volovel'skiĭ, 1957a,b) proved to have antitubercular activity. Tuberculostatic activity has also been demonstrated in 11-aminotigogenin (Jeger, Anner & Kalvoda, 1959) whilst antibacterial properties are characteristic of some 20- and 21-alkyl-amino pregnane derivatives (Nakama & Satake, 1959; Uchino & others, 1960; Varela & Kincl, 1962) and 17-ethylamino-1,3,5(10)-oestratien-3-ol (Misao, Sawai & Suzuki, 1962). *In vitro* activity against Gram-positive organisms, particularly certain strains of *Staphylococcus aureus*, was found for four steroidal [3,2-*d*]- and [17,16-*d*]-2′,6′-diaminopyrimidines (Smith & others, 1963) although these compounds proved incapable of protecting mice against a penicillin-resistant strain of *Staph. aureus*.

Antifungal properties are present in certain hydrazides, disemicarbazones and dithiosemicarbazones derived from hyodeoxycholic acid (Panizo & Laorga, 1958) and in a number of 18-dimethylamino-20-pregnenes (Pappo & Baran, 1959) and *N*-alkyl-17-amino-1,3,5(10)-oestratrien-3-ols (Misao, Sawai & Suzuki, 1962) whilst sterol complexes with piperazine

have proved useful as anthelmintic agents (Robeson, 1963). Antiviral activity is claimed to be present in *p*-toluenesulphonyl-dehydrocholamide (Ueda & others, 1962) and in *N*-dodecanoyl-4-cholylamino-1-naphthalenesulphonamide (Ueda, Kato & Toyoshima, 1958).

The results of attempts to secure antitumour activity in nitrogenous steroids have not been very encouraging. Thus further studies with nitrogen mustard derivatives of oestrogens (Nogrady, Vagi & Adamiewicz, 1962) have indicated that only weak anticancer properties are present in these compounds, although a degree of antitumour activity has been claimed for 6-aza-steroids (Lettre & Knof, 1959), the oxime of lanosta-8,24-dien-3-one (Mori, Gandhi & Schwenk, 1962) and a number of steroidal dihydro-1,3-oxazines (Kuehne & others, 1962). Preliminary biological screening indicated little antitumour activity in several steroidal [2,3-*d*]-isoxazoles (Caspi & Piatak, 1963b). The pteridino-steroids which were prepared as folic acid antagonists with the steroidal supporting moiety designed to confer favourable lipid solubility and cellular transport properties upon the antifolic 2,4-diaminopteridines (Bardos & others, 1963; Raman, Chmielewicz & Bardos, 1963) likewise showed little antitumour activity although 17β-acetoxy-5α-androstano-[4,3-*g*]-2′,4′-diaminopteridine is stated to have given a statistically significant inhibition of approximately 50% in one test—the sarcoma 180 assay—at 150 mg/kg/day (Bardos & others, 1963).



VII

Other biological properties shown by nitrogenous steroids which have been reported include hypoglycaemic activity for a series of aromatic sulphonyl carbamic esters of steroids (Heymons & Liebig, 1961) which are stated to be active on oral administration, and choleretic action for amides formed between various bile acids and amino-acids (Amiard & Heymès, 1958), while some *N*-substituted 17-amino-oestratrienes are claimed to be useful as hair tonics (Suzuki & others, 1963). The 21-glycylglycinate of prednisone has been shown to be without effect on the survival of adrenalectomised male golden hamsters but to increase the level of glutamic-pyruvic-transaminase in the liver (Oriol-Bosch & Voigt, 1961). Similarly 17β-aminoandrost-4-en-3-one has been shown to have very low androgenic and anabolic potencies but to increase the concentration of carbonic anhydrase in the seminal vesicles of intact male rats (Oriol-Bosch & Voigt, 1961). These two compounds thus afford examples of nitrogenous steroids in which there has been a dissociation of the

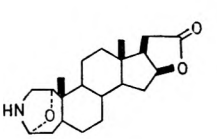
hormonal and enzyme-inducing activities characteristic of the parent compounds and give further examples of action of nitrogenous steroids on enzyme systems (for example, Levey, 1953).

The interest in the combination of steroids with nucleotides which was mentioned in the first of these reviews (Alauddin & Martin-Smith, 1962a) has now been extended to studies of the reaction of steroids with pyridoxine derivatives (Monder & White, 1962) in an attempt to explain how steroids may affect transaminase activity, and it has been shown that on oxidation by cupric ions to the corresponding 21-aldehydes, corticosteroids possessing an hydroxylic function at C-21 form Schiff bases of type VII with pyridoxamine.

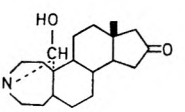
Steroidal alkaloids

SALAMANDER ALKALOIDS

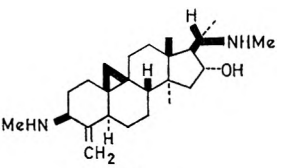
There would seem to have been no further biological investigations reported on the alkaloids of the 2a-aza-A-homoandrostane group although the results of further chemical studies have been published which have led to the elucidation of the structure of samandaridine as VIII (Habermehl, 1963) and to the reassignment of the structure of cycloneosamandione as IX (Habermehl & Goettlicher, 1963).



VIII



IX



X

ALKALOIDS FORMALLY DERIVED FROM PREGNANE

A further review of the occurrence, chemistry and pharmacological properties of the steroidal alkaloids belonging to the pregnane group has appeared (Janot, 1963) and some 20 new members of this group have been characterised.

New mono-acid bases of the pregnane type are irehamine which is 3β-hydroxy-20α-methylaminopregn-5-ene (Truong-Ho & others, 1963b) and thus the *N*-methyl derivative of holafebrine, paravallaridine which is 16α-hydroxyparavallarine (Beugelmans, Kan & Le Men, 1963), latifolinine which is 3-oxoconan-4-ene (Qui Khuong-Huu, Yassi & Goutarel, 1963) and norlatifoline which is 3β-hydroxy-*N*-demethylconan-5-ene (Qui Khuong-Huu, Yassi & Goutarel, 1963). These last two alkaloids, like latifoline (Janot, Qui Khuong-Huu & Goutarel, 1962) therefore represent examples of mono-acid conanine derivatives possessing an oxygen function at C-3 in place of the more usual nitrogen function. In addition, the structure of irehine has been elucidated as 20α-dimethylamino-3β-hydroxypregn-5-ene (Truong-Ho & others, 1963b). New alkaloids belonging to the holarrhimine sub-group of the pregnane diacid bases are epiheteroconessine which is 3α,20α-bis(dimethylamino)-pregn-5-ene (Lábler & Šorm, 1963a; Tschesche & Otto, 1962), irehdiamine A which is 3β,20α-diaminopregn-5-ene, irehdiamine B which is 20α-amino-3β-methylamino-

pregn-5-ene (Truong-Ho, Qui Khuong-Huu & Goutarel, 1963a), the pachysandrines A & B which possess an esterified hydroxyl group at C-4 (Tomita, Uyeo & Kikuchi, 1964) and cyclobuxine (X) Brown & Kupchan 1962a,b) and the cyclomicrophyllines A, B & C (Nikaido & Terao, 1964) which represent examples of a new type of pregnane alkaloid incorporating a cyclopropane ring and retaining additional carbon atoms on C-4 and on C-14. In addition, the structure of kurchessine has been elucidated. It is 3β,20α-bis(dimethylamino)-pregn-5-ene (Lábler & Šorm, 1963a). New di-acid conanine derivatives are concuressine which is 3α-dimethylamino-conan-5-ene (Lábler & Šorm, 1963a), dihydroconcuressine which is 3α-dimethylamino-5α-conanine (Lábler & Šorm, 1963a) and malouphyllamine which is 3β-acetamido-5α-conanine (Janot & others, 1963).

The structure of α-hydroxyconessine has been confirmed as 3β-dimethylamino-4β-hydroxyconan-5-ene (Goutarel, Conreur & Parello, 1963) and kurchamine has been assigned the structure, 3-aminoconan-17-ene with unspecified stereochemistry (Tschesche & Otto, 1962). A new base isomeric with kurchamine and which has been assigned the structure 3-methylamino-*N*-demethyl-conan-17-ene has been termed kurchimine and a further base, designated kurcholessine, isolated (Tschesche & Otto, 1962).

The existence of yet other alkaloids belonging to the pregnane group is suggested by the isolation of the 3α-epimer of conessine and 3α-dimethylamino-5α-conanine together with 3α,20α-(bisdimethylamino)-pregn-5-ene and 3β-,20α-(bismethylamino)-pregn-5-ene from the methylated mother liquors remaining after the isolation of conessine from *Holarrhena antidysenterica* (Lábler & Šorm, 1963b) and by the isolation of a new *Funtumia* alkaloid, funtulatine (Oletta, 1963).

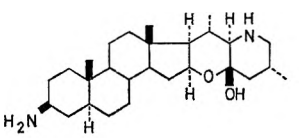
The trimethylammonium quaternary salts derived from funtumine and funtumidine have been claimed to possess 1/75th the neuromuscular blocking potency of tubocurarine (Blanpin & Bretaudeau, 1961; Blanpin & Pierre, 1961) which is of interest since they are monoquaternary, not bisquaternary, salts. Their other pharmacological properties are stated to be similar to those of the parent alkaloids. Pharmacological studies with the three possible isomers of malouetine involving the configurations of the nitrogen atoms, namely the 3β,20β-, 3α,20α- and 3α,20β-bis-(trimethylammonium)-5α-pregnanes, have shown that all three compounds possess neuromuscular-blocking activity comparable to that exhibited by malouetine itself on the rabbit gastrocnemius preparation, all being shorter acting than tubocurarine (Khuong Huu-Lainé & Pinto-Sconamiglio, 1964). The action of all four compounds is abolished by physostigmine and eserine.

ALKALOIDS FORMALLY DERIVED FROM CHOLESTANE

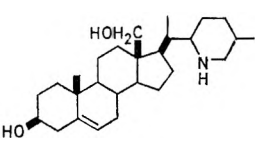
Two new skeletal types are now recognised within the cholestane group and these are represented by solanocapsine (XI) which has been shown to lack the usual spiro-structure (Schreiber & Ripperger, 1962) and by veralkamine (XII) a new alkaloid isolated from *Veratrum album* subspecies *lobelianum* (Tomko & Bendik, 1962).

BIOLOGICAL ACTIVITY IN STEROIDS POSSESSING NITROGEN ATOMS

Several comprehensive surveys of the occurrence of the solanidane and spirosolane alkaloids have been published (Schreiber & others, 1961; Schreiber, 1963a,b). The medicinal applications of extra-European *Solanum* species amongst primitive peoples have also been reviewed (Stopp, 1961) and a detailed study has been reported of the action of the solanum alkaloids on the chemoreceptors of the potato beetle, *Leptinotarsa decemlineata* Say (Stürckow, 1961). Solasodine has been shown to possess cardiotonic and antiphlogistic effects at 5–10 mg/kg in mice in addition to lowering sensitivity to pain stimuli (Turova, Seifulla & Belykh, 1961).



XI



XII

Solasodamine has been shown not to be an individual alkaloid but to be in fact solasonine monohydrate (Briggs, 1961; Briggs, Cambie & Hoare, 1961). Similarly solauricine has proved to be a mixture of solasonine and solamargine whilst the so-called alkamine, solauricidine was a mixture of solasodine and solasodine galactoside (Briggs, 1961; Briggs & others, 1961). Soladulcamaridine, originally classed as an alkamine of the solanidane type (Rasmussen & Boll, 1958) has now been proved to be a mixture of alkamines of the spirosolane type, consisting of solasodine, 5,6-dehydrotomatidine and 3,4 : 5,6-bis(dehydro)tomatidine (Boll, 1962). A number of new glycosidic alkaloids have been reported (for example, Bognár & Makleit, 1962; Makleit, Gaál & Bognár, 1962), including three new representatives from *Solanum dulcamara* which have been termed the α-, β- and γ-solamarines (Boll, 1962) and two new members from *Solanum laciniatum* which have been termed solaradixin and solaradinine (Bite, Jókay & Pongrácz-Sterk, 1962). β_1-Tomatine, which had previously been prepared from tomatine (α-tomatine) by partial acid hydrolysis giving cleavage of the xylose unit (Kuhn, Löw & Trischmann, 1957) has now been found to be of natural occurrence in the leaves of two mutants of *Lycopersicon esculentum* Mill. (Schreiber, 1963c).

The position of attachment of the second hydroxyl group in leptinidine has been shown to be C-23 (Kuhn & Löw, 1962) whilst by means of optical rotatory dispersion studies (Boll & Sjöberg, 1963) it has been shown that the differences existing within the side chains between the tomatidine and 5α-solasodan-3β-ol series are such that tomatidine has the 22βN,25*S* configuration and 5α-solasodan-3β-ol has the 22αN,25*R* configuration.

ALKALOIDS POSSESSING THE JERVI SKELETON

Further detailed pharmacological studies on the anti-accelerator action of veratramine (Hawkins, 1962) have shown that the alkaloid is acting

as a physiological antagonist to adrenaline whilst further careful structure-action studies within the ceveratrum ester group (Kupchan, 1961 ; Kupchan & others, 1961a,c; 1962a) have served to confirm earlier conclusions (Kupchan, Hensler & Weaver, 1961b) concerning the indispensability of esterification of the hydroxyl groups at positions 3 and 15 of protoverine for the presence of high antihypertensive potency. It was further concluded that although the absence of esterification of the alcoholic functions at both the 6 and 7 positions is not disadvantageous, absence of an ester group at position 7 alone does result in marked loss of potency. Esterification of the hydroxyl group at position 16 with either acetic acid or isobutyric acid is disadvantageous as is oxidation of this function to the corresponding ketone, whilst esterification of the hydroxyl at position 4 may lead to a loss of activity. Acetonide formation involving the hydroxyl groups on positions 14 and 15 results in a pronounced fall in potency.

The full chemical structures of sabine and its acetylated (at the hydroxyl group on position 3) derivative sabadine have been elucidated (Kupchan, Gruenfeld & Katsui, 1962). Unlike the related alkamines germine, protoverine, veracevine and zygadenine, sabine lacks a masked α-ketal system in ring A. It is 3β,4α,12α,14α,16β,17α,20β-heptahydroxy-5β-cevane. Similarly, the structure of verticine (*syn* peimine) has been shown to be 3β,6α,20β-trihydroxy-5α-cevane (Ito & others, 1963) whilst sipeimine (*syn* imperialine) has been shown to be a 3β-hydroxy-6-oxo-cevane derivative with an additional unplaced hydroxyl group (Liu & others, 1961).

Theoretical aspects

Recently there has been much speculation about the nature of androgen-receptor interaction, and nitrogenous steroids have played a role in experiments designed to gain more information bearing on this problem. Whereas with glycogenic activity, progestational activity or oestrogenic activity it seems generally accepted that interaction between the steroids concerned, and their receptors, involves the β face of the steroid molecule (Bush, 1961, 1962; Sarett, 1959; Sarett & others, 1963)—since with some exceptions bulky β-substituents attached to the steroid nucleus tend to abolish activity whereas α-substituents do not, thus suggesting that the β-substituents sterically hinder formation of the receptor complex —the position concerning mineralocorticoid or androgenic activity is particularly confused (Bush, 1962). In fact it has been suggested that α-face attachment, as is believed to occur in the binding of steroids to plasma albumin (Westphal & Ashley, 1959), may well be involved in mineralocorticoid and androgenic activity (Bush, 1962; Ringold, 1961) despite many anomalies, but apart from difficulties in interpretation arising from solubility, transport, absorption, distribution and biotransformation factors there remains the uncertainty as to whether a substituent group projecting from the steroid molecule coincides with a hump or a trough in the receptor surface. If the former situation pertains interaction between the steroid molecule concerned and the receptor would be

expected to be difficult or impossible, but if the second situation is the case, interaction with the receptor might even be strengthened due to increased van der Waals bonding.

To test the α-face hypothesis of androgenic attachment, several androgen analogues possessing an oximino group at C-19 or having a nitrile function replacing the C-19 methyl group were prepared and tested for androgenic activity (Wolff & Jen, 1963; Wolff, Jen & Kwok, 1963). Since the van der Waals radius of the nitrile group in the plane perpendicular to the steroid ring system is greater than that of the methyl group and since such a steric change on the β-face would hardly be expected to influence α-face attachment, the fact that the nitriles did not show the activity characteristic of the corresponding methyl compounds was taken to mean that androgens, like the glucocorticoids, progestogens and oestrogens interact with their receptors at the β-face.

A number of interesting vicinal amino-steroidal alcohols have recently been reported (Ponsold, 1963a,b,c) and it will be of some theoretical interest to learn of their biological properties in view of their conformational rigidity and the importance of the β-aminoethanol system as an active moiety or "stripped down" drug (Gero & Reese, 1956; Gero & Withrow, 1957)—being an integral part of the molecules of so many antispasmodic, local anaesthetic, sympathomimetic and antihistaminic drugs.

The potent neuromuscular blocking properties of C-curarine I and toxiferine I (Waser, 1959) which possess fully rigid molecules whose interonium distance can be seen to be *ca.* 9·7 Å from inspection of models (Haining & Johnston, 1962) coupled with the results of conductance studies on the flexible decamethonium molecule which have shown its interonium distance in aqueous solution to be *ca.* 9·5 Å (Elworthy, 1963), make it apparent that the original two-point attachment theory of neuromuscular blockade involving anionic receptor sites separated by *ca.* 14 Å (Paton & Zaimis, 1949, 1951) needs modification, and it is of great interest that several 3β,17β-bisquaternaryammonium androstane derivatives, in which the interonium distance can vary from 10·5 to 11·2 Å (as ring A passes from boat to chair), exhibit potent non-depolarising neuromuscular blockade of short duration (Biggs, Davis & Wien, 1964; May & Baker, 1963). One of these derivatives, 3β,17β-dipyrrolidin-1′-yl-5α-androstane bismethiodide has been found devoid of androgenic, oestrogenic and progestational activity in man. The detailed comparative pharmacological properties of the different members of the series will be of theoretical significance since increase in the size of the substituents on the cationic head in the flexible polymethylene bisquaternary series causes the average interonium distance to increase (Elworthy, 1963) and such variation will be minimised in the steroidal series. Apart from the fact that the possible interonium distances seem significantly higher than the 9·5–9·7 Å previously mentioned (a range also proposed by Carey & others, 1959), the lack of impedence to interaction with the receptors from the angular methyl groups projecting from the β-face of the steroid nucleus at C-10 and C-13, is of theoretical importance; so too is the presence of activity in

3α,17β-bistrimethylammonium-5α-androstane where the quaternary heads lie on opposite sides of the plane of the steroid nucleus. It remains to be seen whether bisquaternary salts having the 3α,17α-configuration in which the interonium distance can vary from 9·3 Å (ring A as chair) to 11·0 Å (ring A as boat) will exhibit similar activity.

Conclusion

If the progress in the nitrogenous steroid field achieved within the two years since the earlier reviews (Alauddin & Martin-Smith, 1962a,b) were completed, as indicated by the present review, can be taken as a criterion of future interest in the field, it may be anticipated that many nitrogenous steroidal drugs will be introduced in the coming years, and that still further contributions to the theoretical aspects of drug action will be forthcoming from this field. The successful development of photochemical reactions applicable to nitrogenous steroids (for example, Barton & Beaton, 1961; Barton & Morgan, 1961, 1962; Barton & others, 1960; Robinson & others, 1961; Tanasescu, Hodosan & Jude, 1960), the successful development of microbiological transformations applicable to nitrogenous steroids (de Flines & others, 1962; Kupchan & others, 1963; Mazur & Muir, 1963; Sato & Hayakawa, 1963, 1964) and the discovery of microbiological amidation of steroids (Smith & others, 1962) open new opportunities for the production of still further nitrogenous steroids and suggest that an active interest in the field will be maintained for some years.

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J. Pharm. Pharmacol., 1964, **16**, 596–602 Received February, 27, 1964

Research Papers

Oxidation of benzaldehyde in some single phase betaine-benzaldehyde-water systems

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The oxidation rates of increasing concentrations of benzaldehyde in ternary systems of the L_1 type have been measured in aqueous solutions of four betaine homologues. On applying corrections for extra-micellar material, the rate becomes independent of the betaine concentration and is related to the micellar ratio of benzaldehyde to betaine when this ratio exceeds 1. The rate of oxidation in an LC system has been determined and compared with that in L_1 systems. The site and mechanism of the oxidation reaction in these systems is postulated.

IN a previous investigation of the oxidation of emulsified and solubilised aldehydes, the rate of oxygen uptake was correlated with the degree of saturation of the dispersion (Carless & Mitchell, 1962). An extension of this work by Carless & Swarbrick (1962) showed that the saturation concept was not applicable to the oxidation of benzaldehyde dispersed in aqueous solutions of an homologous series of ampholytic betaines. It was suggested that only from a consideration of the ternary phase diagrams for these systems might it be possible to relate oxidation rates to the concentration and nature of the oil present.

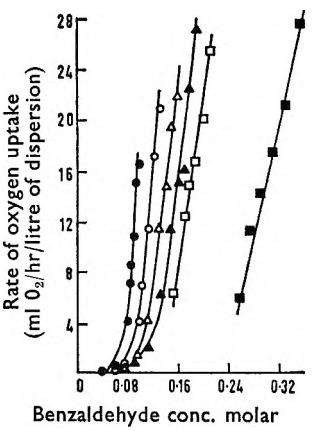

FIG. 1. Variation in the rate of oxygen absorption per litre of dispersion with benzaldehyde concentration in the L_1 phase of the C_{12} system. Betaine C_{12} concentration (molar): ●, 0·0173M; ○, 0·0346M; △, 0·0519M; ▲, 0·0692M; □, 0·0865M; ■, 0·1730M.

The relevant parts of the phase equilibrium diagrams for six betaine-benzaldehyde-water systems were therefore determined by Swarbrick & Carless (1963). On the basis of these studies we present, in this and the

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subsequent paper (Swarbrick & Carless, 1964), the results of oxidation studies made with single phase systems and multiphase systems containing different combinations of the single phases. The terminology used is identical to that defined by Swarbrick & Carless (1963).

Experimental

Materials used. The benzaldehyde and *N*-alkyl *NN*-dimethylglycines (betaines) were as described by Swarbrick & Carless (1963).

Preparation and oxidation of dispersions. All dispersions were prepared on a % w/w basis. The oxygen absorption of freshly prepared 2 ml samples was determined manometrically (Carless & Nixon, 1957), in the presence of 1×10^{-5} M cupric sulphate at a temperature of 25° and a shaking rate of 140 strokes/min.

To calculate readily the rate of oxygen uptake of dispersions prepared on a w/w basis in accord with phase equilibrium diagrams, it was necessary to assume the dispersions had a weight per ml of 1. In view of the relatively dilute dispersions used and the very close correlation between the volumes obtained when dispersions were prepared on a weight basis in flasks calibrated on a volume basis, the error introduced by this approximation is probably less than 2%.

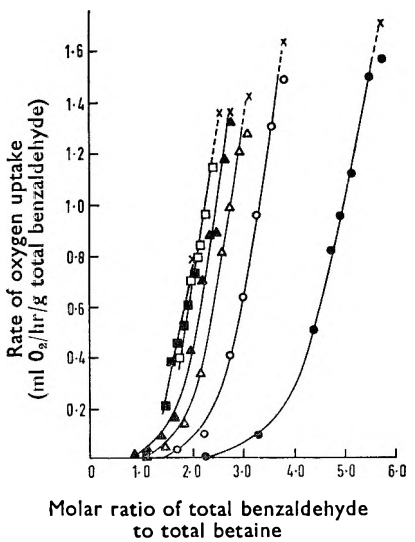

FIG. 2. Variation in the rate of oxygen absorption with the molar ratio of total benzaldehyde to total betaine in the L_1 phase of the C_{12} system. The extrapolated point X denotes the rate at a saturation ratio of 1. Betaine C_{12} concentration (molar): ●, 0·0173M; ○, 0·0346M; △, 0·0519M; ▲, 0·0692M; □, 0·0865M; ■, 0·1730M.

Solubility of benzaldehyde in water. The solubility of benzaldehyde in water at 25° was shown, by refractive index measurements using a Hilger-Raleigh Interferometer, to be 6·9 mg/ml (Carless & Swarbrick 1964; see also Mitchell, Wan & Bjaastad, 1964).

Results

OXIDATION IN L_1 SYSTEMS

The maximum rates of oxygen uptake, expressed as ml 0_2/hr/litre of dispersion (the rate/litre), of ternary L_1 dispersions containing the betaine C_{12} homologue have been plotted in Fig. 1 against the molar concentration of benzaldehyde. Increasing the concentration of the betaines or decreasing the concentration of benzaldehyde, or both, led to an increase in the time elapsing before the attainment of the maximum oxidation rate which we define as the induction period. The induction period of the L_1 dispersions was never less than 10 hr.

Fig. 2 shows the relationship between the rate of oxygen absorption, expressed as ml 0_2/hr/g benzaldehyde (the rate/g), and the molar ratio of total benzaldehyde to total betaine present in the system. The points marked X, obtained by extrapolation, denote the rates/g at molar ratios of benzaldehyde to betaine which exist at the solubility limit of the L_1 phase for the different betaine concentrations used. At these points the Saturation Ratio (Carless & Mitchell, 1962) is equal to 1.

The influence of betaine chain length was studied in dispersions containing 0·0692 M of the C_{10}, C_{12}, C_{14} and C_{16} homologues. The relationship between the molar ratio of the total amounts in the system and the rate/g, shown in Fig. 3, is similar to that found earlier (Fig. 2).

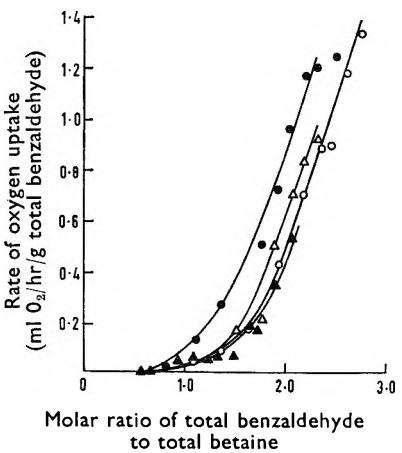

FIG. 3. Variation in the rate of oxygen absorption with the molar ratio of total benzaldehyde to total betaine in the L_1 phase of four systems containing equimolar (0·0692M) betaine concentrations. ●, C_{10}; ○, C_{12}; △, C_{14}; ▲, C_{16}.

OXIDATION IN LC SYSTEMS

The maximum rate of oxidation in ternary C_{14} LC systems containing 21·5% w/w benzaldehyde, which occurred after 7 hr, was 0·23 ml 0_2/hr/g aldehyde. The molar ratio of benzaldehyde to betaine in this system was 3·25.

Discussion

OXIDATION IN L_1 SYSTEMS

For the purposes of this discussion the L_1 phase may be considered as consisting of a micellar pseudo-phase dispersed in, and in equilibrium with, a continuous aqueous phase. The distribution of benzaldehyde between these two phases will depend on the concentration and lipophilic/hydrophilic balance of both the aldehyde and S-amphiphile.

The addition of molecular oxygen to liquid aldehydes involves a free-radical chain reaction which possesses initiation, propagation and termination steps (Bawn & Jolley, 1956). The initiation of benzaldehyde oxidation in the L_1 systems studied is due to photochemical and metal salt catalysis. The rates of oxidation differ however from those found with aldehydes in organic solvents under similar catalytic conditions (Ingles & Melville, 1953; Bawn & Jolley, 1956). This is most probably due to the presence of the aqueous continuous phase which, because of its high dielectric constant, will not favour the free-radical mechanism by which aldehyde oxidation proceeds (Waters, 1948). Carless & Mitchell (1962) considered it unlikely that the amount of aldehyde in the aqueous phase influenced the reaction since they found that aldehydes dissolved below their solubilities in water absorbed no oxygen. Consequently, the site of oxidation in ternary L_1 systems must be the micellar pseudo-phase. Furthermore, the rate of oxidation in these systems is insensitive to catalyst concentration (Swarbrick, 1963) even though the systems are saturated with oxygen. This suggests that the maximum rate is dependent on the rate of propagation of the reaction and not the catalysed initiation reaction which most probably occurs at the interface between the micellar pseudo-phase and the aqueous phase. This contrasts with the situation found in the L_2 phase where the rate is very sensitive to catalyst concentration.

Since the rate of propagation is likely to be dependent on the relative micellar concentrations of benzaldehyde and betaine it is necessary to apply corrections for both the extra-micellar aldehyde and S-amphiphile molecules which take no part in the oxidation reaction. It is reasonable to assume that the extra-micellar S-amphiphile concentration in binary S-amphiphile-water systems is equal to that of the critical micelle concentration (CMC) since the surface tension above this concentration remains fairly constant. However, the presence of benzaldehyde has been shown to lower the CMC in the resultant ternary system to approximately half the value in the binary system (Swarbrick, 1963). Accordingly, this latter value has been used in this work to correct for extra-micellar betaine molecules.

Estimation of the extra-micellar benzaldehyde is more difficult since it is not possible to determine directly the concentrations of aldehyde in the micellar pseudo-phase and the aqueous phase. However, the potentiometric studies of Donbrow & Rhodes (1963; 1964), on the distribution of organic acids and amines in aqueous cetomacrogol solutions, show that the distribution isotherm of benzoic acid is reasonably linear over a wide

range of acid and cetomacrogol concentrations although distribution in favour of the micellar pseudo-phase does increase slightly at high cetomacrogol and low acid concentrations. Evans (1964), using the same technique, has also found the distribution coefficient for *p*-hydroxybenzoic acid in a non-ionic S-amphiphile to be constant over the whole concentration range examined.

Since benzaldehyde is not structurally unlike these compounds it is reasonable to suppose that a comparable situation may exist in the L_1 systems we have examined. Corrections for extra-micellar benzaldehyde have therefore been made on the assumption that (i) the aldehyde concentration in the aqueous phase of the L_1 systems at saturation is equal to its solubility in water, and (ii) the distribution coefficient for benzaldehyde between the aqueous phase and the micellar pseudo-phase is constant over the range of aldehyde and betaine concentrations studied. On this basis the rate of oxygen absorption per g micellar aldehyde in the various C_{12} systems becomes independent of the betaine concentration and proportional to the micellar ratio of benzaldehyde to betaine at values in excess of 1 (Fig. 4). Possible changes in the mean shape and size of

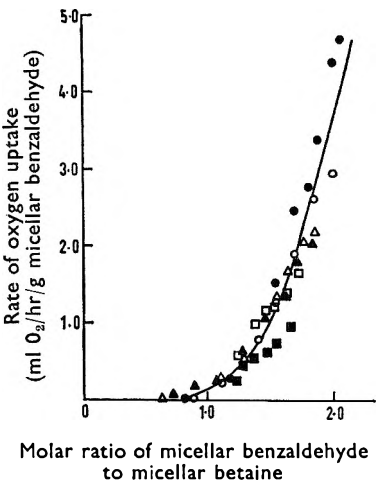

FIG. 4. Dependence of the rate of oxygen absorption on the molar ratio of micellar benzaldehyde to micellar betaine in the L_1 phase of the C_{12} system. Betaine C_{12} concentration (molar): ●, 0·0173M; ○, 0·0346M; ▲, 0·0519M; △, 0·0692M; □, 0·0865M; ■, 0·1730M.

the micelles, as the concentrations of the two amphiphiles are varied, will have no significant effect on the rate of oxidation since the micellar ratio is independent of these factors. The relationship between the rate/g and the micellar ratio using four different betaine homologues (Fig. 5) is similar to that in the C_{12} system although the rates/g in the C_{14} and C_{16} systems are higher than those in the C_{10} and C_{12} systems for micellar ratios greater than 1. This effect may be due to the aldehyde molecules being concentrated more towards the centre of the micelle with the longer chain

betaines. If so, then as Carless & Mitchell (1962) observed with aldehydes of increasing chain length solubilised in cetomacrogol solutions, the oxidation rate increases since collision between the reactive species becomes more frequent.

The application of a saturation ratio to oxidation studies in multiphase systems has been challenged (Carless & Swarbrick, 1962) on the grounds that it takes no account of the phase equilibrium of the system; such a ratio is at best only applicable to oxidation in single phase systems. However Fig. 2 shows that the rate/g at the solubility limit, where the saturation ratio equals 1, falls as the concentration of the S-amphiphile is increased. On the other hand, it is possible to relate the rate to the micellar ratio of benzaldehyde to betaine over at least a tenfold change in betaine concentration.

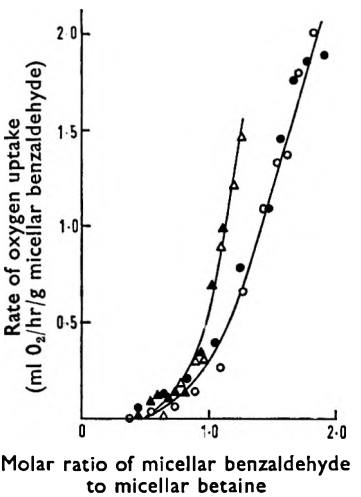

FIG. 5. Dependence of the rate of oxygen absorption on the molar ratio of micellar benzaldehyde to micellar betaine in the L_1 phase of four systems containing equimolar (0·0692M) betaine concentrations. ●, C_{10}; ○, C_{12}; △, C_{14}; ▲, C_{16}.

The explanation for this behaviour appears to lie in the location of the 0-amphiphilic benzaldehyde molecules in the palisade layer of the micelle, an arrangement comparable to that in a highly curved mixed monolayer. The betaine molecules do not oxidise (Swarbrick, 1963) and therefore merely have a diluting effect upon the aldehyde molecules positioned in the "interstitial" spaces between then. Only when these spaces are filled (i.e. when the micellar ratio exceeds 1) do two aldehyde molecules become adjacent and allow the propagation reaction to proceed readily. Since the micelles in the L_1 phase are fluid, deformable structures in equilibrium with the aqueous continuous phase, the micellar ratio is best considered as a measure of the probability of two or more aldehyde molecules being adjacent in the palisade layer. The low rate of oxidation below a micellar ratio of 1 is in accord with this idea since there is still a finite, albeit low, probability of this happening.

This concept is supported by the work of Honn, Bezman & Daubert (1951) who have shown that the rate of oxidation of drying oils, adsorbed on to the surface of finely divided silica gels, is dependent on the average distance between oil molecules. As the distance decreases so the rate of oxidation increases, the most favourable arrangement for promoting oxidation being a closely packed monomolecular layer.

Insufficient results were obtained by Carless & Mitchell (1962) at saturation ratios of 1 and below to allow application of the concept that the rate depends on the micellar ratio of aldehyde to S-amphiphile. It may be, however, that the constant rates observed by these workers at saturation are in fact due to a constant micellar ratio at this point.

OXIDATION IN LC SYSTEMS

The lower oxidation rate/g in the LC phase compared to that in L_1 systems having the same micellar ratio is very likely related to the different micellar structures existing in these phases. Thus the unit structure of the LC phase is a bimolecular layer lattice or leaflet composed of the S-amphiphile and O-amphiphile molecules, the polar heads of which are associated with water sandwiched between these layers. Consequently, the number of aldehyde molecules directly accessible to oxygen and capable of partaking in the initiation reaction is greatly reduced. Furthermore, the LC phase is solid in the plane of the layers containing the benzaldehyde and this will restrict the propagation reaction. The net effect is therefore to produce a lower rate of oxygen uptake than found in the essentially liquid L_1 micelle which has all the polar heads of its constituent aldehyde molecules accessible to oxygen attack.

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Urethane anaesthesia and pituitary-adrenal function in the rat

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Urethane anaesthesia produced a prolonged hypersecretion of ACTH as shown by plasma and adrenal corticosterone and adrenal ascorbic acid changes. There was also a concurrent depletion of the adrenaline content of the adrenal gland. Adrenal demedullation did not prevent the steroid changes induced by the anaesthetic. Hyperactivity of the pituitary-adrenal axis for 24 hr in the urethane-anaesthetised rats did not prevent further alteration of steroid levels in response to a stress.

PENTOBARBITONE treatment has been shown to depress the secretion of ACTH (Egdahl, 1961) whereas ether anaesthesia has been shown to stimulate the secretion of ACTH (Royce & Sayers, 1958). Sayers (1957) found that prolonged ether anaesthesia induced an initial secretion of ACTH in the adrenalectomised rat which was followed by a decline to non-detectable levels. It has been suggested that the transient stimulation of ACTH is compatible with the excitation during the induction period (Sayers & Royce, 1960), but Barrett & Stockham (1963) found that continuous ether anaesthesia produced a continued high level of steroid in the blood. These findings prompted the investigation of the influence of prolonged anaesthesia on the steroid secretion of the adrenal cortex. The effect of urethane anaesthesia was investigated since it is a good anaesthetic in the rat, producing a stable and safe level of anaesthesia.

Urethane has been reported to induce hyperglycaemia (Seuffert & Ullrich, 1925), depression of inflammatory responses (Peng, 1930), leuococytopenia (Stein, 1949) and an increase in the basal metabolic rate (Aub, Bright & Forman, 1922), all conditions which may be associated with hyperactivity of the adrenal gland. The adrenaline content of the adrenal medulla, as well as the plasma and adrenal corticosterone and adrenal ascorbic acid, have been estimated to establish if the cortex and the medulla of the adrenal gland were affected by the anaesthetic and whether there was any inter-relationship.

Methods

Wistar male rats weighing 150–250 g were kept in a room at a constant temperature of 23 ± 2° and stored in single cages. They were fed on a diet of cubes (diet 41B, Lane-Petter & Dyer, 1952) and allowed water *ad lib.* Adrenal demedullation was carried out by the method of Ingle & Griffiths (1942) and the animals were left for 12 weeks before use. Blood samples were obtained from the abdominal aorta and centrifuged at 3,000 rpm for 10 min, and the plasma was analysed for corticosterone. The adrenal glands were removed, cleaned and weighed; one was assayed

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for ascorbic acid content by the method of Roe & Keuther (1943), whilst corticosterone analysis on half of a 0·01 N HCl homogenate was made by the method of Barrett & Stockham (1963). The remaining portion of the adrenal homogenate was estimated for its adrenaline content by the method of Shore & Olin (1958).

Urethane as a 50% w/v solution in water was given intraperitoneally in a dose of 1·5 g/kg. Phenobarbitone sodium in 0·9% w/v saline was given intraperitoneally in a dose of 200 mg/kg. ACTH (Cortrophin, Organon) dissolved in 0·9% w/v saline was given subcutaneously in a dose of 5 units/kg. Some animals were also exposed to ether vapour for 1 min to stimulate the discharge of ACTH from the pituitary.

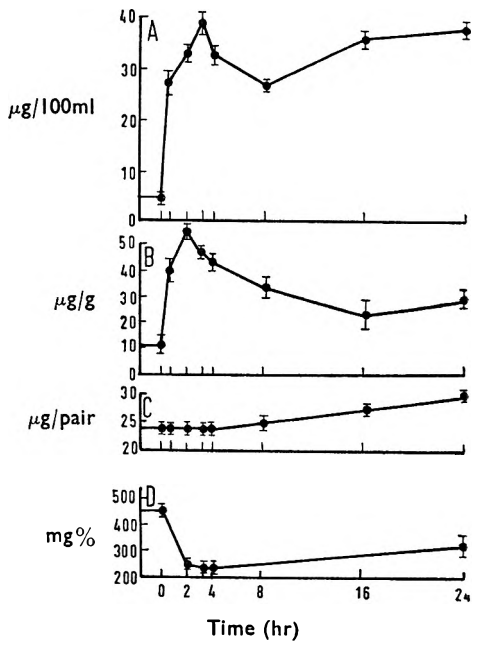

FIG. 1. Changes of indices of pituitary-adrenal cortex function during urethane anaesthesia. Each point is the mean of 6 to 15 observations (± s.e.). A, plasma corticosterone; B, adrenal corticosterone; C, adrenal weight; D, adrenal ascorbic acid.

Results

THE ADRENAL ASCORBIC ACID, CORTICOSTERONE AND ADRENAL WEIGHT CHANGES AFTER URETHANE

The results are summarised in Fig. 1. The plasma and adrenal corticosterone levels were elevated and the adrenal ascorbic acid levels were depleted throughout the period of anaesthesia. The adrenal glands increased in weight by 25%. These changes suggested that there was prolonged hypersecretion of ACTH from the pituitary. The corticosterone in the plasma reached a maximum concentration of 39·4 μg/100 ml in the

first 3 hr and subsequently declined to 26·9 μg/100 ml at 8 hr. The adrenal corticosterone changes exhibited a similar pattern during the first 8 hr. After 8 hr the plasma corticosterone again increased significantly ($P < 0{\cdot}01$) whereas the adrenal level continued to fall. During this period the adrenal ascorbic acid concentration recovered from the minimum level of 250 mg% at 3 hr to 340 mg% at 24 hr. After 8 hr there was marked haemoconcentration.

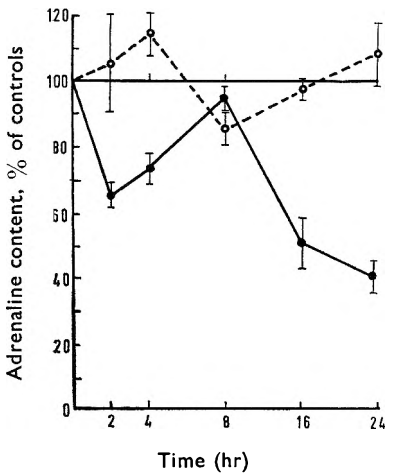

FIG. 2. Adrenal adrenaline levels during either urethane (continuous line) or phenobarbitone (broken line) anaesthesia. Each point is the mean of 6 to 9 observations. Adrenaline content expressed as percentage of control values (± s.e.).

THE ADRENALINE CONTENT OF THE ADRENAL GLANDS DURING URETHANE ANAESTHESIA

Fig. 2 shows that there was a triphasic variation of the adrenaline content during urethane anaesthesia. There was a significant depletion during the first 4 hr which was followed by a significant recovery to normal at 8 hr. Subsequently there was another very large depletion of adrenaline, reaching over 50% at 24 hr. Although blood adrenaline levels were not determined, it is reasonable to suggest that during the first 4 hr and subsequent to 8 hr of urethane anaesthesia there are elevated levels of adrenaline in the blood.

COMPARISON OF PHENOBARBITONE AND URETHANE ANAESTHESIA WITH REGARD TO CORTICOSTERONE AND ADRENALINE CHANGES

To study whether the hormonal changes described for rats under urethane anaesthesia were features of general anaesthesia irrespective of the anaesthetic, the steroid and adrenaline changes were investigated after phenobarbitone. Fig. 3 shows that during the first 4 hr of phenobarbitone treatment, there was only a slight alteration of the plasma level and no alteration of the adrenal steroid level. Fig. 2 shows there was no

alteration of the adrenal adrenaline for the first 4 hr, but subsequently there was a depletion of the amine content. After 8 hr there was an increase of the plasma and adrenal steroid concentrations.

Thus whilst phenobarbitone had little effect on the plasma and adrenal steroid levels and adrenal adrenaline content during the first 4 hr, urethane had a marked effect. Phenobarbitone caused an adrenaline depletion after 4 hr, whereas during urethane anaesthesia the amine level showed a return towards the control value. After 8 hr the picture was even more complicated; however, both urethane and phenobarbitone caused a marked elevation of the plasma steroid concentration. At the same time urethane induced a significant depletion of adrenaline whereas phenobarbitone had no effect.

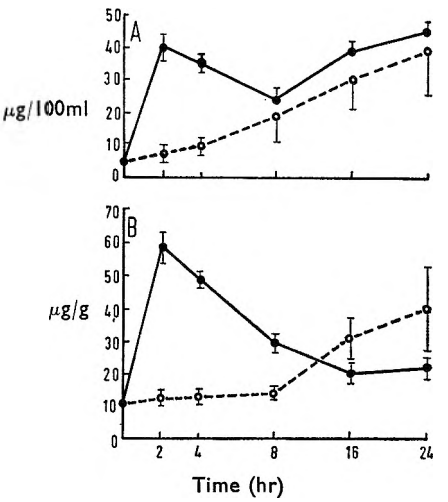

FIG. 3. Changes of plasma and adrenal corticosterone concentrations during either urethane (continuous line) or phenobarbitone (broken line) anaesthesia. Each point is the mean of 4 to 15 observations (± s.e.). A, plasma corticosterone; B, adrenal corticosterone.

CORTICOSTERONE LEVELS DURING URETHANE ANAESTHESIA IN INTACT AND ADRENAL DEMEDULLATED RATS

To determine whether the secretion of adrenaline was responsible for the changes of plasma and adrenal corticosterone, the effects of urethane in adrenal-demedullated rats were examined. There was an initial increase of plasma and adrenal corticosterone in the demedullated rats for the first 4 hr followed by a significant fall at 8 hr. This result was similar to that found in the intact rat (Fig. 4) and indicated that the release of adrenaline from the adrenal medulla played no major role in the release of corticosterone during the first 8 hr of urethane anaesthesia. Subsequent to 8 hr, the fall in plasma level continued in the demedullated rats whereas the intact rats showed a further rise. However, the adrenal corticosterone concentration returned towards the control level in both groups.

THE EFFECT OF A STRESSFUL STIMULUS AFTER 24 HR URETHANE ANAESTHESIA

The effects of injection of ACTH and exposure to ether vapour after prolonged activation of the pituitary-adrenal axis were investigated (Fig. 5). Both stimuli caused significant ($P < 0{\cdot}05$) increases in both the plasma and adrenal corticosterone concentrations. Thus 24 hr of

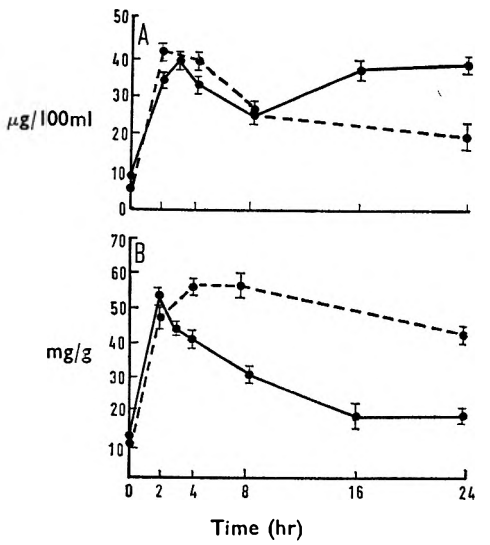

FIG. 4. Corticosterone changes in intact (continuous line) and adrenal demedullated (broken line) rats during urethane anaesthesia. Each point is the mean of 4 to 15 observations (± s.e.). A, plasma corticosterone; B, adrenal corticosterone.

urethane anaesthesia did not prevent the release of ACTH from the pituitary or prevent the adrenal gland from synthesising or releasing the steroid. However, neither ACTH nor the ether stimulus depleted the adrenal ascorbic acid level despite this having recovered by 100 mg% from the maximally depleted value. This suggested that the sensitivity of the adrenal ascorbic acid index to ACTH during the repletion phase was altered and therefore not suitable as a measure of pituitary-adrenal activation.

Discussion

Urethane anaesthesia produced a remarkably prolonged hypersecretion of ACTH from the pituitary gland as measured by all the indices of pituitary-adrenal activity used. Hermansky, Pudlach & Dienster (1955) also showed that urethane induced a release of adrenocortical hormones as indicated by the peripheral lytic effect on leucocytes.

As continuous anaesthesia had been shown to cause adrenocortical activation (Barrett & Stockham, 1963) it was possible that the hormonal changes were due to general anaesthesia. Phenobarbitone anaesthesia did not change the plasma or adrenal corticosterone level during the first 8 hr, but after this period there was an increase in toxicity, 16 out of

32 died by 8 hr and 47 out of 51 by 24 hr, and the animals which survived showed a rise in plasma and adrenal steroid concentrations. Gorby, Leonard, Ambrus & Harrison (1953) reported that corticoids increased the toxicity of phenobarbitone. The change in adrenal corticosterone was very variable and there was no significant increase in adrenal weight. These factors suggested that the hormonal changes after 8 hr treatment were related to the toxic effect of the drug. These results cast doubt on the validity of using a rise in plasma corticosterone as an index of ACTH release in these circumstances. It was concluded that the steroid changes during urethane anaesthesia were not simply resultant of general anaesthesia.

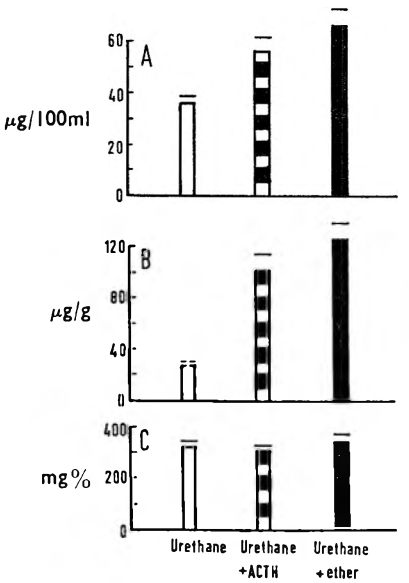

FIG. 5. The effect of the ether stimulus or ACTH on corticosterone levels (A, plasma; B, adrenal) and adrenal ascorbic acid levels (C) after 24 hr urethane anaesthesia. Each value is the mean of 7 to 11 observations. The horizontal bars represent one standard error.

No direct evidence for a central action of urethane on the anterior pituitary was presented since the drug was extremely toxic in hypophysectomised rats. Urethane was also toxic in adrenalectomised rats.

Urethane activated the adrenal medulla, which was in agreement with Kodama (1930), who recorded that the secretion of amines was prevented by section of the splanchnic nerves. Aub, Bright & Forman (1922) showed that the hyperglycaemia during urethane anaesthesia was due to the secretion of adrenaline from the adrenal gland, and De (1946) reported that this persistent hyperglycaemia was reduced after section of the hypothalamus. This evidence implied that there was a central excitation under the anaesthetic which induced the secretion of adrenaline from the adrenal gland.

The changes in plasma and adrenal corticosterone were concurrent with the depletion of adrenal adrenaline for both urethane and phenobarbitone anaesthesia. The stimulation of the adrenal cortex may have followed an increase in blood levels of adrenaline. However, the increments in plasma and adrenal corticosterone under urethane anaesthesia were not altered by adrenal demedullation. Therefore it was likely that the activation of both divisions of the adrenal gland was of central origin. Additional evidence for a central effect on the anterior pituitary was presented by Fuhrmann (1950) who described an increased release of pituitary gonadotrophins.

It was probable that the changes in the plasma steroid level were not mediated by one mechanism since the response was triphasic in nature. During the second rise in the plasma level after 8 hr there was no concomitant increase in adrenal corticosterone concentration and the adrenal ascorbic acid concentration recovered by 100 mg%, but there was a further adrenaline depletion from the gland. After 8 hr there was a pronounced haemoconcentration and Doljanski & Rosin (1944) reported that the liver underwent serious degenerative changes with extended anaesthesia. These observations indicated that the second rise in the plasma corticosterone was not due to a further release of ACTH from the pituitary but was mediated by general deterioration of the animal.

24 hr after injection of urethane the rats were still anaesthetised, the plasma corticosterone was 960%, and the adrenal corticosterone was 230% above the control values. Both ACTH injection and the exposure to the ether vapour produced significant increases in the plasma and adrenal corticosterone levels. Therefore prolonged hyperactivity of the pituitary-adrenal system had not reduced the capacity to respond to a stress. It was of interest that a high plasma corticosterone level over a long period had not depressed the pituitary or adrenal gland by a feedback mechanism. The increments of adrenal corticosterone concentrations for both stimuli were significantly ($P < 0{\cdot}01$) greater than in the previously untreated rat, which may be related to the 25% increase in adrenal weight over 24 hr. However, the increments in the plasma corticosterone were 20% smaller than in the untreated rat. The reason for this is not clear, but recently Kolthoff, Macch & Wyman (1963) demonstrated that rats with regenerated adrenal glands, although synthesising corticosteroids in response to a stress in a normal manner, failed to exhibit a large rise in plasma steroid level due to adrenal circulatory changes. The hypersecretion of adrenaline with urethane anaesthesia may therefore have affected the circulation of blood through the adrenal gland and thus modified the response to a stress.

Acknowledgements. We wish to thank Dr. J. S. Tindall of Organon Laboratories for the generous gift of a sample of corticosterone. The investigation was made during the tenure by T.L.B.S. of a grant from the Medical Research Council and by M.A.S. of a grant from the Pharmaceutical Society of Great Britain.

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J. Pharm. Pharmacol., 1964, **16**, 611–617 Received March 13, 1964

Inactivation of a bacteriophage by chemical antibacterial agents

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An extinction method has been used to examine the inactivation of coliphage T6r by a number of chemical antibacterial agents. A marked difference in the effect of concentration on inactivation efficiency has been shown for different agents, chloramine-T and formaldehyde having concentration exponents of approximately 2 and 3 respectively; crystal violet, cetrimide and phenol having concentration exponents of approximately 11, 13 and 15 respectively. It is suggested that a low concentration exponent is associated with inactivation of the phage by an effect on the protein coat of the particle and a high concentration exponent with an effect on its internal structure.

AN extinction method of examining the inactivation of phage has been previously described (Cook & Brown, 1963). This method has now been applied to a study of the effect of a number of chemical antibacterial agents on a coliphage.

Experimental

Bacteriophage and bacterial host. The phage used was a coliphage with the cultural characteristics of strain T6r (Demerec & Fano, 1945; Hershey, 1946) and the bacterial host a laboratory strain of *Escherichia coli* sensitive to the phage. The methods and media used for cultivating the host and preparing phage stocks were those previously described (Cook & Brown, 1963). A single phage stock suspension, stored at 5°, was used throughout the experiments on phage inactivation. The titre of this stock, as determined by periodic plaque counts using a modification of the method described by Williams-Smith (1951, 1953), was $4{\cdot}06 \pm 0{\cdot}29 \times 10^{10}$ ($P = 0{\cdot}95$) phage particles per ml.

Antibacterial agents. From a number of preliminary experiments, 5 antibacterial agents showing relatively high viricidal activity against phage were selected. The substances used were cetrimide and crystal violet of the British Pharmacopoeia 1958, phenol (Analar) and formaldehyde solution (Analar, 36·83% CH_2O), and chloramine-T (British Drug Houses Laboratory Regent). Experimental solutions of chloramine-T and formaldehyde were prepared daily as required, those of the other agents were prepared immediately before use by diluting stock solutions stored in the dark at ambient room temperature (20–25°). All solutions were prepared using sterile distilled water.

Inhibition of phage growth. The bacteriostatic activity of each agent for the bacterial host was determined using a serial tube dilution method. The effect of sub-bacteriostatic concentrations of the agents on the rate of mass lysis in phage cultures was examined by the same method using phage inocula of various titres (Cook & Brown, 1963).

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Inactivation of extracellular phage. The method used was that previously described in detail (Cook & Brown, 1963), and consisted, essentially, of adding a standard phage inoculum (4×10^8 phage particles per ml of reaction mixture) to a solution of the agent under test, immediately removing a number of samples of the mixture, diluting these samples with peptone water after suitable time intervals, inoculating the dilution with bacterial host cells and incubating the cultures at 37°. The presence of surviving infective phage was shown by visible lysis in the incubating cultures. The time of contact of the phage and antibacterial agent after which no infective phage can be detected is now defined as the Extinction Time.

Each agent was used in a series of ranging tests until the concentration range giving conveniently measurable Extinction Times was established. The Mean Extinction Time (M.E.T.) for each of several concentrations in this range was then determined, each estimate of M.E.T. being based on not less than 5 replicate determinations. (The term "Mean Extinction Time" is now preferred to "Mean Inactivation Time", used previously, Cook & Brown, 1963).

In all tests the reaction mixture sample volume taken was 6 drops (approximately 0·1 ml) from a standardised dropping pipette. To ensure dilution of the antibacterial agent to a concentration which would permit the detection of small numbers of infective phage particles, the samples were normally diluted with 20 ml of peptone water. In the tests on formaldehyde 100 ml volumes of peptone water were used to achieve the required dilution.

Results

Inhibition of phage growth. The inhibitory action of the antibacterial agents on the growth of the bacterial host is shown in Table 1.

TABLE 1. GROWTH OF *E.COLI* IN THE PRESENCE OF ANTIBACTERIAL AGENTS (IN PEPTONE WATER AT 37°)

Antibacterial agents	Maximum concentration permitting		Minimum concentration inhibiting growth in all replicates (M)
	approximately normal rate of growth (M)	growth in all replicates after 48 hr (M)	
Cetrimide* ..	$2{\cdot}7 \times 10^{-6}$	$5{\cdot}5 \times 10^{-6}$	$2\,7 \times 10^{-5}$
Chloramine-T ..	$8{\cdot}9 \times 10^{-4}$	$2{\cdot}5 \times 10^{-3}$	$2\,8 \times 10^{-3}$
Crystal violet ..	$1{\cdot}0 \times 10^{-6}$	$2{\cdot}0 \times 10^{-5}$	$5\,0 \times 10^{-6}$
Formaldehyde ..	$3{\cdot}3 \times 10^{-4}$	$1{\cdot}2 \times 10^{-3}$	$1\,8 \times 10^{-3}$
Phenol	$2{\cdot}7 \times 10^{-3}$	$2{\cdot}1 \times 10^{-2}$	$2\,7 \times 10^{-2}$

* Molarity calculated assuming sample to be pure cetyltrimethylammonium bromide

Mass lysis occurred in phage cultures containing all concentrations of the antibacterial agents permitting growth of the host cells. Where the concentrations of the agents were low enough to permit an approximately normal rate of growth of host cells, mass lysis occurred at a normal rate but in those concentrations giving a slow host growth rate lysis was correspondingly delayed.

Inactivation of extracellular phage. The results of the later experiments on the inactivation of extracellular phage are illustrated in Fig. 1. The results for phenol have previously been reported in detail (Cook & Brown, 1963) but have been summarised and included here for comparison.

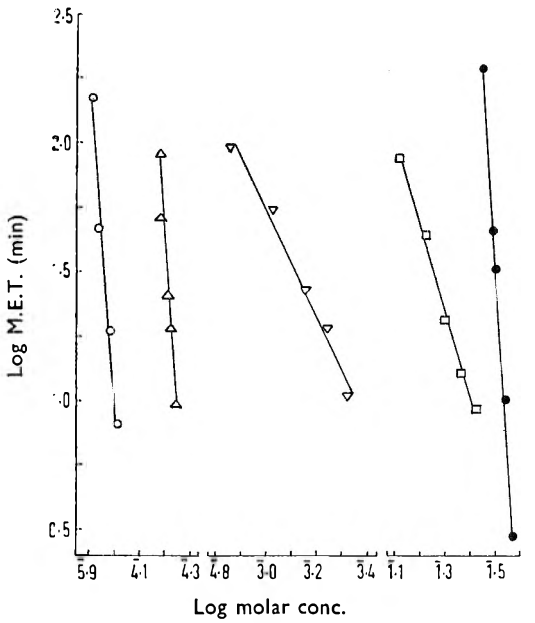

FIG. 1. Relation between concentration of antibacterial agent and Mean Extinction Time (M.E.T.) for the inactivation of coliphage T6r (25°). Cetrimide △—△; Chloramine-T ▽—▽; Crystal Violet ○—○; Formaldehyde □—□; Phenol ●—●.

For each antibacterial agent, the regression of log M.E.T. upon log molar concentration was shown to be linear (Table 2). The slope of such a

TABLE 2. REGRESSION OF LOG M.E.T. UPON LOG MOLAR CONCENTRATION FOR THE INACTIVATION OF COLIPHAGE T6r

Antibacterial agent	Correlation coefficient	Regression coefficient	Standard deviation of regression coefficient
Cetrimide ..	0·9972	− 13·28	0·57
Chloramine-T ..	0·9896	− 2·00	0·17
Crystal violet ..	0·9964	− 10·85	0·65
Formaldehyde ..	0·9966	− 3·35	0·16
Phenol	0·9986	− 15·20	0·47

(The regression coefficient here equals the concentration exponent, n.)

regression represents the concentration exponent (n) which can be defined (Watson, 1908) by the expression,

$$n = \frac{\log t_2 - \log t_1}{\log c_1 - \log c_2}$$

where c_1 and c_2 are concentrations giving M.E.T. of t_1 and t_2. The calculated value of the regression coefficients (or slopes) are shown in Table 2 together with their standard deviations.

Comparison of the slopes of the regressions of log M.E.T. upon log molar concentration for different agents indicated that they could not be considered parallel (Table 3).

TABLE 3. COMPARISON OF REGRESSION COEFFICIENTS FOR THE REGRESSION OF LOG M.E.T. UPON LOG MOLAR CONCENTRATION

Comparing regression coefficients of:	Variance of difference in regression coefficients	t	Degrees of freedom	Corresponding probability
Cetrimide and phenol	0·6222	3·092	6	0·02 to 0·05
Cetrimide and crystal violet ..	0·8504	2·860	5	0·02 to 0·05
Phenol and crystal violet	0·6009	7·283	5	about 0·001
Chloramine-T and formaldehyde ..	0·06729	20·11	6	less than 0·001

Discussion

In the experiments on the inhibition of mass lysis in phage cultures, no evidence of the selective inhibition of phage growth was found with any of the antibacterial agents tested. The selective inhibition of phage growth reported by Graham & Nelson (1954) for lactic streptococcus phages, was not found to occur with coliphage T6r as tested here.

The reduced rate of growth of host cells and the consequent reduction in the rate of lysis in phage cultures containing concentrations of antibacterial agents approaching bacteriostatic concentrations has a decided significance in the present work. The level of dilution of the reaction mixture samples in the tests for phage inactivation was selected so as to ensure the reduction of the concentration of antibacterial agents to one which had been shown to permit a normal rate of growth of host cells and mass lysis by phage. Under these conditions small numbers of surviving infective phage can be readily detected.

The 5 antibacterial agents examined can be arranged in ascending order of efficiency in inactivating extracellular phage as phenol, formaldehyde, chloramine-T, cetrimide and crystal violet.

The most interesting feature, however, of the results is the difference found in the effect of changes of concentration on their activity, expressed as the concentration exponent (n).

The validity of using n as a basis for speculation on the mechanisms of the inactivation of bacteria by bactericides has in the past, been the subject of some argument (Rahn, 1945). Much of the early criticism of this use of n was based on its variability, resulting largely from the use of imprecise methods of determination such as those used by Tilley (1939) on whose results Rahn's arguments were largely based. It can now be accepted that, provided the determination of n is sufficiently precise, significant variations in values of n between the action of different

bactericides on one organism at least gives an indication that the mechanism of inactivation in each case is different. It has been concluded from the present work that the value of n has an equal significance in the inactivation of phage by antibacterial agents.

If this significance of n is accepted the 5 antibacterial agents examined in detail fall into 2 general groups; chloramine-T and formaldehyde with relatively low values of n (approximately 2 and 3 respectively) in one group, and crystal violet, cetrimide and phenol with values of n greater than 10 (approximately 11, 13 and 15 respectively) in the other. It should be noted that, within each group, the slopes of the regression from which the values of n were derived have been found to show significant departure from parallelity when tested statistically. The possibility therefore exists that there are differences in the mechanism of inactivation among the members of each group.

The question which now arises is, can any similarity be found between the possible general mechanisms of inactivation of phage by the substances in each group?

The action of formaldehyde on extracellular phage has been extensively studied and, while the mode of action has not been fully elucidated, the consensus of opinion is that the phage is inactivated by the combination of the formaldehyde with the protein coat of the phage particle. Sauerbier (1960) has suggested that formaldehyde inactivates T1 phage, not by protein damage but by reacting with the phage deoxyribonucleic acid (DNA) and the demonstration by Mutsaars (1957a, b, 1959) that multiplicity reactivation occurs in formaldehyde inactivated coliphage-N also implies DNA damage. On the other hand it was clearly demonstrated by Hershey & Chase (1952) that T2 coliphage, inactivated by formaldehyde, is still adsorbed on to its host but the phage DNA is not released. A similar reaction was reported by Bourgaux (1957) for coliphage-N treated with formaldehyde. It has further been clearly established (Staehelin, 1958; Berns & Thomas, 1961; Grossman, Levine & Allison, 1961) that formaldehyde will not react with amino-groups involved in strong hydrogen bondings as is the case in DNA. That formaldehyde forms an unstable combination with phage is indicated by the ease with which formaldehyde inactivated T-group phages are reactivated by storage in the presence of histidine (Heicken & Spicher, 1959). Such an unstable combination implies that the formaldehyde combines with some part of the protein coat of the phage.

No attempts have been made to investigate the mechanism of action on phage of compounds liberating chlorine. Indeed, little information is available on the mechanism of action of these compounds on bacteria although it seems clear that "chlorine compounds" including the chloramines, attack bacteria through the undissociated hypochlorous acid formed by the interaction of the liberated chlorine with water (Marks, Wyss & Strandskov, 1945; Hadfield, 1957; Brazis, Leslie, Kabler & Woodward, 1958). Chlorine is known to have an intense reactivity with proteinous material (Sykes, 1958) and it seems likely that the site of the attack on phage by chloramine-T is the protein coat.

Both formaldehyde and chlorine react strongly with organic matter so that the inactivation time estimates for these two agents will be particularly affected by the presence of peptone in the phage inoculum. The effect will be to make the M.E.T., determined experimentally, longer than the true inactivation time. This effect will be most pronounced with low concentrations of the agents so that the value of n obtained will be greater than the true value although the size of the effect is a matter for speculation. Since the arguments on the significance of the values of n found here are based on the smallness of n for formaldehyde and chloramine the effect, in any case, does not invalidate the argument.

The mode of inactivation of phage by crystal violet has not been investigated but it seems likely, from the known affinity of crystal violet for DNA (Stearn, 1930; Mirsky & Ris, 1951; Graham & Nelson, 1954) that it occurs by combination with phage DNA. That ability to combine with free DNA does not necessarily confer the ability to produce marked inactivation of intact phage is shown by the acridines, for which combination with free DNA has been proved (Peacocke & Skerret, 1956; Luzzati, Masson & Lerman, 1961) but which have little or no effect on free phage (Hotchin, 1951).

The prevalent theory of the mode of action of the quaternaries (including cetrimide) on bacteria is that their main effect is to cause an increase in the permeability of the cytoplasmic membrane and a lethal loss of intracellular constituents (Salton, 1951; Stedman, Kravitz & King, 1957). This effect has been suggested by Gilby & Few (1957) to be caused by the interaction of the compounds with phospholipids in the cytoplasmic membrane. The action of phenol on bacteria has been shown to be very similar to that of cetrimide (Gale & Taylor, 1947; Maurice, 1952; Stedman & others, 1957).

It is therefore suggested that both cetrimide and phenol act on phage in a similar manner by affecting the association of the protein coat of the phage with the DNA (without necessarily causing complete dissociation of the two) or by denaturing both the protein coat and the "internal protein" which has been shown by Spizizen (1957) to play an essential part in initiating phage multiplication within the host cells.

It is suggested that, when the inactivation of a phage by an antibacterial agent has a low value of n, the agent is attacking the protein coat of the particle. When the value of n is high then the attack is on the internal structure of the phage, either by combination with the DNA, as in the case of crystal violet, or by affecting the association of the DNA with the protein content of the phage, as with cetrimide and phenol. The determination of an accurate value for n may be a useful preliminary indication of the site of action of an antibacterial agent on a phage.

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J. Pharm. Pharmacol., 1964, **16**, 618–626 Received February 27, 1964

Synthesis of aliphatic amino-acid hydrazides as potential tuberculostatic agents

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Some dialkylamino-aliphatic acid hydrazides and bis(hydrazinocarbonyl) compounds have been prepared and tested for antituberculosis activity. None of these compounds was found to possess tuberculostatic activity of the same order as that of isoniazid.

THE success of isoniazid in the treatment of tuberculosis has led to the preparation and testing for antituberculosis activity of many other acid hydrazides and related compounds (Steenken & Wolinsky, 1952; Bernstein, Jambor, Lott, Pansy, Steinberg & Yale, 1953; Bavin, James, Kay, Lazare & Seymour, 1955). In the aliphatic series the most effective acid hydrazide tested was cyanacethydrazide (Hartyl, 1954), which was claimed to be comparable to isoniazid in the treatment of human pulmonary tuberculosis (Kirshner, 1957). *In vitro* experiments on other aliphatic acid hydrazides and related compounds showed that some of them possessed similar or greater activity than cyanacethydrazide (Mukherjee, Naha, Raymahasaya, Laskar & Gupta, 1955).

Long-chain fatty acids are known to have a marked inhibitory effect on *Mycobacterium tuberculosis*, and replacement of the carbonyl group by a dialkylaminoalkyl group has also produced active compounds. Furthermore, displacement of the carbonyl group toward the centre of the molecule has been shown to increase the activity of the fatty acid (Stanley, Coleman, Greer, Sacks & Adams, 1932).

In view of these observations, it was decided to synthesise a series of ω-dialkylamino-aliphatic acid hydrazides (I ; R = Me, Et, or Pr, and n = 1, 2, 3, or 4), by means of which the effect, on tuberculostatic activity, of a dialkylamino-group and also that of varying chain-length, could be studied. The effect of introducing a second hydrazide group with the amino-group in the centre of the molecule was later investigated. For this purpose, a smaller series of compounds (II ; R = H, Me, or Et)

$R_2N{\cdot}[CH_2]_n{\cdot}CO{\cdot}NH{\cdot}NH_2$ (I) $RN(CH_2{\cdot}CH_2{\cdot}CO{\cdot}NH{\cdot}NH_2)_2$ (II)

was prepared, in which the distances between the amino-group and each of the hydrazide groups corresponded with the chain-length of the most active of the monohydrazides.

All the hydrazides were prepared by the interaction of the corresponding ethyl or methyl ester with hydrazine hydrate in ethanolic solution. The routes by which these esters were prepared depended on the chain-lengths

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of the compounds. Ethyl diethylaminoacetate and the corresponding dimethylamino- and dipropylamino- compounds were obtained by the interaction of ethyl chloroacetate and the appropriate dialkylamine.

Ethyl β-diethylaminopropionate (III; R = Et) was prepared in good yield from ethyl acrylate and diethylamine by the method of Adamson (1949). Ethyl β-dimethylaminopropionate (III; R = Me) and ethyl

$$R_2N{\cdot}CH_2{\cdot}CH_2{\cdot}CO{\cdot}O{\cdot}C_2H_5$$

(III)

β-dipropylaminopropionate (III; R = Pr) were prepared similarly. Ethyl γ-diethylaminobutyrate (IV; R = Et, $n = 1$) was prepared by chain extension according to the following scheme:

$$R_2N{\cdot}[CH_2]_n{\cdot}CO{\cdot}O{\cdot}Et \xrightarrow{LiAlH_4} R_2N{\cdot}[CH_2]_{n+1}{\cdot}OH \xrightarrow[(2)\ NaOH]{(1)\ SOCl_2} R_2N{\cdot}[CH_2]_{n+1}{\cdot}Cl \xrightarrow[NaOC_2H_5]{CH_2(CO{\cdot}O{\cdot}Et)_2} R_2N{\cdot}[CH_2]_{n+1}{\cdot}CH(CO{\cdot}O{\cdot}Et)_2$$

$$\xrightarrow[reflux]{HCl} R_2N{\cdot}[CH_2]_{n+2}{\cdot}CO_2H{\cdot}HCl \xrightarrow[H_2SO_4]{EtOH} R_2N{\cdot}[CH_2]_{n+2}{\cdot}CO{\cdot}O{\cdot}Et$$

(IV)

Ethyl γ-dimethylaminobutyrate (IV; R = Me, $n = 1$), ethyl γ-dipropylaminobutyrate (IV; R = Pr, $n = 1$) and ethyl δ-diethylaminovalerate (IV; R = Et, $n = 2$) were similarly prepared.

The method of preparing the last named compound gave poor yields in the final stages of hydrolysis, decarboxylation and re-esterification. An alternative scheme was therefore adopted for the syntheses of dimethylamino- and dipropylamino-valerates. The route shown below led to the formation of the methyl, instead of the ethyl, esters.

$$HO_2C{\cdot}[CH_2]_4{\cdot}CO{\cdot}O{\cdot}Me \xrightarrow{AgON_3} AgO_2C{\cdot}[CH_2]_4{\cdot}CO{\cdot}O{\cdot}Me \xrightarrow{Br_2} Br{\cdot}[CH_2]_4{\cdot}CO{\cdot}O{\cdot}Me \xrightarrow{R_2NH} R_2N{\cdot}[CH_2]_4{\cdot}CO{\cdot}O{\cdot}Me$$

Di(2-ethoxycarbonylethyl)methylamine (V; R = Me) was prepared by the reaction of methylamine in ethanol with two equivalents of ethyl

$$RN(CH_2{\cdot}CH_2{\cdot}CO{\cdot}O{\cdot}Et)_2$$

(V)

acrylate. Di(2-ethoxycarbonylethyl)amine (V; R = H) was similarly prepared, using a solution of ammonia in ethanol in place of the solution of methylamine, and di(2-ethoxycarbonylethyl)ethylamine (V; R = Et) was prepared from it by reaction with ethyl iodide.

The dihydrochloride of δ-dipropylaminovalerohydrazide, monohydrochlorides of dimethylaminoacethydrazide and β-dimethylaminopropionhydrazide, and the salicylate of diethylaminoacethydrazide were obtained as colourless crystalline solids. No crystalline condensation products with either acetone or benzaldehyde were obtained from any of the hydrazides.

Infra-red spectra were obtained for all the hydrazides prepared, as an aid to characterisation of these compounds. As no information was available on the absorption due to the hydrazide grouping, formhydrazide, acethydrazide and butyrohydrazide were prepared and their spectra obtained. In the infra-red spectrum of formhydrazide, shown in Fig. 1,

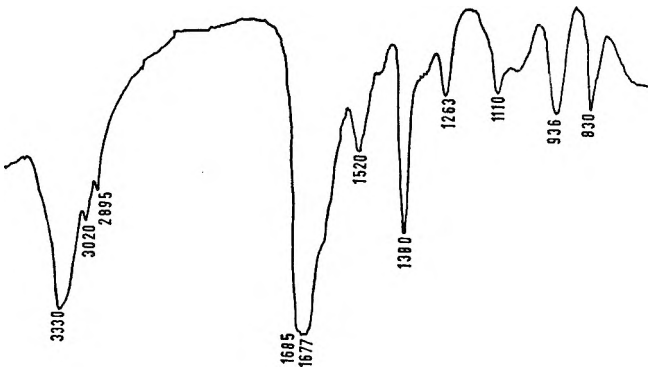

FIG. 1. Infra-red spectrum of formhydrazide, KCl disc.

the only bands due to $\rightarrow$CH occur at 2895 cm^{-1} and 1380 cm^{-1}. The other bands are therefore due to vibrations occurring in the hydrazide grouping of this compound. The spectrum of δ-dipropylaminovalerohydrazide is shown in Fig. 2, and the other hydrazides prepared gave very similar spectra.

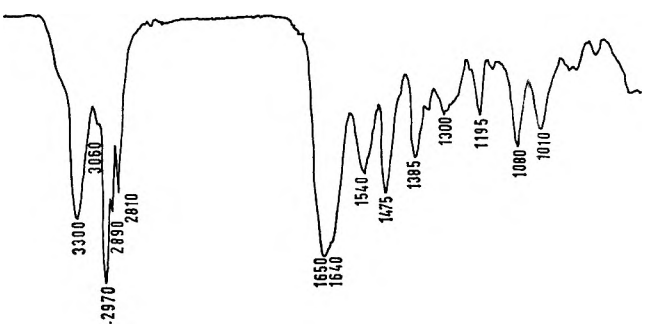

FIG. 2. Infra-red spectrum of δ-dipropylaminovalerohydrazide. Liquid film.

In the range 3,010–3,360 cm^{-1} two bands, one strong and one weak, usually occurred in the spectra of these compounds. There were also two strong bands in the range 1,635–1,685 cm^{-1}, and another between 1,505 and 1,550 cm^{-1}. All the dimethylaminohydrazides showed a band at 1,170–1,175 cm^{-1}, the diethylaminohydrazides at 1,200–1,205 cm^{-1}, and the dipropylamino-hydrazides at 1,190–1,195 cm^{-1}. With several compounds, strong and medium bands occurred below 1,000 cm^{-1}, but had no apparent correlation with the structures of the compounds.

BACTERIOLOGICAL RESULTS

Preliminary assessment of the tuberculostatic activity of the hydrazides prepared was obtained against *Mycobacterium tuberculosis* var. *hominis*, (H37 Rv) in Peizer and Schechter medium. Some of the results are shown in Table 1.

TABLE 1. TUBERCULOSTATIC ACTIVITY OF HYDRAZIDES PREPARED

Compound	Inoculation (days	Minimum inhibitory concentration (μg/ml)	
		Test 1	Test 2
Dimethylaminoacethydrazide	14	—	—
	21	>100	>100 (±100)
γ-Diethylaminobutyrohydrazide	14	—	—
	21	>100 (±100)	>100 (±100)
δ-Diethylaminovalerohydrazide	14	—	—
	21	>100	>100 (±100)
δ-Dipropylaminovalerohydrazide	14	—	—
	21	>100 (±100)	>100
Di(2-hydrazinocarbonylethyl)amine	14	100 (±25)	100 (±50)
	21	100	100
Di(2-hydrazinocarbonylethyl)methylamine ..	14	>100 (±100)	>100 (±100)
	21	>100	>100 (±100)
Di(2-hydrazinocarbonylethyl)ethylamine ..	14	100 (>50)	100
	21	>100	>100
Isoniazid	14	—	—
	21	0·02	—

(±) signifies concentrations at which there was partial inhibition of growth.

None of the compounds examined was found to possess tuberculostatic activity of the same order as that of isoniazid. The dialkylamino-aliphatic acid hydrazides are less active than the bis(hydrazinocarbonyl) compounds. Alkylation of the amino-group appears to decrease the activity to some extent, the secondary amino-compound di(2-hydrazino-carbonylethyl)amine being the most active of the compounds examined.

Experimental

Ethyl dimethylaminoacetate was prepared by the method of Viscontini & Meier (1950).

Ethyl diethylaminoacetate. Ethyl chloroacetate (100 g), benzene (350 ml) and diethylamine (120 g) were mixed and allowed to stand for 18 hr. The reaction mixture was extracted with dilute hydrochloric acid, and the benzene layer containing neutral ethyl chloroacetate was discarded. The aqueous layer was treated with a slight excess of sodium hydroxide solution, extracted with ether, and the ethereal solution dried (Na_2SO_4). Ethyl diethylaminoacetate was obtained, after removal of solvent, as a colourless oil, b.p. 67–68°/13 mm, $n_D^{21.5}$ 1·4230 (75·6 g).

Ethyl dipropylaminoacetate was prepared from ethyl chloroacetate (56 g) by the method used in the preparation of ethyl dimethylamino-acetate, and was obtained as a colourless oil, b.p. 96°/14 mm, $n_D^{20.5}$

1·4271 (65·5 g). Found: equiv. 192·9; N, 7·2%. $C_{10}H_{21}NO_2$ requires equiv. 187·2; N, 7·5%.

Ethyl β-dipropylaminopropionate. Ethyl acrylate (100 g) and dipropylamine (100 g) were mixed and allowed to stand for 8 days. Fractional distillation yielded ethyl β-dipropylaminopropionate as a colourless oil, b.p. 100°/13 mm, n_D^{21} 1·4310 (170 g).

Di(2-ethoxycarbonylethyl)amine. An ethanolic solution of ammonia (170 ml 5%) was slowly added (1 hr) to ethyl acrylate (100 g), chilled in an ice-bath, and allowed to stand for 7 days. After evaporation of solvent, fractional distillation yielded the product as a colourless oil, b.p. 105–108°/0·3 mm, n_D^{16} 1·4429 (15·9 g). Found: N, 6·3%. $C_{10}H_{19}NO_4$ requires: N, 6·5%.

Di(2-ethoxycarbonylethyl)methylamine. A solution of methylamine in ethanol (55 ml 33%) was slowly added (45 min) to ethyl acrylate (100 g) cooled in an ice-bath, and allowed to stand for 4 days. Fractional distillation yielded the product as a colourless oil, b.p. 104–105°/0·5 mm, n_D^{19} 1·4407 (53·9 g). Found: N, 6·1%. $C_{11}H_{21}NO_4$ requires: N, 6·1%.

Di(2-ethoxycarbonylethyl)ethylamine. Di(2-ethoxycarbonylethyl)amine (20 g) and ethyl iodide (20 g) were mixed and heated under reflux for 30 min. Excess ethyl iodide was removed under reduced pressure, and the yellow viscous residue was dissolved in water, made alkaline with sodium hydroxide solution, and extracted with ether. After drying (Na_2SO_4), the solvent was evaporated to yield on distillation, the product as a colourless oil, b.p. 96–100°/0·35 mm, n_D^{20} 1·4410 (10·9 g). Found: N, 5·7%. $C_{12}H_{23}NO_4$ requires: N, 5·7%.

2-*Dipropylaminoethanol* was prepared by lithium aluminium hydride reduction of ethyl dipropylaminoacetate (134·5 g) and was obtained as a colourless oil, b.p. 81–82°/13 mm, $n_D^{20·5}$ 1·4378 (83·1 g).

3-*Dipropylaminopropanol* was prepared by lithium aluminium hydride reduction of ethyl β-diethylaminopropionate (150 g) and was obtained as a colourless oil, b.p. 76–77°/10 mm, n_D^{21} 1·4340 (96 g).

2-*Chloroethyldimethylamine.* Thionyl chloride (135 ml) in ether was added slowly (3 hr) to a stirred solution of 2-dimethylaminoethanol (150 g) in ether, and the solvent was carefully evaporated. The solid residue was dissolved in water, chilled in an ice-bath, and the solution made alkaline with sodium hydroxide solution. The oil which separated was extracted with ether, the ethereal solution dried (Na_2SO_4), and the solvent removed under reduced pressure to yield an amber-coloured oil, n_D^{16} 1·4287 (105 g). The product was not further purified.

2-*Chloroethyldiethylamine* was prepared from 2-diethylaminoethanol (31 g) by the above method and was obtained as a colourless oil, b.p. 50°/20 mm, n_D^{17} 1·4379 (22·3 g).

2-*Chloroethyldipropylamine* was prepared from 2-dipropylaminoethanol (83 g) by the above method and was obtained as a brown oil, n_D^{21} 1·4396 (85 g), which was not further purified.

3-*Chloropropyldiethylamine was prepared from* 3-diethylaminopropanol (96 g) by the above method and was obtained as an amber-coloured oil, n_D^{21} 1·4408 (70 g), which was not further purified.

3,3-*Diethoxycarbonyl)propyldimethylamine.* Diethyl malonate (160 g) was added slowly (30 min) to a solution of sodium (23 g) in dry ethanol (720 ml). To the cooled solution of sodium diethyl malonate, 2-chloroethyldimethylamine (105 g) was added slowly, and the mixture was heated under reflux for 3 hr. After evaporation of the solvent the residue was cooled, dissolved in water (200 ml), and extracted with ether. The ethereal solution was dried (Na_2SO_4), the solvent evaporated, and the residual liquid fractionally distilled to yield the product as a colourless oil, b.p. 131–133°/13 mm, n_D^{15} 1·4352 (98·8 g). Found: N, 6·0%. Calc. for $C_{11}H_{21}NO_4$: N, 6·1%. Selleri & Chiti (1957) report b.p. 106°/5 mm.

3,3-*Di(ethoxycarbonyl)propyldiethylamine* was prepared from 2-chloroethyldiethylamine (22 g) by the above method and was obtained as a colourless oil, b.p. 90–96°/0·45 mm, $n_D^{22·5}$ 1·4370 (19·3 g). Selleri & Chiti (1957) report b.p. 108–109°/4 mm. Magidson & Strukov (1933) report b.p. 136–142°/12 mm, n_D^{20} 1·4386.

3,3-*Di(ethoxycarbonyl)propyldipropylamine* was prepared from 2-chloroethyldipropylamine (85 g) by the above method and was obtained as a straw-coloured oil, b.p. 154°/12 mm, n_D^{22} 1·4420 (80 g). Found: C, 62·1; H, 10·2; N, 4·7%; $C_{15}H_{29}NO_4$ requires C, 62·7; H, 10·2; N, 4·9%.

4,4-*Di(ethoxycarbonyl)butyldiethylamine* was prepared from 3-chloropropyldiethylamine (77·5 g) by the above method and was obtained as a colourless oil, b.p. 160–162°/13 mm, $n_D^{21·5}$ 1·4407 (45 g). Magidson & Strukov (1933) report b.p. 149–151°/4 mm, n_D^{20} 1·4416. Marvel, Zartman & Bluthard (1927) report b.p. 163–170°/23 mm, n_D^{25} 1·4380.

Ethyl γ-dimethylaminobutyrate. 3,3-Di(ethoxycarbonyl)propyldimethylamine (98·5 g) was heated under reflux for 3·5 hr with hydrochloric acid (600 ml), and the mixture evaporated to dryness under reduced pressure. The semi-solid residue was heated under reflux with ethanol (720 ml) and sulphuric acid (72 ml) for 5 hr, and the excess of ethanol was evaporated. Water (70 ml) was added to the residue, which was then made alkaline with sodium hydroxide solution and extracted with ether and benzene. After drying (Na_2SO_4) and removal of solvent, ethyl γ-dimethylaminobutyrate was obtained by distillation of the mixed extracts as a colourless oil, b.p. 79–80°/16 mm, n_D^{16} 1·4232 (6 g). Prelog (1931) reports b.p. 78–90°/18 mm.

Ethyl γ-diethylaminobutyrate was prepared from 3,3-di(ethoxycarbonyl)propyldiethylamine (19·3 g) by the above method and was obtained as a colourless oil, b.p. 98–99°/13 mm, n_D^{20} 1·4337 (7·2 g). Found: equiv., 186·2. Calc. for $C_{10}H_{21}NO_2$: equiv. 187·2. Magidson & Strukov (1933) report b.p. 103–105°/16–17 mm, n_D^{20} 1·4342. Reppe & Mitarbeiter (1955) report b.p. 98–103°/14 mm.

Ethyl γ-dipropylaminobutyrate was prepared from 3,3-di(ethoxycarbonyl)propyldipropylamine (80 g) by the above method and was obtained as a colourless oil, b.p. 120–122°/14 mm, n_D^{22} 1·4328 (14·7 g). This compound without further characterisation was used in the preparation of γ-dipropylaminobutyrohydrazide.

Ethyl δ-diethylaminovalerate was prepared from 4,4-di(ethoxycarbonyl)-butyldiethylamine (10·5 g) by the above method and was obtained as a

colourless oil, b.p. 118–120°/12 mm, $n_D^{19.5}$ 1·4349 (3·3 g). Magidson & Strukov (1933) report b.p. 130–131°/25 mm, n_D^{20} 1·4354.

Methyl δ-bromovalerate was prepared from methyl hydrogen adipate as described in Organic Synthesis (1946), b.p. 57–58°/0·7 mm, n_D^{19} 1·4663.

Methyl δ-dimethylaminovalerate. Methyl δ-bromovalerate (45·5 g), dimethylamine (35 g) and benzene (100 ml) were heated under reflux for 5 hr. The reaction mixture was extracted as described in the preparation of ethyl diethylaminoacetate and methyl δ-dimethylaminovalerate was obtained as an amber-coloured oil, n_D^{20} 1·4310 (5·4 g). Solov'ev, Arendaruk & Skoldinov (1961) report n_D^{20} 1·4322.

Methyl δ-dipropylaminovalerate was prepared from methyl δ-bromovalerate (35 g) and dipropylamine as described above, and was obtained as a straw-coloured oil, b.p. 125–126°/12 mm, $n_D^{15.5}$ 1·4400 (18·8 g). Found: equiv., 211·1. $C_{12}H_{25}NO_2$ requires equiv. 215·3.

Dimethylaminoacethydrazide. Ethyl dimethylaminoacetate (11·5 g) in ethanol (21 ml) was heated under reflux with hydrazine hydrate (5 ml) for 3 hr and allowed to stand for 18 hr. After evaporation under reduced pressure the residual oil was distilled and *dimethylaminoacethydrazide* was obtained as a colourless, viscous oil, b.p. 86–88°/0·6 mm, n_D^{17} 1·4886 (7·8 g). Found: equiv., 122·9; C, 40·7; H, 9·2; N, 35·1%. $C_4H_{11}N_3O$ requires equiv., 117·1; C, 41·0; H, 9·5; N, 35·9%. The *monohydrochloride* was precipitated with dry hydrogen chloride from a dry ethereal solution of the hydrazide and recrystallised (ethanol-ether), m.p. 180–182°. Viscontini & Meier (1950) reported m.p. 181–183°.

Diethylaminoacethydrazide was prepared from ethyl diethylaminoacetate (20 g) by the above method and was obtained as a colourless, viscous oil, b.p. 84–87°/0·45 mm, $n_D^{27.5}$ 1·4763 (16·3 g). Found: equiv., 149·4; C, 49·5; H, 10·2; N, 29·2%. Calc. for $C_6H_{15}N_3O$: equiv., 145·1; C, 49·6; H, 10·4; N, 29·0%. Momose & Tanaka (1953) report b.p. 105°/2 mm. The *salicylate* was prepared by mixing ethanolic solutions of the hydrazide and salicylic acid, and evaporation of solvent. The product was obtained (from acetone-ether) as colourless crystals, m.p. 126–128°. Found: N, 14·3%. $C_{13}H_{21}N_3O_4$ requires N, 14·8%.

Dipropylaminoacethydrazide was prepared from ethyl dipropylaminoacetate (30 g) by the above method, and was obtained as a colourless, viscous oil, b.p. 116–117°/0·55 mm, $n_D^{21.5}$ 1·4696 (21 g). Found: equiv., 178·4; C, 56·1; H, 10·9; N, 24·4%. $C_8H_{19}N_3O$ requires equiv., 173·3; C, 55·4; H, 11·0; N, 24·3%.

β-Dimethylaminopropionhydrazide was prepared from ethyl β-dimethylaminopropionate (Adamson, 1949) (42 g) by the above method, and was obtained as a colourless, viscous oil, b.p. 112–113°/0·5 mm, n_D^{17} 1·4928 (25·3 g). Found: equiv., 138·8; C, 45·3; H, 9·6; N, 32·3%. $C_5H_{13}N_3O$ requires equiv., 131·1; C, 45·8; H, 10·0; N, 32·1%. The *monohydrochloride* (from ethanol-ether), m.p. 124–125°. Found: Cl, 21·5%. $C_5H_{14}ClN_3O$ requires Cl, 21·2%.

β-Diethylaminopropionhydrazide was prepared from ethyl β-diethylaminopropionate (Adamson, 1949) (30·3 g) by the above method, and was

obtained as a colourless, viscous oil, b.p. 121–123°/1·4 mm, n_D^{21} 1·4879 (18·4 g). Found: equiv., 172·3; C, 52·2; H, 10·7; N, 26·6%. $C_7H_{17}N_3O$ requires equiv., 159·2; C, 52·8; H, 10·8; N, 26·4%.

β-Dipropylaminopropionhydrazide was prepared from ethyl β-dipropylaminopropionate (30 g) by the above method and was obtained as a colourless, viscous oil, b.p. 142–144°/1·2 mm, n_D^{18} 1·4819 (17·4 g). Found: equiv., 193·2; C, 56·8; H, 11·1; N, 21·8%. $C_9H_{21}N_3O$ requires equiv., 187·2; C, 57·7; H, 11·3; N, 22·5%.

Di(2-hydrazinocarbonylethyl)amine was prepared from di(2-ethoxycarbonylethyl)amine (11·7 g) by the above method and was obtained as a white solid. Recrystallisation from ethanol yielded *di(2)-hydrazinocarbonylethyl)amine* as colourless crystals, m.p. 124° (6·7 g). Found: equiv., 191·7; C, 37·9; H, 7·3; N, 37·1%. $C_6H_{15}N_5O_2$ requires equiv., 189·2; C, 38·1; H, 7·9; N, 37·0%.

Di(2-hydrazinocarbonyl)ethylmethylamine was prepared from di(2-ethoxycarbonylethyl)methylamine (22·7 g) by the above method, and was obtained as a very viscous, amber-coloured oil, n_D^{20} 1·5300, which solidified on refrigeration. *Di(2-hydrazinocarbonylethyl)methylamine* was recrystallised from ethanol as yellowish-white crystals, m.p. 114° (9·3 g). Found: equiv., 213·0; C, 41·4; H, 8·5; N, 33·6%. $C_1H_{17}N_5O_2$ requires equiv., 203·2; C, 41·4; H, 8·4 N, 34·5%.

Ethyldi (2-hydrazinocarbonylethyl)amine was prepared from di(2-ethoxycarbonylethyl)ethylamine (10·8g) by the above method, and was obtained as a colourless, viscous oil, b.p. 134–135°/0·45 mm, $n_D^{19·5}$ 1·5036 (3·6 g). Found: equiv., 193·0; C,44·7; H, 9·2; N, 31·5%. $C_8H_{19}N_5O_2$ requires equiv., 217·3; C, 44·2; H, 8·8; N, 32·3%.

γ-Dimethylaminobutyrohydrazide was prepared from ethyl γ-dimethylaminobutyrate (6 g) by the above method, and was obtained as a colourless, viscous oil, b.p. 119°/0·4 mm, $n_D^{20·5}$ 1·4852 (4·8 g). Found: equiv., 147·1; C, 49·6; H, 10·2; N, 27·8%. $C_6H_{15}N_3O$ requires equiv., 145·1; C, 49·6; H, 10·4; N, 29·0%.

γ-Diethylaminobutyrohydrazide was prepared from ethyl γ-diethylaminobutyrate (7·2 g) by the above method, and was obtained as a colourless, viscous oil, b.p. 120–121°/0·55 mm, n_D^{16} 1·4837 (5·7 g). Found: equiv., 182·3; C, 55·6; H, 11·3; N, 23·6%. $C_8H_{19}N_3O$ requires equiv., 173·2; C, 55·4; H, 11·1; N, 24·3%.

γ-Dipropylaminobutyrohydrazide was prepared from ethyl γ-dipropylaminobutyrate (14·5 g) by the above method, and was obtained as a colourless, viscous oil, b.p. 150–152°/0·35 mm, $n_D^{20·5}$ 1·4735 (4·6 g). Found: equiv., 205·2; C, 59·3; H, 11·5; N, 20·2%. $C_{10}H_{23}N_3O$ requires equiv., 201·2; C, 59·7; H, 11·5;N, 20·9%.

δ-Diethylaminovalerohydrazide was prepared from ethyl δ-diethylaminovalerate (3·3 g) by the above method, and was obtained as a colourless, viscous oil, b.p. 155–156°/0·9 mm, n_D^{18} 1·4846 (2 g). Found: equiv., 188·5; C, 57·4; H, 11·3; N, 22·4%. $C_9H_{21}N_3O$ requires equiv., 187·2; C, 57·7; H, 11·3; N, 22·5%.

δ-Dimethylaminovalerohydrazide was prepared from methyl δ-dimethylaminovalerate (5·4 g) by the above method and was obtained as a colourless,

viscous oil, b.p. 148–150°/0·45 mm, $n_D^{20·5}$ 1·4826 (3·5 g). Found: equiv., 169·1; C, 48·6; H, 10·9; N, 26·6%. $C_7H_{17}N_3O$ requires equiv., 159·2; C, 52·8; H, 10·8; N, 26·4%.

δ-*Dipropylaminovalerohydrazide* was prepared from methyl δ-dipropylaminovalerate (18 g), by the above method and was obtained as a colourless, viscous oil, b.p. 154–156°/1·3 mm, n_D^{18} 1·4780 (11·8 g). Found: equiv., 220·0; C, 61·1; H, 11·8; N, 19·4%. $C_{11}H_{25}N_3O$ requires equiv., 215·2; C, 61·4; H, 11·7; N, 19·5%. The *dihydrochloride* was prepared by passing dry hydrogen chloride into a dilute solution of the hydrazide in dry ether. It was obtained (from ethanol) as colourless crystals, m.p. 194°. Found: equiv., 148·0; C, 46·0; H, 9·6; Cl, 24·4; N, 14·4%. $C_{11}H_{27}Cl_2N_3O$ requires equiv., 144·1; C, 45·8; H, 9·4; Cl, 24·7; N, 14·6%.

Acknowledgement. We would like to thank Dr. S. R. M. Bushby of the Wellcome Research Laboratories for carrying out the bacteriological tests.

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J. Pharm. Pharmacol., 1964, **16**, 627–629 Received March 16, 1964

A phytochemical investigation of *Trigonella corniculata* Linn.

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The extraction of ground seeds of *Trigonella corniculata* with light petroleum has yielded triacontane and 22,23-dihydrostigmasterol. Further extraction of the seeds with ethanol yielded choline and betaine. Trigonelline, the major alkaloid of *Trigonella foenum-graecum* was not found.

THE seeds and leaves of *Trigonella corniculata* Linn. commonly known in Punjab and Kashmir as "Kasuri Methi" are an important article of commerce. The bitter fruit is astringent and styptic. It is applied to swellings and bruises (Kirtikar & Basu, 1933; Chopra, Nayar & Handa, 1956). The seeds are hot and dry and are used as a household remedy for backaches and in diseases of puerperal women. The plant is cultivated extensively in many parts of India, particularly in Northern India and is used as a green vegetable. The seeds and leaves have a characteristic pleasant odour and are used as flavouring agents and spice.

Experimental

The powdered drug (2·5 kg) was extracted with light petroleum (b.p. 60–80°) in a soxhlet apparatus for 60–70 hr. Removal of the solvent furnished a dark green lipid residue (147·5 g). 50 g of this was saponified (2 hr) with 0·5N ethanolic potassium hydroxide (500 ml) on a water-bath. The yellowish orange unsaponifiable matter (6·4 g) was extracted with cold light petroleum to remove the carotenoid material. The residual amorphous mass (4 g) was applied to a column of alumina (125 g, activity grade I) in light petroleum (b.p. 60–80°) (100 ml). Elution proceeded as follows: (i) Light petroleum (b.p. 60–80°) (250 ml). (ii) Benzene (500 ml). (iii) Benzene: ether (95:5 progressively increasing to 50:50) (300 ml). (iv) Ether (550 ml). (v) Ether: absolute ethanol (80:20) (150 ml).

Fraction (i) yielded triacontane m.p. 64–65°, crystallised twice from acetone (Heilbron & Banbury 1953 report m.p. 66°).

Fraction (iv) crystallised twice from ethanol gave 2·04 g of colourless crystals m.p. 134°. Chromatography of this on alumina (50 g) yielded 22,23-dihydrostigmasterol m.p. 137° $[\alpha]_D$ 36·60° ($CHCl_3$) giving no depression on admixture with an authentic specimen. Its identity was further confirmed by preparing its acetate m.p. 126–127° and benzoate m.p. 145–146°. (Merck Index, 1952, reports 22,23-dihydrostigmasterol acetate m.p. 127–128°, benzoate m.p. 146–147° and $[\alpha]^{25}_D$–37·0 ($CHCl_3$).

EXTRACTION OF ALKALOIDS

The powdered drug (2·5 kg) previously exhausted with light petroleum, ether and chloroform was extracted further with ethanol (95% v/v) in a

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soxhlet apparatus for 140 hr. There resulted 250 g of reddish-brown semi-solid mass which on paper chromatography showed the presence of two alkaloids.

The residue was treated with cold ethanol (95% v/v) (500 ml) and the solution filtered from insoluble gummy matter. The ethanolic solution was then treated with a concentrated solution of lead acetate until no further precipitation occurred. The precipitate was filtered off and filtrate and washings were freed from lead salts with hydrogen sulphide. The residual solution was concentrated under reduced pressure at 60° to 200 ml.

The above concentrated solution (200 ml) was acidified to congo red with 3% sulphuric acid. Overnight at 0° a gelatinous precipitate was deposited: this was rejected. To the clear pale solution, a 4% aqueous solution of ammonium reineckate was slowly added with constant stirring, until a sample of the supernatant solution no longer gave any turbidity. A 10% excess was then added to ensure completeness of precipitation. The rose-coloured precipitate was washed with water, sucked dry and finally dried in a desiccator to a pink chalky powder (21 g). This was decomposed by the method of Kapfhammer as modified by Wieland (Dutcher, 1946), and the syrupy solution of base hydrochlorides thus obtained, dried in a vacuum desiccator (6·2 g).

PURIFICATION OF CRUDE ALKALOIDAL MIXTURE

Part of this mixture was soluble in ethanol (Fraction A), part was apparently insoluble (Fraction B). Paper chromatography indicated that Fraction A was a mixture of two alkaloids while Fraction B was a single alkaloid.

ISOLATION AND CHARACTERISATION OF ALKALOID A

Fraction A was purified further by column chromatography over Merck alumina (50 g, activity grade I) in absolute ethanol. Elution proceeded as follows: (i) Absolute ethanol (120 ml). (ii) Absolute ethanol: methanol (120 ml). (iii) Methanol (180 ml). (iv) Distilled water (60 ml).

Fraction (i) was light yellow in colour. Because of the hygroscopic nature of the residue, the melting-point could not be determined. The residue was redissolved in absolute ethanol and decolourised with charcoal. The residue from the colourless filtrate, disolved in a small quantity of absolute ethanol, on addition of dry ether deposited colourless needle-shaped crystals m.p. 288–290°.

Found for Alkaloid 'A': C, 42·4; H, 10·5; Cl, 24·2; N, 10·5. Calculated for $C_5H_{14}ClNO$(choline chloride): C, 43·0; H, 10·2; Cl, 25·4; N, 10·0%. Alkaloid A gave an Rf value of 0·41 on a paper buffered to pH 7·4 using n-butanol: hydrochloric acid: water (5:2:1, upper phase) as the solvent system. Mixed application of alkaloid A and authentic choline chloride gave only a single spot on paper chromatograms. Using n-butanol; acetic acid: water (4:1:5, upper phase) and unbuffered papers, alkaloid A gave Rf value 0·67 and authentic choline chloride 0·68.

Picrate, picrolonate and reineckate of alkaloid A prepared by the usual methods gave m.p. 239–240°, 178–179° and 250–252° respectively. It appears that the melting-points of picrate and picrolonate of choline are not recorded in literature, these salts, prepared from authentic choline chloride gave melting-points identical with those of the isolated sample.

ISOLATION AND CHARACTERISATION OF ALKALOID B

Fraction (iii) obtained from column chromatography of Fraction A. with methanol as eluant, yielded almost colourless residues m.p. 224–225°, This was combined with the ethanol-insoluble Fraction B (m.p. 226°). The combined alkaloid, on recrystallisation from methanol gave colourless, shining crystals m.p. 227° (Heilbron & Banbury, 1953 report m.p. 227–228°).

Found for Alkaloid B: C, 39·2; H, 7·7; Cl, 22·9; N, 9·1%. Calculated for $C_5H_{11}NO_2$.HCl(betaine hydrochloride) C, 39·1; H, 7·8; Cl, 23·1; N, 9·1%.

The base betaine was prepared from the hydrochloride by rubbing it with freshly prepared moist silver oxide, taking up the liberated base with methanol (Merck) and repeatedly crystallizing it from absolute ethanol, when colourless crystals were obtained, m.p. 264–265° with decomposition (Mulliken, 1916 reported, for betaine 273° ± 3°, with decomposition).

The Rf value of alkaloid B using the solvent system butanol: hydrochloric acid: water (5: 2: 1, upper phase), and *n*-butanol; acetic acid; water (25: 1: 6, upper phase) was 0·26 and 0·14 respectively. These values correspond with those obtained for authentic betaine hydrochloride.

Picrate, picrolonate and reineckate of alkaloid B prepared by usual methods gave m.p. 181–182°, 200°, and 148–150° (with decomposition) respectively. These melting-points are in close agreement with the values reported for these derivatives.

Acknowledgement. Our thanks are due to Mr. K. Sahni of F.R.I., Dehradun for identification of the plant material, to Dr. Harkishan Singh of Saugor University for helpful suggestions and to Dr. W. G. Bywater of S. B. Penick and Company for elemental analysis of isolated samples.

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J. Pharm. Pharmacol., 1964, **16**, 630–631 Received February 28, 1964

Action of *N*-*o*-Chlorobenzyl-*N'N''*-dimethylguanidine (BW 392C60), a bretylium-like drug in lowering the intraocular pressure of rabbit eyes

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N-*o*-Chlorobenzyl-*N'N''*-dimethylguanidine (BW392C60), a potent bretylium-like drug, lowers the intraocular pressure in rabbits either when injected intravenously or when instilled into the conjunctival sac. The lowering of the intraocular pressure is accompanied by miosis and relaxation of the nictitating membrane.

THE role of the sympathetic nervous system in the regulation of the intraocular pressure has been widely studied. However, conflicting results have been obtained by the use of sympathomimetic and sympatholytic agents, of cervical sympathetic nerve stimulation or surgical sympathectomy. (For a literature review see Grant, 1955.)

Recently the effect of guanethidine in lowering the intraocular pressure in man and animals has been demonstrated (Oosterhis, 1962). The mechanism of action may be due to initial active release of catecholamines from tissue stores or to catecholamine depletion (Bonomi & Comite, 1963).

The Compound *N*-*o*-chlorobenzyl-*N'N''*-dimethylguanidine (BW392C60) is an adrenergic neurone blocking agent, 20 times more potent than bretylium in inhibiting the release of noradrenaline caused either by sympathetic stimulation, or by drugs such as guanethidine (Boura & Green, 1963; Costa, Kuntzman, Gessa & Brodie, 1962). It seemed to us that a study of the effect of this compound on intraocular pressure would contribute to the understanding of the role of the sympathetic nervous system in the regulation of this property.

TABLE 1. EFFECT OF BW 392C60 (15 mg/kg i.v.) ON THE INTRAOCULAR PRESSURE OF ANAESTHETISED AND UNANAESTHETISED RABBITS

Treatment	No. of animals	Intraocular pressure mm Hg (mean ± s.d.) at various times (min) after treatment							
		0	15	45	70	110	140	190	255
Saline	13	29·8±1·9	—	29·4±1·7	—	—	—	29·8±1·8	29·7±2·0
BW 392C60	5	30·6±1·5	—	24·4±2·9	—	—	—	20·9±4·4	20·6±3·3
Chloralose + saline	11	29·4±2·3	29·4±2·2	—	29·8±1·8	29·8±1·8	—	29·2±1·7	29·7±1·5
Chloralose + BW 392C60	21	29·8±1·8	23·9±3·5	23·5±4·2	18·5±4·2	18·5±3·0	20·4±4·8	20·5±3·1	20·6±3·3

Methods and results

Adult, male, pigmented rabbits weighing between 1·6 and 2·5 kg were used. In some experiments, the animals were anaesthetised with chloralose (80 mg/kg i.v. in 3 ml H_2O). In other experiments, the animals were held still. Handling did not cause the intraocular pressure to change. It was measured from the right eye with a Schötz tonometer after anaesthetising the cornea with 2 drops of a solution of 0·5% amethocaine. Each measurement was made with 2 tonometer weights (5·5 and 10g) and corrected for scleral rigidity. BW 392C60 was administered either

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by slow intravenous injection of 15 mg/kg or by topical application into the conjunctival sac using 3 drops of a 10% solution. In control experiments the animals were given saline. The intraocular pressure in the right eye of treated animals was compared with that in the right eye of the control group.

Table 1 shows the results obtained after intravenous injection of the drug in anaesthetised and unanaesthetised rabbits. Within 15 min there was a marked fall in the intraocular pressure accompanied by miosis and relaxation of the nictitating membrane which persisted. Table 2

TABLE 2. EFFECT OF TOPICAL APPLICATION OF BW 392C60 10% ON UNANAESTHETISED RABBITS, WITH OR WITHOUT PRECEDING TONOMETRY (p.t.)

Treatment	No. of animals	Intraocular pressure mm Hg (mean ± s.d.) at various times (hr) after treatment			
		0	3	7	24
Saline with p.t.	13	29·5 ± 1·5	29·8 ± 1·9	29·6 ± 1·8	29·2 ± 1·9
Saline without p.t. ..	5	—	—	—	29·6 ± 2·8
BW 392C60 with p.t. ..	20	23·2 ± 2·2	26·6 ± 4·6	23·2 ± 4·4	20·6 ± 2·6
BW 392C60 without p.t...	5	—	—	—	22·8 ± 1·9

summarises the results obtained after the drug was applied topically, either with or without preceding tonometry. Eyes treated with BW 392C60 showed a lowering of the intraocular pressure lasting more than 24 hr, and relaxation of the nictitating membrane. Usually these effects were accompanied by miosis but in some animals a mydriasis occurred which was over in 8 hr. No significant changes were observed in the untreated eyes. Stimulation of the cervical sympathetic nerve failed to evoke a mydriatic response in eyes treated with BW 392C60.

Discussion

These results suggest that BW 392C60 lowers the intraocular pressure by an action on sympathetically innervated ocular structures. It might be concluded that the blockade of release of noradrenaline leads to a lowering of the intraocular pressure. The effect is exerted by BW 392C60 applied topically, but its effects were limited to the treated eyes. The initial mydriasis sometimes observed after topical application of BW 392C60 might be related to the monoamine oxidase-inhibiting action of BW 392C60 recently demonstrated by Gessa, Cuenca & Costa (1962).

Studies are in progress to ascertain whether the lowering of the intraocular pressure is caused by a decreased rate of formation of aqueous humour or by an increase in its rate of absorption.

Acknowledgements. We are indebted to Drs. J. Burns and R. Kuntzman from the Wellcome Research Laboratories, Tuckahoe, N.Y., U.S.A. for a supply of BW 392C60.

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Letters to the Editor

The solubility of benzaldehyde in water

SIR,—During investigations into factors controlling the oxidation of benzaldehyde solubilised in aqueous solutions of various polyoxyethylene glycol ethers we found the rate of reaction to depend mainly on the concentration of aldehyde in the micelles and not on the total concentration. To estimate the distribution of benzaldehyde between the micelles and true aqueous "phase" it was essential to know the solubility of benzaldehyde in water. In International Critical Tables (1928) the water-solubility of benzaldehyde is given as 3 g/litre at room temperature and in the Merck Index (1960) as 1 in 350 parts of water. The oxidation of benzaldehyde dispersed in aqueous solutions of surface-active agents has been investigated by Nixon (1958) and Swarbrick (1964) who estimated the water-solubility of benzaldehyde at 25° to be 3·49 and 3·5 g/litre respectively. We have found that the water-solubility is considerably higher than these values.

Benzaldehyde was distilled at low pressure under oxygen-free nitrogen using a Towers fractional distillation unit fitted with automatic reflux ratio control. The aldehyde was packed in ampoules under oxygen-free nitrogen and stored protected from light in a refrigerator. Samples were examined for the presence of impurities and decomposition products by infra-red spectroscopy and gas chromatography. Double distilled water from an all-glass still was used throughout.

Saturated solutions were prepared by shaking together an excess of aldehyde in water in stoppered cylinders in a water-bath thermostatically controlled at 25° ± 0·01°. Excess aldehyde was removed by filtration through Whatman No. 3 filter paper. The first portion of the filtrate was rejected. The amount of aldehyde in an aliquot of saturated solution was determined gravimetrically as the 2,4-dinitrophenylhydrazone according to the method of Iddles & Jackson (1934). The accuracy of the method was verified using known weights of benzaldehyde. An alternative method for removing excess aldehyde from the saturated solution was to centrifuge samples at a controlled temperature of 25° and then to remove a sample of saturated solution by means of a pipette. Both methods were satisfactory and gave concordant results.

The value for water-solubility obtained by the gravimetric method was checked by gas chromatography using methyl salicylate as an internal standard (Burchfield & Storrs, 1962). A Pye Panchromatograph equipped with a flame ionization detector was used as the gas chromatograph. The column was prepared by packing a 210 cm by 3 mm glass column with 25% Carbowax 20-M on 60–80 mesh Chromosorb W solid support. Operating conditions were as follows: column temperature, 200°; argon flow rate, 60 ml/min; detector voltage, 400 V.

TABLE 1. THE WATER-SOLUBILITY OF BENZALDEHYDE AT 25°

Method	Solubility, g/litre	Standard deviation*
Gravimetric	6·55	0·004
Gas chromatography	6·58	0·070

* 2 determinations on each of 3 saturated solutions.

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The solubility of benzaldehyde in water as determined by refractive index measurements

SIR,—Mitchell Wan & Bjaastad (1964) have recently determined the solubility of benzaldehyde in water at 25° and found a value of 6·55 mg/ml, a figure considerably higher than the previous literature values which range from 3·0 to 3·5 mg/ml. As a result of this finding we have determined the solubility of benzaldehyde in water by measurement of the refractive index of a range of benzaldehyde dispersions.

The sample used was redistilled Analar benzaldehyde that had been stored, refrigerated, in glass ampoules under nitrogen in the dark. Varying concentrations of benzaldehyde were dispersed in freshly boiled and cooled distilled water; the air above the dispersions was then displaced with nitrogen and the sealed flasks shaken overnight in a water-bath at 25° (±0·5°). The dispersions were then left for a further 2–4 hr without shaking to allow sufficient separation of a clear supernatent in those dispersions containing benzaldehyde in excess of its aqueous solubility. This technique was used in preference to centrifuging, where the control of temperature was found to be inadequate, even with a temperature-controlled centrifuge. The refractive index of each benzaldehyde solution, in terms of instrument scale reading, was determined against water in the reference cell using a Hilger-Rayleigh interference refractometer (Model M154) maintained at 25° by circulating water from a thermostat bath. 1 cm cells were used throughout and the supernatent aqueous solutions of benzaldehyde, pipetted from the dispersions kept in the water-bath, were allowed to equilibrate in the instrument for 20 min. Duplicate readings, accurate to one scale division, were taken for each of the ten dispersions prepared. The whole procedure was then repeated using a second sample of benzaldehyde.

The solubility of benzaldehyde, taken as the intercept of the two straight lines shown in Fig. 1, is 6·9 to 7·0 mg/ml, as compared with the value of 6·55 mg/ml found by Mitchell using a gravimetric method confirmed by gas chromatography.

The refractive index method described herein has the advantage that it is independent of any coefficients or factors, involves no manipulative techniques

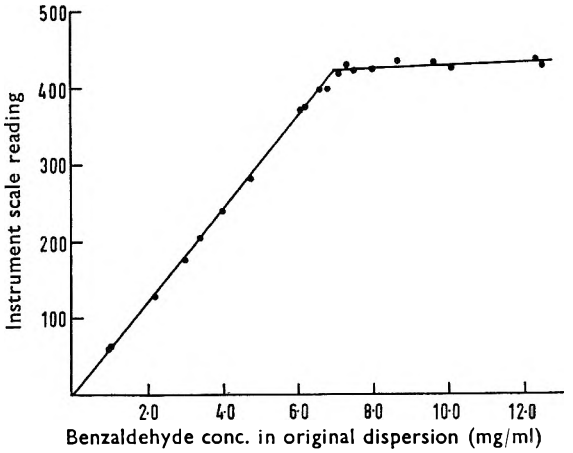

FIG. 1. Variation in refractive index, in terms of instrument scale reading, of aqueous solutions containing increasing concentrations of benzaldehyde at 25°.

such as filtration, ignition or dilution which may introduce errors and is capable of rigid temperature control, an obvious necessity in solubility determinations. As such it appears to be an ideal method for measuring the solubility of benzaldehyde in water and is also applicable to many other materials.

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Some newer anti-inflammatory agents

SIR,—Lightbody & Reid (1960) demonstrated the hypoglycaemic effect of *o*-cresotinic acid. The hypoglycaemic effects of some newer salicylic acid congeners were reported by Luthera & Tayal (1962). Since salicylates possess potent anti-inflammatory activity, the anti-inflammatory effects of these newer salicylic acid congeners, 2,4-diacetoxybenzoic acid, *m*-cresotinic acid and 5-ethyl-2-hydroxybenzoic acid, on formaldehyde-induced arthritis (Brownlee, 1950) was compared with that of hydrocortisone.

Albino rats weighing between 100–110 g were divided into five groups of six animals each. The anteroposterior diameters of the ankle joints were measured daily for 10 consecutive days and 0·1 ml of 2% formaldehyde solution (v/v) was injected in each foot subcutaneously under the plantar aponeurosis on first and third days. One group of animals served as control. Other groups were treated with daily intraperitoneal injections of 2,4-diacetoxybenzoic acid, *m*-cresotinic acid, 5-ethyl-2-hydroxybenzoic acid (2·0 mg/100 g body weight) and hydrocortisone (0·5 mg/100 g body weight) respectively. The results are shown in Table 1.

TABLE 1. EFFECT OF HYDROCORTISONE AND SALICYLIC ACID CONGENERS ON FORMALDEHYDE-INDUCED ARTHRITIS IN RATS

	Dose mg/100 g i.p.	Initial diameter mm	Average ten day diameter mm	% anti-inflammatory effect	P
Control	—	6·1 ± 0·07	7·53 ± 0·04	—	—
Hydrocortisone	0·5	6·04 ± 0·09	7·1 ± 0·09	30·7	<0·001
2,4-Diacetoxybenzoic acid	2·0	6·1 ± 0·05	7·08 ± 0·08	31·5	<0·001
m-Cresotinic acid	2·0	6·15 ± 0·06	7·22 ± 0·09	21·7	0·02–0·01
5-Ethyl-2-hydroxybenzoic acid	2·0	5·92 ± 0·06	7·11 ± 0·08	29·3	<0·001

The drugs showed potent anti-inflammatory activity similar to that of hydrocortisone. Quantitatively, *m*-cresotinic acid was found to be a less potent anti-inflammatory agent (21·7%) than 2,4-diacetoxybenzoic acid (31·5%) and 5-ethyl-2-hydroxybenzoic acid (29·3%).

Acknowledgement. The authors are grateful to Dr. J. N. Tayal of Defence Research Laboratories, Kanpur, India, for the supply of drugs used in present study.

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Anti-erythemic effectiveness of some metabolic inhibitors in guinea-pigs

SIR,—Among the methods applied to the measurement of the anti-inflammatory activity of nonsteroid compounds is the inhibition of ultra-violet light-induced erythema in guinea-pigs. The advantages of the method are its sensitivity and specificity and the close relation between clinical and anti-erythemic doses. Having subjected more than a hundred compounds to careful analysis, Winder (1958) concluded that only the well known antiphlogistics used clinically have a significant anti-erythemic effect.

The existence of a correlation of the antiphlogistic and metabolism-inhibiting (uncoupling) effect of nonsteroid agents is becoming an accepted hypothesis (Whitehouse, 1963). Thus, to clarify the role of the major energy producing processes—glycolysis and oxidation—in the development of ultra-violet-induced erythema we examined the influence of some enzyme inhibitors, of known biological mechanisms of action, in guinea-pigs. The effect of these compounds has not been investigated in this way before.

Our method was identical with that of Winder (1958). The depilated skin of the guinea-pig's back was irradiated with a 1000 W mercury lamp. Heat rays were filtered by cold water in a quartz tube. Each spot was irradiated for 80 sec. The spots were scored by marks 0, 0·5 and 1, the maximum score for the total of the three spots irradiated being 3 per animal. If the effect scored was below or equal to 1·5 in an animal, this was considered as an inhibition.

Table 1 shows that the substances when administered in non-toxic doses, intensely inhibited the appearance of erythema. 2-Deoxyglucose inhibits

TABLE 1. EFFECT OF SOME METABOLIC INHIBITORS ON ULTRA-VIOLET ERYTHEMA IN GUINEA-PIGS

Inhibitor	Dose* mg/kg	Anti-erythemic effect (animals scored $\leq$ 1·5) (total animals)				Effective dose (ED50) mg/kg
		Control	Hr after exposure 2	3	4	
Monoiodoacetic acid	15 (s.c.)	0/7	2/7	1/7	1/7	21
	25 (s.c.)	0/8	4/8	3/8	1/8	
	75 (s.c.)	0/8	8/8	8/8	7/8	
Sodium fluoride	25 (s.c.)	0/8	2/8	0/8	0/8	40
	50 (s.c.)	0/11	7/11	5/11	3/11	
2-Deoxyglucose	25 (i.p.)	0/6	1/6	0/6	0/6	47
	50 (i.p.)	0/8	5/8	3/8	2/8	
	100 (i.p.)	0/16	13/16	10/16	1/16	
	300 (i.p.)	0/7	7/7	5/7	5/7	
2-Deoxyglucose†	100 (i.p.)	0/9	1/9	1/9	0/9	
Arsenate	2 (i.p.)	0/5	2/5	2/5	0/5	2·4
	5 (i.p.)	0/8	7/8	3/8	2/8	

* 30 min before the ultraviolet exposure.
† All animals received 4 U/100 g insulin (protamine-zinc) by the subcutaneous route simultaneously with 2-deoxyglucose.

glucolysis far beyond the glucose-6-phosphate level (Sols & Crane, 1954). The effect of 2-deoxyglucose in inhibiting the release of histamine *in vitro* (Chakravarty, 1962) and of dextran oedema *in vivo* (Goth, 1959) is generally known. The effect of 2-deoxyglucose in inhibiting oedema produced by dextran can be prevented by insulin (Goth, 1959). Monoiodoacetic acid stops glycclysis primarily by the inhibition of phosphoglycerinealdehyde dehydrogenase; fluoride suspends it by inhibiting the enolase. It is well-known that arsenate causes "arsenolysis" which results in the suspension of the oxidative phosphorylation. Strenger (1959), has found monoiodoacetic acid and fluoride to have an inhibiting effect upon the dextran and formalin oedema in rats.

The dextran oedema-inhibiting effect of fluoride and arsenate was discussed in our previous paper (Görög & Szporny, 1964). The results of our present experiments permit us also to attribute an important role to increasing glycolysis and oxidation in the mechanism of the development of ultra-violet-induced erythema. The present experiments show such erythema in guinea-pigs to be inhibited by compounds which have an inhibitory effect upon the metabolism and which are not applied in therapy. The effectiveness of nonsteroid antiphlogistics in this test, (Winder, 1958) may also be attributed to their inhibition of metabolism and adenosine triphosphate synthesis.

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Mode of action of vancomycin

SIR,—Vancomycin, an antibiotic produced by *Streptomyces orientalis* (McCormick & others, 1956), has been found to produce osmotically sensitive spheres in *Pseudomonas fluorescens* (Durham, 1963). No reports have appeared on the induction of similar forms in *Escherichia coli.*

Experiments have been made on the effect of 50, 100, 250 500, and 1000 μg/ml of vancomycin hydrochloride on *E. coli* at 35° in nutrient broth, containing 0·33 M sucrose and 0·25% w/v magnesium sulphate, $MgSO_4 \cdot 7H_2O$, of final pH 7·4. Microscopical examination showed that spherical forms were induced within 5 hr with 500 and 1000 μg/ml of the antibiotic, but not with the lower concentrations. The spheres (spheroplasts) lysed if the osmotic pressure of the surrounding medium was suddenly reduced, indicating cell wall fragility, but not necessarily the complete absence of cell wall constituents (Brenner & others, 1958).

In a further series of experiments, vancomycin was tested against *E. coli* at 35° when Mg^{++} was omitted from the medium. Spheroplasts were not induced with any concentration of the antibiotic. Such results are in accord with those obtained with penicillin (Hugo & Russell, 1960) and with glycine (John & Russell, 1963), when it was shown that Mg^{++} ions were essential for stabilisation of the cytoplasmic membrane.

It is known that substances which inhibit bacterial cell wall synthesis will induce spheroplast formation (see review by McQuillen, 1960). Vancomycin can thus be added to those substances, which include the penicillins, cycloserine, glycine and certain D-amino acids, which produce spheroplasts in susceptible bacteria. It might thus be expected that the cell wall is the primary site of action of vancomycin, and Jordan (1961) and Jordan & Inniss (1961) have stated this to be so, although the action of this antibiotic was not entirely similar to benzylpenicillin (Jordan & Innis, 1961; Reynolds, 1961, 1962). From his studies on the effect of some antibiotics on the incorporation of three radioactive amino-acids into the protein, and of (^{14}C)-glycerol into the lipid, of the protoplast membrane of *Bacillus megaterium*, Yudkin (1963) concluded that the bacterial cell wall was not the primary site of action of vancomycin.

Spheroplast induction in *Ps. fluorescens* (Durham, 1963) and in *E. coli* could thus be the result of a secondary effect of the drug.

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The comparative activity of arecoline and arecoline *N*-metho salt

SIR,—Arecoline is well known as one of the relatively few tertiary amines that have high activity as a muscarinic agent. Indeed this activity has been underestimated in the past because the pKa of arecoline is 7·61 (35°) so that it is incompletely ionised at blood pH and even more so in Ringer-Locke or Tyrode's. Recently, we have found that the activity of the arecolinium ion on the guinea-pig ileum was 1·4 times as great as carbachol and had a similar slope and maximum and it was also about equal to acetylcholine although differing in slope. We thought it of interest to see whether the quaternary arecolinium *N*-metho salt (the methiodide prepared by the method of Wilstätter, 1887) had an even greater activity. However, it was only one fortieth of the activity of the tertiary arecolinium ion itself. On the other hand, when tested on the frog rectus abdominis, the tertiary arecolinium ion had only 1·1% of the activity of carbachol whereas the quaternary *N*-metho salt was a little more active than carbachol (Table 1).

TABLE 1. RELATIVE ACTIVITY OF ARECOLINE AND ITS *N*-METHO SALT.

	Molar potency relative to carbachol		Relative potency
	Arecolinium	*N*-methylarecolinium	Arecolinium / *N*-Methylarecolinium
Guinea-pig ileum	1·42	0·034	42
Frog rectus abdominis ..	0·011	1·39	0·008

These results point to the conformation of the nitrogen in tetrahydropyridine being such that at the muscarinic receptor a better fit is obtained when the free groups are a methyl and a hydrogen than when both free groups are methyl. This suggests that when the nitrogen is constrained within the ring the additional methyl prevents optimal interaction possibly by increasing the separation of the positive nitrogen from an anionic group in the receptor and so reducing the coulombic attraction. This behaviour is in contradistinction to the effect of quaternisation in acetylcholine, muscarine, dioxolanes and other substances where the quaternary group is in an aliphatic chain capable of assuming several rotational conformations in solution and in which the addition of a methyl group increases muscarinic potency by a factor of 10^2—10^3.

On the other hand at the nicotinic receptor arecoline and its *N*-metho derivative behave in conformity with other tertiary-quaternary pairs and the increase in activity on quaternising is not dissimilar to the difference between 2-dimethylaminoethyl acetate and acetylcholine at this receptor. However, nicotine is actually slightly more active than *N*-methyl nicotinium (Hamilton, 1963). It seems as though the geometry of the nicotinic receptor is less stringent in the conformation required around the nitrogen than is the muscarine receptor. Study of the activity of acetylcholino-mimetic substances derived from heterocycles differing in ring size and conformation seems to offer a fruitful way of studying the detailed geometry of the cation combining site at these receptors.

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August 6, 1964

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Book Reviews

LEHRBUCH DER ALLGEMEINEN PHARMAKOGNOSIE. By E. Steinegger and R. Hänsel. Pp. xii + 595 (including Index). Springer-Verlag, Berlin, 1963. DM. 69.00.

It is not always appreciated that pharmacognosy, having undergone considerable evolution during the past twenty years, is no longer confined to a study of the botanical characteristics of a number of dried drugs. It is a living science and collates those aspects of distribution, taxonomy, biochemistry, genetics and chemical analysis which concern medicinal plants. This more complete approach has been evident in some American textbooks for a number of years but Steinegger and Hansel's work is probably the first of its kind in Europe.

The book is divided into twelve parts. The first considers the aims of the subject and the second (82 pages) the basic sciences involved in its study. In the latter are discussed the application of systematic botany, morphology, genetics, plant physiology and phytochemistry. The genetics deals with selection, cross-breeding, polyploidy and genemutation with appropriate examples. In the phytochemistry section the main groups of plant constituents are discussed together with their biosynthetic origins. This is a rapidly developing field of research and some summaries given are not entirely up-to-date, for example, no mention appears to be made of the role of malonate in fatty acid and anthraquinone biosynthesis.

The remaining ten parts of the book are devoted to a consideration of individual drugs arranged according to the chemical nature of their active constituents. Ten chemical groups are considered one of which concerns medicinal material derived from micro-organisms and another a miscellaneous collection of drugs, the active constituents of which are improperly known. Each group is usually introduced by a general discussion, as for example for essential oils, their composition, occurrence and extraction are mentioned. Large diversified chemical groups, like the alkaloids and glycosides, are further sub-divided according to chemical structure. In the treatment of individual drugs, most emphasis is given to chemical constituents and one feature of the book is the numerous structural formulae. Large groups of closely related compounds, like the alkaloids of *Veratrum* and the *Digitalis* cardioactive glycosides are conveniently tabulated for easy study. The botanical and geographical sources of each drug are considered but the reader will find no microscopy and little detail concerning macroscopical descriptions or adulteration. There are only five illustrations in the entire book. A number of literature references, which cover limited aspects of the subject matter, are given.

This textbook is probably the most complete, unified approach to the chemical aspects of pharmacognosy yet available and, as such, it should prove a useful source of information to those connected with the active constituents of medicinal plants. For students, including those with little facility in the German language, it will serve as a useful book of reference.

W. C. EVANS

EXCITATION. By Georges Ungar. American Lectures in Pharmacology series. Pp. xiii + 437 (including index). Charles C. Thomas, Springfield, Ill., U.S.A., 196 . $13.50.

Two main problems present themselves to any writer on the concept of biological excitation. Firstly, the great volume of literature available and, secondly, a problem of definition, for excitation is neither the state of excitability shown by all living cells, nor is it the final response of a cell to a stimulus.

On the first problem, Dr. Ungar's own work has obviously made him familiar with a large number of papers covering many aspects of excitation as it affects both the cell and the whole organism. The second problem he avoids by giving a no more precise definition of excitation than that it is the response of living matter to stimulation.

The historical development of the concept of excitation and the basic problems of any study of living matter in its environment are dealt with in a short introductory section which was designed for "any person with a minimum of scientific culture"—a surprising aim since the remaining three hundred pages require specialist knowledge. From this introduction the author goes on to discuss what he calls "primary manifestations" of excitation, including not only the too familiar ionic fluxes but physical and metabolic events also. In this part of the book a successful attempt has been made to correlate the work of electrophysiologists, biochemists, biophysicists and physical chemists in order to give a better perspective of the problems and present state of knowledge of excitation at a cellular level.

Single cells are, however, only of limited interest to the pharmacologist, and the book goes on to show, of necessity only briefly, the importance of excitation to the whole animal from its embryology to its adult behaviour. Inhibition, with its obvious importance to the nervous system, is introduced at this point as is the author's satisfying concept of effector cell differentiation throughout the animal kingdom. He proposes that the basic manifestations of excitation, previously discussed, are possessed by all cells but that effector cells are differentiated by the emphasis of one of these components. Thus, changes in protein configuration are prominent in muscle cells and the expulsion of chemical material in secretory cells. This unifying concept is further developed in a study of excitation in disease, as typified by the inflammatory response.

Finally the book relates excitation to drug action. Despite the fact that this is one of a series of "Lectures in Pharmacology," this is the shortest part of the book. While, as a pharmacologist, one feels that this section could profitably have been expanded, Dr. Ungar has attempted, and succeeded, in bringing together many aspects of work on excitation in a book that yet remains interesting, easy to read and, I think, of use to pharmacologist, physiologist and biochemist alike. He has treated acceptable and unorthodox theories with equal frankness and, if nothing else, the book serves to show how far we have come since the beginnings of the concept of excitation and how far we still have to go before we can pretend to have unravelled the complexities of biological excitation.

M. G. TWEEDDALE

Journal of Pharmacy and Pharmacology

SEPTEMBER 1964 VOL. 16. No. 9

Review Article

569–595 M. MARTIN-SMITH, M.SC., PH.D., M. F. SUGRUE, M.SC.
Biological activity in steroids possessing nitrogen atoms: recent advances

Research Papers

596–602 J. SWARBRICK, J. E. CARLESS
Oxidation of benzaldehyde in some single phase betaine-benzaldehyde-water systems

603–610 T. L. B. SPRIGGS, M. A. STOCKHAM
Urethane anaesthesia and pituitary-adrenal function in the rat

611–617 A. M. COOK, W. R. L. BROWN
Inactivation of a bacteriophage by chemical antibacterial agents

618–626 D. EDWARDS, D. HAMER, W. H. STEWART
Synthesis of aliphatic amino-acid hydrazides as potential tuberculostatic agents

627–629 C. K. ATAL, S. P. SOOD
A phytochemical investigation of *Trigonella corniculata* Linn

630–631 G. L. GESSA, G. SANGIORI
Action of *N-o*-Chlorobenzyl-*N'N"*-dimethylguanidine (BW 392C60), a bretylium-like drug lowering the intraocular pressure of rabbit eyes

Letters to the Editor

632–633 A. G. MITCHELL, LUCY S. C. WAN, S. G. BJAASTAD
The solubility of benzaldehyde in water

633–634 J. E. CARLESS, J. SWARBRICK
The solubility of benzaldehyde in water as determined by refractive index measurements

634–635 K. K. TANGRI, K. P. BHARGAVA
Some newer anti-inflammatory agents

635–636 P. GÖRÖG, L. SZPORNY
Anti-erythemic effectiveness of some metabolic inhibitors in guinea-pigs

637 A. D. RUSSELL
Mode of action of vancomycin

638 A. S. V. BURGEN
The comparative activity of arecoline and arecoline *N*-metho salt

639–640 Book Reviews

PRINTED BY W. HEFFER & SONS LTD., CAMBRIDGE, ENGLAND.