# RESEARCH PAPERS

## AMINO-ACID DECARBOXYLASE ACTIVITIES IN RAT HEPATOMA

By D. Mackay, J. F. Riley and D. M. Shepherd

*From the Department of Pharmacology and Therapeutics, University of St. Andrews, Queen's College, Dundee, and the Radiotherapy Department, Dundee Royal Infirmary*

Received January 26, 1961

The effects of pH, substrate concentration and addition of benzene on the rate of histamine production by extracts of rat hepatoma and guinea-pig kidney have been determined, and manometric studies of the DOPA and 5-HTP decarboxylase activities of these extracts have also been made. The histidine decarboxylases of the two tissues have quite different properties, and the histidine decarboxylase of the rat hepatoma is not associated with 5-HTP and DOPA decarboxylase activities. Whereas the urinary histamine output of hepatoma-bearing female August rats is greatly raised, the 5-HIAA output lies within the normal range.

Mackay, Marshall and Riley (1960) have recently shown that the urine of female August rats implanted with a transplantable hepatoma, F-HEP, contains large amounts of histamine, and that extracts of these tumours are rich in histidine decarboxylase. These results fit in well with those of Kahlson (1960) on the histamine-forming capacity of regenerating rat liver. Telford and West (1960), who incidentally have been unable to confirm Kahlson's results for regenerating liver, have also reported that the histidine decarboxylase of the liver of the foetal rat differs in its pH optimum from that of the post-natal rat.

Further studies on the urine of hepatoma-bearing rats, on rat hepatoma extracts, and on extracts of guinea-pig kidney are now reported. The urinary output of 5-hydroxyindolyl-3-acetic acid (5-HIAA) by female August rats, implanted with F-HEP, has been followed simultaneously with the histamine output. The effects of pH, substrate concentration and the addition of benzene on the rate of histamine production by extracts of rat hepatoma and guinea-pig kidney have been determined, and manometric studies of the $\beta$-(3,4-dihydroxyphenyl)-$\alpha$-alanine (DOPA) and 5-hydroxytryptophan (5-HTP) decarboxylase activities of rat hepatoma have also been carried out.

### Experimental

*Urinary Output of Histamine and* 5-HIAA

Pooled 24-hr. urine samples were collected from a group of three female August rats implanted with F-HEP. The daily volume of urine (8–20 ml.) was collected in flasks containing 0·5 ml. of a mixture of toluene and glacial acetic acid (3 ml. toluene : 25 ml. glacial acetic acid), and was thus suitable for the determination of both 5-HIAA and histamine. The histamine was assayed directly on the atropinised guinea-pig ileum. The 5-HIAA was determined by the method of Macfarlane, Dalgliesh, Dutton, Lennox, Nyhus and Smith (1956) except that, instead of 5-HT creatinine sulphate, the standard solutions contained 5-HIAA. The standards consisted of appropriate dilutions of a stock solution (5 mg. 5-HIAA in 20 ml. glacial

acetic acid). To obtain reproducible results it was necessary to adjust the concentration of acetic acid in all samples, urines and standards, to a constant value.

*Enzyme Studies*

For measurement of histidine decarboxylase activity the tissue extracts and incubation media were prepared basically as described by Mackay and Shepherd (1960), the volume of saline used in the preparation of the extract being 3 ml./g. of tissue and not 30 ml./g. of tissue as erroneously stated. The iso-osmotic phosphate buffers were of various pH values, and the substrate concentrations were also varied. Aminoguanidine to give a

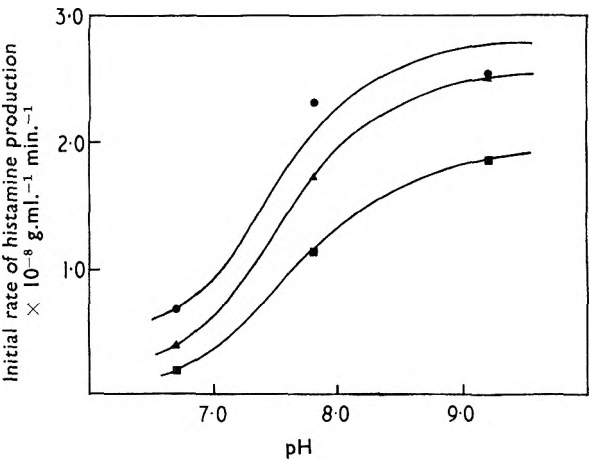



FIG. 1. The initial rate of production of histamine by the histidine decarboxylase of guinea-pig kidney as a function of pH and substrate concentration. Incubations at 37°. L-Histidine concentrations—● 15 mg./ml. ▲ 10 mg./ml. ■ 5 mg./ml.

final concentration of $6 \times 10^{-5}$ M was added routinely to inhibit any histaminase, and in all experiments a final concentration of 40 $\mu$g./ml. of pyridoxal-5′-phosphate was also present. The rate of histamine production was followed by withdrawing samples at various times after the beginning of the reaction, and the histamine was assayed on the atropinised guinea-pig ileum. When benzene was added to the incubation medium the volume added was small (0·3 ml./25 ml. of medium). All incubations were carried out at 37°.

For the manometric studies the volume of saline used in the preparation of the tissue extracts was 2 ml./g. of tissue, and the Warburg flasks contained 1·0 ml. of extract in 2·0 ml. of final incubation medium. The concentration of 5-HTP or DOPA in the final media was always 0·5 mg./ml. Iso-osmotic phosphate buffers of various pH values were used and the reaction was stopped as required by tipping 0·3 ml. of 2N sulphuric acid into the flask from the centre well. The incubations were carried out in an atmosphere of oxygen-free nitrogen at 37°.

## Results and Discussion

The effects of pH and substrate concentration on the initial rate of formation of histamine, from L-histidine, by extract of guinea-pig kidney are shown in Fig. 1. The optimum pH of this enzyme appears to be close to 9, and the concentration of histidine required to saturate the enzyme is at least 15 mg./ml. On the other hand, the optimum pH for histamine production by rat hepatoma extract is close to 7·0, as shown in Fig. 2. Preliminary experiments showed that the enzyme is practically saturated at a substrate concentration of 1 mg./ml. The effect of benzene on the

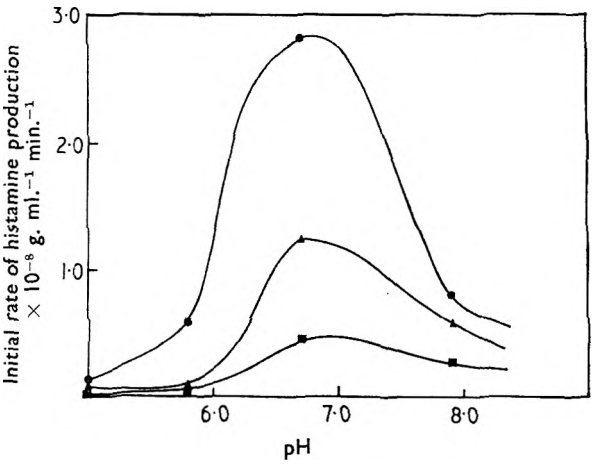



Fig. 2. The initial rate of production of histamine by the histidine decarboxylase of rat hepatoma as a function of pH and substrate concentration. Incubations at 37°. L-Histidine concentrations—● 0·25 mg./ml. ▲ 0·05 mg./ml. ■ 0·01 mg./ml.

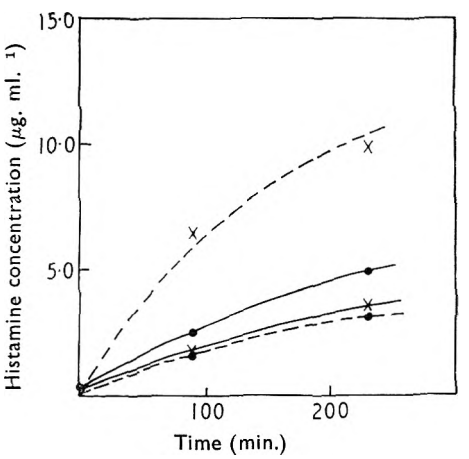



Fig. 3. The effects of benzene on the rates of production of histamine by the histidine decarboxylases of guinea-pig kidney and rat hepatoma, at 37°. Unbroken lines—experiments without benzene. Broken lines—experiments with benzene. x—guinea-pig kidney extract. ●—rat hepatoma extract.

activity of the two extracts is illustrated in Fig. 3, from which it will be seen that while the rate of histamine production by guinea-pig kidney extract is greatly increased in the presence of benzene, that by rat hepatoma extract is actually reduced. It is clear from these results that the enzymes present in the two tissues are quite different.

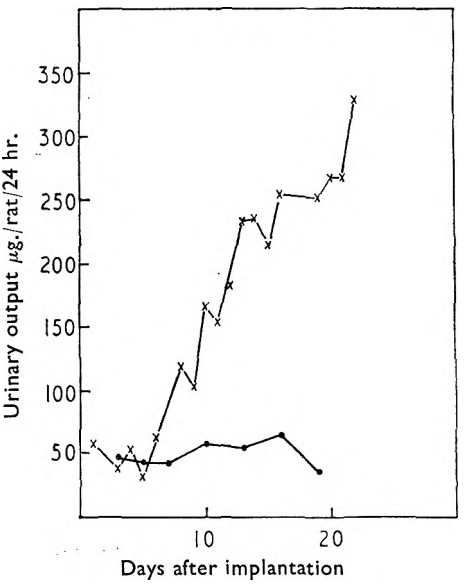



FIG. 4. Urinary histamine and 5-HIAA output of female August rats implanted with the transplantable hepatoma, F-HEP. x—histamine output per rat per day (μg.). ●—5-HIAA output per rat per day (μg.).

If the Michaelis-Menten treatment is assumed to apply to these systems, in spite of the crude nature of the enzyme-containing tissue extracts, then the Michaelis constant ($K_m$) and the maximum rate of histamine production (V), under the various experimental conditions,

TABLE I

EFFECT OF pH ON THE MAXIMUM RATE OF HISTAMINE PRODUCTION (V), AND ON THE VALUE OF THE MICHAELIS CONSTANT ($K_m$) OF HISTIDINE DECARBOXYLASES AT 37°

| Tissue extract | pH | $K_m$ mole. litre$^{-1}$ | V mole. litre$^{-1}$ min.$^{-1}$ |
|---|---|---|---|
| Guinea-pig kidney .. .. .. .. | 9·2 | $4{\cdot}5 \times 10^{-2}$ | $3{\cdot}8 \times 10^{-7}$ |
| | 7·8 | $7{\cdot}2 \times 10^{-2}$ | $3{\cdot}3 \times 10^{-7}$ |
| | 6·7 | — | — |
| Rat hepatoma .. .. .. .. | 7·8 | $1{\cdot}4 \times 10^{-4}$ | $0{\cdot}77 \times 10^{-7}$ |
| | 6·7 | $6{\cdot}8 \times 10^{-4}$ | $3{\cdot}6 \times 10^{-7}$ |
| | 5·8 | $68 \times 10^{-4}$ | $2{\cdot}0 \times 10^{-7}$ |

can be determined from a plot of the reciprocal of the initial reaction rate against the reciprocal of the substrate concentration (Lineweaver and Burk, 1934). The results obtained are given in Tables I and II. The value of $K_m$ for the enzyme of guinea-pig kidney is approximately $5 \times 10^{-2}$ mole litre$^{-1}$ as compared with a value of $7 \times 10^{-4}$ mole litre$^{-1}$

for that of rat hepatoma, showing that the enzyme in the tumour has the greater affinity for L-histidine. From Table II it will be seen that for both enzymes the values of $K_m$ appear to be raised in the presence of benzene. However benzene greatly increases V in the case of guinea-pig kidney, but has little effect in the case of rat hepatoma. This suggests that benzene exerts its main influence on guinea-pig kidney extract either by increasing the rate of breakdown of the enzyme-substrate complex or by increasing the concentration of enzyme molecules available (Waton, 1956).

We have confirmed the finding that guinea-pig kidney extracts have high DOPA and 5-HTP decarboxylase activities when studied manometrically (Blaschko, 1942; Smith, 1960). However, using the same method we

TABLE II

EFFECT OF BENZENE ON THE MAXIMUM RATE OF HISTAMINE PRODUCTION (V), AND ON THE VALUE OF THE MICHAELIS CONSTANT ($K_m$) OF HISTIDINE DECARBOXYLASES AT 37°

| Tissue extract | pH | $K_m$ mole litre$^{-1}$ | | V mole litre$^{-1}$ min.$^{-1}$ | |
|---|---|---|---|---|---|
| | | without benzene | with benzene | without benzene | with benzene |
| Guinea-pig kidney .. .. | 9·2 | $3{\cdot}3 \times 10^{-2}$ | $5{\cdot}8 \times 10^{-2}$ | $3{\cdot}3 \times 10^{-7}$ | $25{\cdot}0 \times 10^{-7}$ |
| Rat hepatoma .. .. .. | 6·7 | $8{\cdot}7 \times 10^{-4}$ | $17{\cdot}4 \times 10^{-4}$ | $5{\cdot}5 \times 10^{-7}$ | $5{\cdot}9 \times 10^{-7}$ |

have been unable to detect these two enzymes in extracts of rat hepatoma at pH values of 5·0, 5·8 or 6·7. The apparent absence of 5-HTP decarboxylase from rat hepatoma is consistent with the unchanged urinary output of 5-HIAA from female August rats implanted with F-HEP, as shown in Fig. 4. On the other hand, in keeping with the high histidine decarboxylase activity of the tumours, the urinary histamine rose dramatically as previously reported (Mackay, Marshall and Riley, 1960). The histidine decarboxylase activity of rat hepatoma, unlike that of guinea-pig kidney, does not therefore appear to be associated with DOPA and 5-HTP decarboxylase activities. The above results indicate that the histidine decarboxylase present in rat hepatoma is a completely different enzyme from that which occurs in guinea-pig kidney.

*Acknowledgement.* The authors are grateful to Dr. K. S. Kirby and Professor A. Haddow for supplying the August rats and hepatoma transplant. One of the authors (D. M.) also wishes to acknowledge the financial support of Smith, Kline and French Laboratories Ltd.

REFERENCES

Blaschko, H. (1942). *J. Physiol.*, **101**, 337–349.
Kahlson, G. (1960). *Lancet*, **1**, 67–71.
Lineweaver, H., and Burk, D. (1934). *J. Amer. chem. Soc.*, **56**, 658–666.
Macfarlane, P. S., Dalgliesh, C. E., Dutton, R. W., Lennox, B., Nyhus, L. M., and Smith, A. N. (1956). *Scot. med. J.*, **1**, 148–155.
Mackay, D., Marshall, P. B., and Riley, J. F. (1960). *J. Physiol.*, **153**, 31P.
Mackay, D., and Shepherd, D. M. (1960). *Brit. J. Pharmacol.*, **15**, 552–556.
Smith, S. E. (1960). *Ibid.*, **15**, 319–327.
Telford, J. M., and West, G. B. (1961). *J. Physiol.*, in the press.
Waton, N. G. (1956). *Biochem. J.*, **64**, 318–322.

# HYDROGENATION OF DIGITALIS GENINS AND ANHYDROGENINS

By B. T. Brown and S. E. Wright

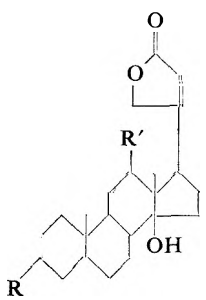
*From the Department of Pharmacy, University of Sydney, Sydney, Australia*

Received November 24, 1960

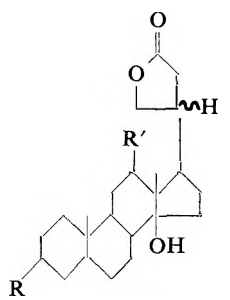
Hydrogenation of the 14,15-unsaturated linkage in the digitalis β-anhydrogenins without simultaneous reduction of the cyclobutenolide group is described. The reduced compounds have an α configuration for the hydrogen at C(14). Hydrogenation of the cyclobutenolide ring produces two isomeric compounds which have been separated and assigned to the 20α and 20β steroid series respectively.

To investigate the influence of the 14β-hydroxyl group characteristic of cardiac glycosides on the pharmacological action of these compounds we have eliminated this group from digoxigenin (Ia) and digitoxigenin acetate (Ic) by dehydration followed by hydrogenation of the anhydrogenin so produced without at the same time reducing the unsaturated 20,22 linkage of the cyclobutenolide ring. It was necessary to avoid reduction of this ring as Jacobs and Hoffman (1927), and Chen and Elderfield (1940) have shown that 20,22-dihydroglycosides have much reduced activity. The C/D ring juncture of the 14-deoxygenins so produced was however *trans* instead of *cis* in the original genins.

The reduction of the unsaturated 20,22 linkage of the cyclobutenolide ring has also been investigated. Hydrogenation of this linkage introduces a centre of asymmetry at C(20) and the isomers produced may be related




Ia. R = R′ = OH
Ib. R = OH, R′ = H
Ic. R = OAc, R′ = H

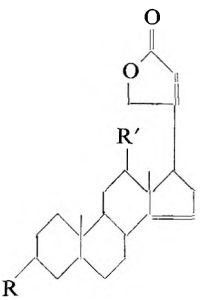



IIa. R = R′ = OH
IIb. R = OH, R′ = H

to the 20α or 20β series of steroids as typified by the 20-isonorallocholanic acids (20α) and the slightly more dextrorotatory norallocholanic acids (20β) (Plattner, 1951). We have hydrogenated digoxigenin (Ia) and 3β-acetoxy-14α-card-20(22)-enolide (IVb) (14-deoxydigitoxigenin acetate) with platinum catalyst, separated the isomers by crystallisation and related each isomer to the 20α or 20β series on the basis of optical rotation and melting points.

14-*Deoxygenins*

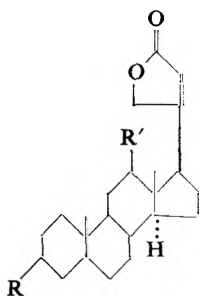
Preferential reduction of the 14,15-unsaturated linkage in β-anhydrodigoxigenin (IIIa) and β-anhydrodigitoxigenin acetate (IIIb) without the simultaneous reduction of the cyclobutenolide ring was achieved by hydrogenation in ethanolic solution with palladium catalyst at room temperature. Under these conditions one molecular equivalent of hydrogen was absorbed and the reduced products showed absorption



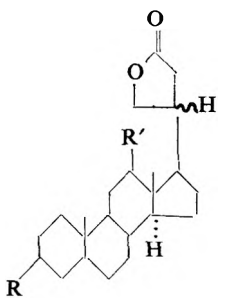
IIIa. R = R′ = OH
IIIb. R = OAc, R′ = H

spectra in the ultra-violet similar to the parent genins (14-deoxydigitoxigenin acetate max. Log $\epsilon = 4{\cdot}236$ at 216 mμ and 14-deoxydigoxigenin max. Log $\epsilon = 4{\cdot}195$ at 217 mμ). Colour reactions with alkaline *m*-dinitrobenzene and tetranitromethane confirmed the presence of the cyclobutenolide ring and the absence of the 14,15-unsaturated linkage.

The α configuration of the hydrogen atom at C(14) (C/D ring *cis*) of the 14-deoxygenins has been assigned on the following evidence.



IVa. R = R′ = OH
IVb. R = OAc, R′ = H



Va. R = R′ = OH
Vb. R = OAc, R′ = H

Complete reduction of 14-deoxydigoxigenin (IVa) by catalytic hydrogenation with platinum produced a substance with no absorption at 217 mμ and which gave no colour with alkaline *m*-dinitrobenzene. The melting point and optical rotation were identical with that of tetrahydro-β-anhydrodigoxigenin (Va) prepared by Plattner, Ruzicka and Pataki (1945) by complete reduction of β-anhydrodigoxigenin (IIIa). Chromic acid oxidation of tetrahydro-β-anhydrodigoxigenin (Va) by these workers gave the diketone 3,12-dioxo-14α-cardanolide which was identical with the diketone

obtained by oxidation of 3α,12β-dihydroxy-14α-cardanolide prepared by synthesis from 3α,12β-dihydroxypregnan-20-one which has a 14α (C/D *trans*) configuration (Ruzicka, Plattner and Pataki, 1944). By analogy, 14-deoxydigitoxigenin acetate (IVb) would have a 14α (C/D *trans*) configuration.

20,22-*Dihydrogenins*

The cyclobutenolide double bond of digoxigenin (Ia) was hydrogenated in ethanolic solution with platinum catalyst. By repeated crystallisation of the 20,22-dihydrodigoxigenin (IIa) from ethyl acetate two crystalline isomerides which differed in melting point and optical rotation were separated. The more soluble material, which had the higher dextrorotation, was designated the 20β isomer and the isomeride with lower dextrorotation, 20α. Constants for both isomers are recorded in Table I.

TABLE I

| Compound | α Isomer | | β Isomer | | Reference |
|---|---|---|---|---|---|
| | m.p. | $[\alpha]_D$ | m.p. | $[\alpha]_D$ | |
| 3α,12β-Dihydroxy-14α-cardanolide | 243° | +56·8° | 225° | +62·8° | Plattner and others (1945) |
| 3α,12β-Diacetoxy-14α-cardanolide | 209° | +116° | 188° | +122° | *Ibid.* |
| 3,12-Dioxo-14α-cardanolide .. | 312° | +123° | 297° | +132° | *Ibid.* |
| 3β-Acetoxy-21-oxo-nor*allo*cholanic acid lactone (23,21) .. .. | 243° | +5·9° | 204° | +19° | Ruzicka and others (1941) |
| 21-Oxonorallocholanic acid lactone (23,21) .. .. .. .. | 178° | +11·3° | 180° | +24·3° | Tschesche (1933) |
| 20,22-Dihydrodigitoxigenin acetate | 186° | +8° | 178° | +14° | Meyer (1946) |
| 20,22-Dihydrodigoxigenin (IIa) .. | 225° | +11° | 209° | +15° | This paper |
| 3β-Acetoxy-14α-cardanolide (Vb) | 139° | +24° | 128° | +51° | This paper |

3β-Acetoxy-14α-card-20(22)-enolide (IVb) was similarly hydrogenated using platinum oxide catalyst. The dihydro-compound (3β-acetoxy-14α-cardanolide (Vb) was repeatedly crystallised from aqueous ethanol and gave two crystalline isomers. The more soluble material, which also had a higher dextrorotation, was designed 20β, and the isomeride with lower dextrorotation, 20α. Melting points and rotations for both isomers are shown in Table I. None of the 20,22-dihydro-compounds prepared showed absorption at 218 mμ in ethanol, nor at 235 mμ in sulphuric acid. This is the absorption maxima of an unsaturated cyclobutenolide in sulphuric acid (Repke, 1960; Brown and Wright, 1960).

Plattner and others (1945) have hydrogenated the 20,22-double bond of synthetically prepared 3α,12β-dihydroxy-14α-card-20(22)-enolide and after acetylation and oxidation have, by crystallisation, obtained the C(20) isomers of 3α,12β-dihydroxy-14α-cardanolide, the 3,12-diacetate and the 3,12-diketone. These workers have related the higher dextrorotatory isomer of these isomeric pairs to the 20β series of steroids, and the lower dextrorotatory to the 20α series. In Table I the melting points and rotations of these isomers are recorded, together with values for a number of other 20α- and 20β-saturated 23,21 steroid lactones.

In each of the C(20) isomeric pairs listed in Table I, with the possible exception of 21-oxo-norallocholanic acid lactone (23,21), the melting point

of the $\beta$ isomer (higher dextrorotation) is lower than the melting point of the corresponding $\alpha$ isomer. Both the isomeric pairs prepared in the present study have a similar relationship of melting point and optical rotation, that is, the $\beta$ isomer has a lower melting point and a higher dextrorotation than the corresponding $\alpha$ isomer.

Smith (1930) prepared 20,22-dihydrodigoxigenin which melted at 170°, resolidified and remelted at 215°, $[\alpha]_D + 19°$. From the melting point and rotation it would appear that this compound is predominately the 20$\beta$ isomer.

We have also prepared 20,22-dihydrodigitoxigenin (IIb) from digitoxigenin (Ib) by hydrogenation with platinum catalyst. We could obtain only one 20,22-dihydro product, which was similar in melting point and rotation to the 20,22-dihydrodigitoxigenin obtained by Cardwell and Smith (1954) (m.p. 226°, $[\alpha]_D + 17°$). These workers assigned a 20$\beta$ configuration to this compound, as its rotation agreed more with that of the 20$\beta$-dihydrodigitoxigenin acetate ($[\alpha]_D + 14°$) than with that of the 20$\alpha$ isomeride ($[\alpha]_D + 8°$). (Meyer, 1946).

We have also hydrogenated the glycoside digoxin. Using palladium-carbon catalyst in ethanol, reduction occurred only when the material was heated to 50°, while hydrogenation using platinum oxide catalyst proceeded quite rapidly at room temperature. In each instance, reduction was complete as judged by the absence of an absorption maxima at 218 m$\mu$ in ethanol. The major portion of the material was amorphous, though a small number of crystals were obtained from methanol-water. Attempts to separate 20$\alpha$ and 20$\beta$ isomers were unsuccessful.

## Experimental

*Effect of hydrogenation of digoxigenin using palladium catalyst.* Digoxigenin (103 mg.) was dissolved in ethanol (10 ml.) and palladium-carbon catalyst (5 per cent) (190 mg.) added. At room temperature and atmospheric pressure there was no absorption of hydrogen over 8 hr., and the material was recovered unchanged.

14-*Deoxydigoxigenin (IVa).* $\beta$-Anhydrodigoxigenin (m.p. 180°, 259 mg., prepared by treatment of digoxigenin with sulphuric acid (Smith, 1930) was hydrogenated with palladium-carbon catalyst (5 per cent) (208 mg.) in 50 ml. ethanol. Hydrogenation proceeded rapidly for the first 6 hr. and was complete after 16 hr., absorbing 17·7 ml. hydrogen (1 mol. requires 16·6 ml.). Recrystallisation from ethyl acetate gave 102 mg. material, m.p. 114°, which on further recrystallisation was raised to m.p. 117–118°, $[\alpha]_D + 20°$ ($c = 0{\cdot}97$ in MeOH). Found: C, 72·65, H, 9·00. $C_{23}H_{34}O_4$ requires C, 73·76; H, 9·15 per cent. Absorption maximum in ethanol, Log $\epsilon$ 4·197 (217 m$\mu$); in sulphuric acid at 235 m$\mu$. A tetranitromethane test was negative, and with alkaline *m*-dinitrobenzene the blue colour characteristic of the unsaturated cyclobutenolide ring was observed.

3$\alpha$,12$\beta$-*Dihydroxy*-14$\alpha$-*cardanolide* (*Va*) (*Tetrahydro-*$\beta$*-anhydrodigoxigenin*). 14-Deoxydigoxigenin (30 mg.) was dissolved in ethanol (10 ml.)

and platinum oxide (8 mg.) added. Complete hydrogenation required 2 hr. After recrystallisation from ethyl acetate, the material melted 116°, $[\alpha]_D$ + 20° ($c$ = 0·88 in $CHCl_3$). The material showed no absorption maxima at 217 m$\mu$ in ethanol and no maxima at 235 m$\mu$ in sulphuric acid and gave no colour with alkaline *m*-dinitrobenzene.

14-*Deoxydigitoxigenin acetate* (*IVb*). $\beta$-Anhydrodigitoxigenin acetate (m.p. 185°, 279 mg. prepared from digitoxigenin acetate by dehydration) (Hunziker and Reichstein, 1945) was hydrogenated with palladium-carbon catalyst (5 per cent) (307 mg.) in 50 ml. ethanol. Complete reduction required 7 hr. and 20·4 ml. were absorbed (1 mol. requires 19·7 ml.). On recrystallisation from ethyl acetate and then methanol-water, the material melted 176–178°,$[\alpha]_D$ + 18·4° ($c$ = 0·49 in $CHCl_3$). Found: C, 74·93; H, 9·14. $C_{25}H_{36}O_4$ requires C, 74·96; H, 9·06 per cent.

20,22-*Dihydrodigoxigenin* (*IIa*). Digoxigenin (412 mg.) was dissolved in ethanol (20 ml.) and hydrogenated with platinum oxide (67 mg.) at room temperature. Complete reduction required 6 hr. and 31·0 ml. of hydrogen were absorbed (1 mol. requires 29·8 ml.). After removal of the catalyst, the solution was evaporated to dryness and the solid (405 mg.) dissolved in ethyl acetate. The first crop of crystals were large and granular and melted 220°. On further recrystallisation from ethyl acetate the melting point was raised to 223–225°, $[\alpha]_D$ + 11° ($c$ = 0·99 in $CHCl_3$). Found: C, 70·78; H, 9·13. $C_{23}H_{36}O_5$ requires C, 70·37; H, 9·25 per cent. This material was designated the 20$\alpha$ isomer.

The more soluble material crystallised from ethyl acetate in white, feathery crystals. On recrystallisation from ethanol-water, the material melted 145°, resolidified about 160°, and remelted 209–211°, $[\alpha]_D$ + 15° ($c$ = 0·82 in $CHCl_3$). (Found C, 70·30; H, 9·00, $C_{23}H_{36}O_5$ requires C,70·37; H, 9·25 per cent). This material was designated the 20$\beta$ isomer.

20,22-*Dihydrodigitoxigenin.* Digitoxigenin (408 mg.) was hydrogenated with platinum oxide (50 g.) in ethanol (25 ml.). Reduction required 7 hr. and 31·3 ml. hydrogen were absorbed (1 mol. requires 29·0 ml.). After recrystallisation from ethanol-water the material melted 222–224°, $[\alpha]_D$ + 16·5 ($c$ = 1·0 in MeOH). Found: C, 74·68; H, 9·78. $C_{23}H_{36}O_4$ requires C, 73·36; H, 9·64 per cent.

3$\beta$-*Acetoxy*-14$\alpha$-*cardanolide* (*Vb*). 14-Deoxydigitoxigenin acetate (85 mg.) was dissolved in ethanol (40 ml.) and hydrogenated at room temperature and atmospheric pressure with platinum oxide (79 mg.). Reduction required 1 hr. and 4 ml. hydrogen were absorbed (1 mol. requires 4·8 ml.). The first crop of crystals from ethanol were large and needle-like, melted 103–105°, resolidified and finally melted 115–116°. Further recrystallisation raised the melting point to 137–139° with no lower melting point, $[\alpha]_D$ + 24° ($c$ = 0·8 in $CHCl_3$). This isomer was designated 20$\alpha$. Found: C, 73·62; H, 9·69. $C_{25}H_{38}O_4$ requires C, 74·59; H, 9·52 per cent. The more soluble material crystallised on concentration with m.p. 120–126° which was raised to 126–128° on further crystallisation. This was designated the 20$\beta$ isomer. $[\alpha]_D$ + 51° ($c$ = 0·6 in $CHCl_3$). Neither of the above compounds gave a colour reaction with alkaline *m*-dinitrobenzene.

20,22-*Dihydrodigoxin*

*Palladium Reduction.* Digoxin (248 mg.) in ethanol (25 ml.) was hydrogenated with palladium-carbon (5 per cent) (535 mg.) at room temperature. Over a period of 8 hr. no absorption of hydrogen was observed. The solution was then heated to 50° and complete hydrogenation as judged by the absence of a colour reaction with *m*-dinitrobenzene required 8 hr. The major portion of the material would not crystallise, but from methanol-water some crystals were obtained m.p. 148°, which on further recrystallisation melted 160–166°, $[\alpha]_D + 12°$ ($c = 1{\cdot}28$ in MeOH). The material showed no absorption at 217 m$\mu$ in ethanol and no absorption at 235 m$\mu$ in sulphuric acid.

*Platinum reduction.* Digoxin (251 mg.) in ethanol (25 ml.) was hydrogenated with platinum oxide (25 mg.) at room temperature. Reduction required 8 hr. and 8·2 ml. hydrogen were absorbed (1 mol. requires 8·0 ml.). After recrystallisation from ethanol-water the material melted 155–162°, resolidified 200°, and finally melted 252–254°. Found: C, 61·03; H, 8·52. $C_{41}H_{66}O_{14}.H_2O$ requires C, 61·50; H, 8·54 per cent.

A further sample of digoxin (273 mg.) was reduced with platinum oxide (28 mg.) and 10·8 ml. of hydrogen were absorbed (1 mol. requires 9·0 ml.). After recrystallisation from ethyl acetate the material melted 268–270°, with no lower melting point. $[\alpha]_D + 13°$ ($c = 1{\cdot}4$ in MeOH). Found: C, 61·39; H, 8·54. $C_{41}H_{66}O_{14}.H_2O$ requires C, 61·50; H, 8·54 per cent. Neither of the above compounds showed absorption in ethanol at 217 m$\mu$. Both samples showed absorption in sulphuric acid at 320 m$\mu$, 390 m$\mu$ and 490 m$\mu$ but not at 235 m$\mu$. Attempts to separate possible 20$\alpha$ and 20$\beta$ isomers were not successful.

## References

Brown, B. T. and Wright, S. E. (1960). *J. Amer. pharm. Ass. Sci. Ed.*, **49**, 777–779.
Cardwell, H. M. E. and Smith, S. (1954). *J. chem. Soc.*, 2012–2023.
Chen, K. K. and Elderfield, R. C. (1940). *J. Pharmacol.*, **70**, 338–346.
Hunziker, F. and Reichstein, T. (1945). *Helv. chim. Acta.*, **28**, 1472–1479.
Jacobs, W. A. and Hoffman, A. (1927). *J. biol. Chem.*, **74**, 787–794.
Meyer, K. (1946). *Helv. chim. Acta.*, **29**, 718–726.
Plattner, Pl. A. (1951). *J. chem. Soc.*, 3536–3537.
Plattner, Pl. A., Ruzicka, L. and Pataki, J. (1945). *Helv. Chim. Acta.*, **28**, 389–395.
Repke, K. (1960). *Arch. exp. Path. Pharmak.*, **239**, 131–143.
Ruzicka, L., Plattner, Pl. A. and Furst, A. (1941). *Helv. chim. Acta.*, **24**, 716–724.
Ruzicka, L., Plattner, Pl. A. and Pataki, J. (1944). *Ibid.*, **27**, 988–994.
Smith, S. (1930). *J. chem. Soc.*, 2478–2482.
Tschesche, R. (1933). *Z. physiol. Chim.*, **222**, 50–57.

# SOME OBSERVATIONS ON THE CURARISING ACTIVITY OF *GONIOMA KAMASSI*, E. MAY

## PRELIMINARY OBSERVATIONS

BY J. A. LOCK

*From the Department of Pharmacology, Makerere College Medical School, Kampala, Uganda*

Received January 3, 1961

The total bases from *Gonioma kamassi* have been divided into tertiary and quaternary fractions and electrophoretic patterns of these are shown. The LD50 values for mice of the various fractions have been determined. The curariform action has been located in the quaternary fractions, and potencies estimated in comparison with tubocurarine. The curariform action has been shown to be truly neuromuscular, and strong evidence presented that the action is depolarising. Effects on respiration and duration of blocking action, compared with tubocurarine have been shown, and the presence of a prolonged parasympathetic ganglion blocking activity in the quaternary fraction is also demonstrated.

IN 1911 Dixon reported the pharmacology of an alkaloidal fraction of *Gonioma kamassi*, South African boxwood, and showed the presence of weak curarising activity. The description given of the preparation of Dixon's sample suggests that it may well have been a mixture of tertiary and quaternary alkaloids. In 1951, Schlittler and Gellért isolated from the bark of *G. kamassi* a tertiary alkaloid which they called Kamassin; Gellért and Witkop (1952) have since demonstrated the identity of this alkaloid with quebrachamine, and it is probable that Dixon in fact investigated a mixture of this alkaloid and curarising substances.

It seemed pertinent to reinvestigate the alkaloidal fractions from *G. kamassi* particularly with relation to the curarising activity and the possibility of the presence of quaternary alkaloids. Investigation has shown, as might be expected, that curarising activity occurs in the quaternary fraction. This fraction presents a complexity similar to those from the South American *Strychnos* species and gives rise to similar difficulties of isolating the individual alkaloids.

## EXPERIMENTAL METHODS

*Preparation of plant material.* All materials were air dried and reduced to moderately fine powder in a disintegrator. The samples were defatted by continuous extraction with isohexane.

### *Extraction of Alkaloids*

*General.* All evaporations and concentrations were made under reduced pressure. All representative samples were adjusted to contain the equivalent of 0·5 g. plant material per ml.

*Method.* The defatted material was continuously extracted with methanol, the methanol removed and the residue repeatedly extracted with hot water. The combined aqueous extracts were then filtered and concentrated.

*Separation of total base chlorides.* Basic compounds present in crude extracts were precipitated as reineckates from dilute acid solution. The reineckates were dried, dissolved in acetone, filtered and reconverted to chlorides by the Kapfhammer method using silver sulphate and barium chloride.

*Separation of* (*a*) *tertiary and* (*b*) *quaternary base chlorides.* (*a*) (i) The solution of total base chlorides was made alkaline with ammonia and repeatedly extracted with ether freed from peroxides. The ether fractions were combined and extracted with dilute hydrochloric acid to yield tertiary base chlorides.

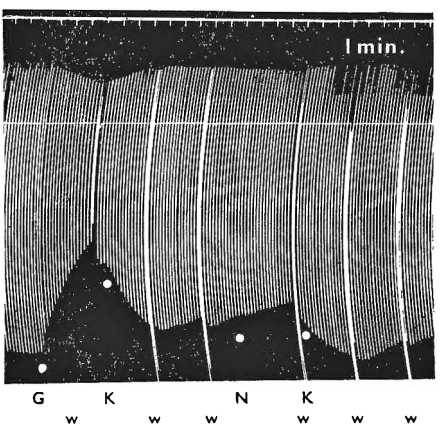



FIG.1. The effect of potassium chloride on the block induced by gonioma. Isolated rat diaphragm. 50 ml. bath. At G, 10 mg. gonioma, at w, wash, at K, 30 mg. KCl. N, normal dose position: no dose given; kymograph running continuously.

(ii) Material precipitated by ammonia and insoluble in ether was filtered off during the last ether extraction, washed with ether and water, dissolved in acid and the bases separated as chlorides by the reineckate process.

(*b*) The ammoniacal aqueous liquid was made acid and the quaternary bases separated as chlorides by the reineckate process.

*Electrophoresis on paper.* Whatman No. 1 paper was used with 15 per cent acetic acid as electrolyte. A potential gradient of 30 V./cm. was applied for one hr.

*Pharmacological*

*Toxicity determinations.* White mice, 16–20 g. were injected intraperitoneally in groups of 10 per dose. Mortalities per cent were converted to probits and approximate LD50 determinations obtained graphically.

*Isolated rat diaphragm—phrenic nerve preparation.* This preparation was arranged according to the method described by Chou (1947). Doses of drug were added by an automatic syringe device, Lock (1961).

*Gastrocnemius nerve-muscle preparation.* Cats and grey monkeys were anaesthetised by chloralose, 80 mg./kg., and sodium phenobarbitone, 120 mg./kg. respectively, and arranged according to the method of Bülbring and Burn (1941). In these preparations doses of drug were given either into the external iliac artery or the jugular vein. Respiration was recorded by Gaddum's (1941) method. Some cats were arranged for recording contractions of the nictitating membrane after supra-maximal square-wave stimulation of the preganglionic fibres at a frequency of 10/sec.

*Assay method.* The isolated rat diaphragm was used for approximate potency determinations of the curarising activity. It was found that samples of gonioma, in amounts producing more than a 30 per cent inhibition, resulted in a very slow rate of recovery compared with tubocurarine.

The addition of KCl to the bath during the recovery period, as suggested by West (1947), resulted in a rapid expansion of the trace, but after removal of the KCl the inhibition increased and tended to return to its

TABLE I

THE TOXICITIES TO MICE, CURARISING POTENCIES AND TOTAL ACTIVITIES OF GONIOMA LEAF, BARK AND WOOD CRUDE EXTRACTS. SAMPLES WERE STANDARDISED TO CONTAIN 0·5 G. OF CRUDE DRUG PER ML.

| Sample | Wt. residue mg./ml. | mg. residue equivalent to 1 mg. tubocurarine | LD50 to mice mg./kg. i.p. | Total activity per kg. orig. material in terms of tubocurarine |
|---|---|---|---|---|
| Leaves .. .. | 47·0 | 490 | 135 | 660 |
| Bark .. .. | 80·4 | 142 | 71 | 1133 |
| Wood .. .. | 26·0 | 1270 | 557 | 39 |

expected position as if the KCl had not been added (Fig. 1). Two applications of KCl during the wash periods are shown after a dose of gonioma sufficient to produce a 48 per cent inhibition. On each occasion the trace expanded during the application of KCl and contracted to above the base line during the subsequent wash period. Full recovery was reached only after two complete cycles, that is, 26 min. Without the addition of KCl a similar dose of gonioma took much the same time for recovery.

In view of these observations, curarising potencies of various fractions were determined on the rat diaphragm by matching, doses being added in the order ABBA, and in amounts to produce not more than a 30 per cent inhibition. It is with these limitations that curarising potencies are presented in this paper.

## RESULTS

The relative amounts of curarising activity in leaves, bark and wood is shown in Table I. Not unexpectedly, samples of bark showed the highest activity, and in view of the relatively low potency of wood, further work has been confined to leaves and bark.

Table II shows relative activities of tertiary and quaternary fractions and the ether and water insoluble fraction obtained during separation (see methods). The relatively low oral toxicity to mice of the quaternary fraction from bark is also included.

TABLE II

THE TOXICITIES TO MICE, CURARISING POTENCIES AND TOTAL ACTIVITIES OF TOTAL BASES, QUATERNARY AND ETHER: WATER INSOLUBLE FRACTIONS OF GONIOMA LEAF AND BARK

| | Sample | Wt. residue mg./ml. | mg. residue equivalent to 1 mg. tubocurarine | LD50 to mice mg./kg. i.p. | Equivalent mg. tubocurarine per kg. |
|---|---|---|---|---|---|
| Leaf | Total bases | 8·0 | 34·5 | 12·8 | 490 |
| | Quaternary | 2·0 | 35·0 | 12·6 | 166 |
| | Ether: water insoluble | 2·5 | 30·0 | 10·6 | 166 |
| Bark | Total bases | 23 | 38·2 | 22·4 | 1200 |
| | Quaternary | 8·4 | 45·8 | 14·0 oral 840 | 366 |
| | Ether: water insoluble | 7·8 | 25·0 | 13·0 | 624 |

The tertiary bases contained no curarising activity, although difficulty was initially experienced in obtaining a fraction uncontaminated with quaternary substances. The ether and water insoluble complex, initially discarded during filtrations to prevent emulsions while extracting the tertiary components from ammoniacal bases with organic solvents was found to contain as much curarising activity as the quaternary fraction.

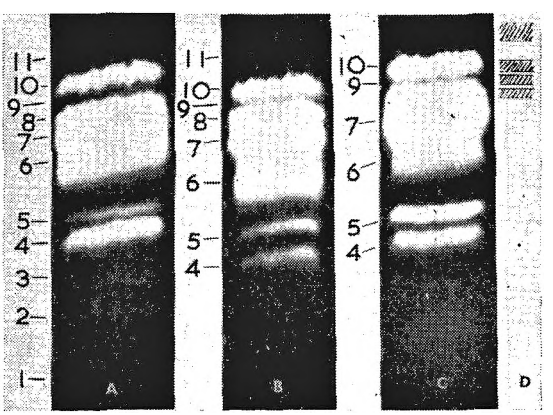



FIG. 2. The electrophoretic patterns of fractions from *G. kamassi* bark. U.V. light 360 Å; electrolyte, acetic acid 15 per cent; potential gradient 30 V/cm. for 1 hr.; paper Whatman No. 1. A, total base; B, ether : water insoluble; C, quaternary; D, Dragendorff positive zones. Fluorescent colours 1, 2 and 3, whitish; 4 blue; 5 yellow; 6 blue; 7 yellow; 8 green; 9 blue; 10 yellow; 11 dark purple. No. 11 does not show on the photograph; it is present in A and B but not in C.

*Identification of components*

Paper chromatography, with a large number of solvent mixtures including Karrer's A, B, D & E (Schmid and Karrer, 1950; Schmid, Kebrle and Karrer, 1952) have produced indifferent results with gonioma extracts and fractions. Paper electrophoresis has, however, made evident the presence of a large number of positively charged components most of

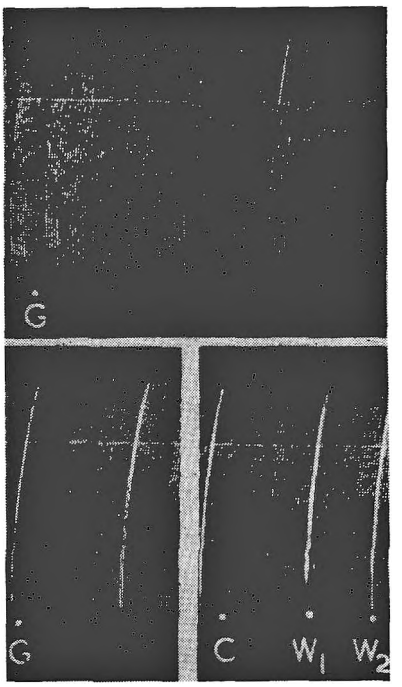



FIG. 3. The effect of direct muscular and neural stimulation of the isolated rat diaphragm exposed to gonioma, and also the application of gonioma to nerve alone, Upper Fig., at G, 0·16 mg./ml. gonioma in the bath. Ten stimulations per min. alternately to nerve and muscle. Lower Fig. a, at G, 0·8 mg./ml. gonioma applied to the nerve. The nerve from the diaphragm was passed through a rubber seal into a glass tube which contained the electrodes. Lower Fig. b, at C, 10 per cent cocaine applied to the nerve; W, wash in the main bath; $W_2$ replacement of the cocaine by Tyrode, kymograph off 15 min.

which show strong fluorescence and some a positive reaction with Dragendorff's reagent. Fig. 2A, B, and C, shows the electrophoretic patterns of total bases, ether: water insoluble, and quaternary fractions from bark under ultra-violet light of wavelength 360 Å, and at D the position of bands acting with Dragendorff's reagent.

A deep purple zone (at 11 in Fig. 2A and B), which had the greatest rate of migration, showed a transient violet coloration with ceric sulphate. This fluorescence had not sufficient actinic power to show in the photograph. This band was found to be identical with the tertiary component

identified by Gellért and Witkop (1952) as quebrachamine and the reaction with ceric sulphate (Kebrle, Schmid, Waser and Karrer, 1953) is in accord with the indol group in its structure. It also gave a positive reaction with Draggendorff's reagent. It is of interest that no other component showed a similar colour reaction, in marked contrast with the

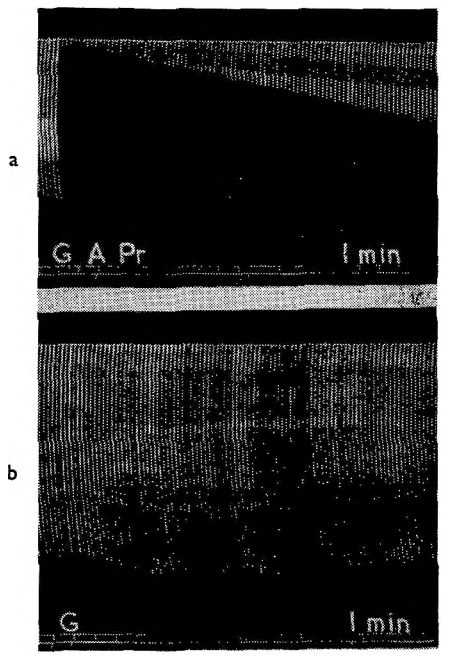



FIG. 4. The effect of a small dose of gonioma on the sciatic-gastrocnemius preparation, and also the effect of neostigmine on a gonomia produced block in the same preparation.

Vervet monkey, 6·2 kg., sodium phenobarbitone:

(a) At G, 28 mg. gonioma; at A, 1·5 mg. atropine; at Pr. 2 mg. neostigmine, all intra-arterially.

(b) At G, 11 mg. gonioma intra-arterially.

curarising strychnos alkaloids, most of which show colours with this reagent (Lederer, 1959).

A notable difference between leaf (not shown) and bark is the low intensity in the former of the two pronounced yellow bands at 4 and 6 in the bark. Electrophoretic patterns of the quaternary bases (Fig. 2C) show only five groups of components with three Dragendorff positive zones in contrast to eleven groups in the total bases. The ether: water insoluble fraction (Fig. 2B) is similar to the former except for the absence of band 2, tending to confirm the suggestion that quaternary bases are rendered insoluble by the presence of resinous substances.

From Table II it will be seen there is an appreciable apparent loss of activity during the division of the total bases into tertiary and quaternary components. This activity, however, was found to be present as a contaminant of the tertiary alkaloidal fraction. Repeated transference

between aqueous and non-aqueous solvents with acid and alkali did not achieve separation of these fractions, but chromatography of the crude tertiary fraction on alumina in isohexane allowed the passage through the column of the tertiary alkaloids; the curarising activity was retained at the top of the column and was elutable with ethanol.

*Pharmacological Properties of the Quaternary Fraction from Bark*

As the activities of quaternary fractions of leaves and bark appeared qualitatively similar, results reported below are confined to the quaternary fraction from bark. During the presentation of results this sample will be referred to as gonioma.

*Site of blocking effect.* While Dixon appreciated the similarity of the paralysis resulting from gonioma to that of "curare", he did not investigate the site of the blocking action. It was therefore important to ascertain whether gonioma is in fact a neuromuscular blocking agent.

In Fig. 3 will be seen the effect of the application of gonioma to the isolated rat diaphragm during alternate stimulations of the nerve and

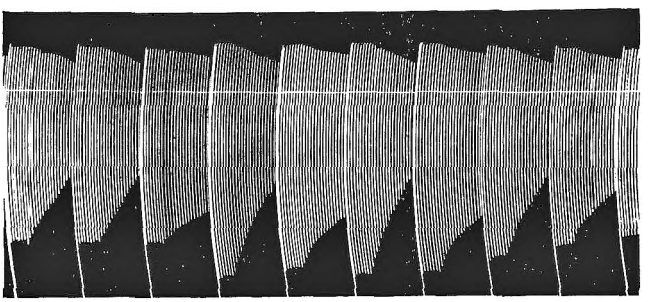



FIG. 5. The effect of neostigmine on alternate doses of tubocurarine and gonioma on the isolated rat diaphragm-phrenic nerve preparation. At T, 100 μg., tubocurarine; at G, 3 mg. gonioma; at Pr., 0·25 μg./ml. neostigmine in the Tyrode; at N, normal Tyrode resumed.

muscle. After sufficient time to produce a complete block to neural stimulation, there is no appreciable loss of excitability of the muscle to direct stimulation. The application of gonioma to the nerve alone, in a concentration five times greater than that necessary to produce the block shown in the first part of this experiment, produced no block in 3½ min. (Fig. 3a). The application of 10 per cent cocaine hydrochloride to the nerve showed an inhibition not removed by washing the main bath, but removed by replacing the cocaine solution by Tyrode, thus showing the validity of the method (Fig. 3b). It is concluded that the action of gonioma is that of a true neuromuscular blocking agent.

*Type of blocking action.* Tubocurarine, the toxiferines and the ethroidine alkaloids have in common the property of being antagonised by neostigmine, and small doses do not cause an exaggerated response to nerve stimulation in the normal muscle. This is in contradistinction to

the depolarising agents such as decamethonium. With gonioma the onset of paralysis of the sciatic-gastrocnemius preparation is rapid and doses large enough to produce an appreciable degree of inhibition do not normally show an initial expansion of the trace. When, however, a suitable small dose is given an expansion is marked, as will be seen in Fig. 4b.

When a dose sufficiently large to produce a 95 per cent inhibition was given to the same animal, after atropine, the administration of neostigmine produced a slight but definite increase in the degree of inhibition. This is shown in Fig. 4a.

A similar enhancement by neostigmine of the inhibition by gonioma was also demonstrable on the isolated rat diaphragm (Fig. 5), tubocurarine being antagonised in the expected way. Percentage inhibitions are shown in Table III.

Further evidence in this direction was obtained using chicks according to the method of Buttle and Zaimis (1949).

After the intravenous injection of 2·4 mg./kg. of gonioma, spastic paralysis occurred, the characteristic opisthotonic attitude being assumed.

TABLE III

THE EFFECT OF NEOSTIGMINE ON THE INHIBITIONS PRODUCED BY TUBOCURARINE AND GONIOMA ON THE ISOLATED RAT DIAPHRAGM (TAKEN FROM FIG. 5)

| Drug | Per cent inhibition | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | P | P | P | P | | | | |
| Tubocurarine 100 μg. .. | 41·5 | | 20 | | 36·2 | | 33·3 | | 34·9 | |
| Gonioma 3·3 mg. .. .. | | 44·5 | | 50 | | 52·3 | | 50 | | 45·3 |

At P, neostigmine, 0·25 μg./ml. was added to the Tyrode solution.

These observations present strong presumptive indications that unlike the other natural alkaloids mentioned above, the blocking activity of gonioma is of a depolarising nature.

*Duration of action of the blocking effect.* Reference to the more prolonged effect of gonioma compared with tubocurarine has already been made in connection with the isolated rat diaphragm assay of gonioma fractions. On gastrocnemius-sciatic nerve preparations both in monkeys and cats, gonioma produced a more prolonged blocking effect than tubocurarine. Fig. 6 shows that an 83 per cent inhibition in the gastrocnemius of a vervet monkey is followed by recovery in 20 min., whereas recovery from a 70 per cent inhibition produced by gonioma required 32 min. Results on cats gave a similar slower rate of recovery from gonioma.

*Effect on respiratory muscles.* Also in Fig. 6 will be seen the effect of the two drugs on respiration. Gonioma produced a slight decrease in amplitude of the respiratory movements, but the amount of air passing through the lungs was practically unchanged. Gonioma appears to have little, if any, relatively greater effect on the respiratory muscles than tubocurarine.

*Effect on vagus stimulation.* Doses of gonioma, well below those required to produce neuromuscular block, cause a profound and long lasting inhibition of the response of the blood pressure to stimulation of the peripheral end of the cut vagus.

Fig. 7 shows that an intravenous dose of gonioma insufficient to produce either expansion or contraction of the gastrocnemius twitch produced a complete block to vagal stimulation, although the fall in blood

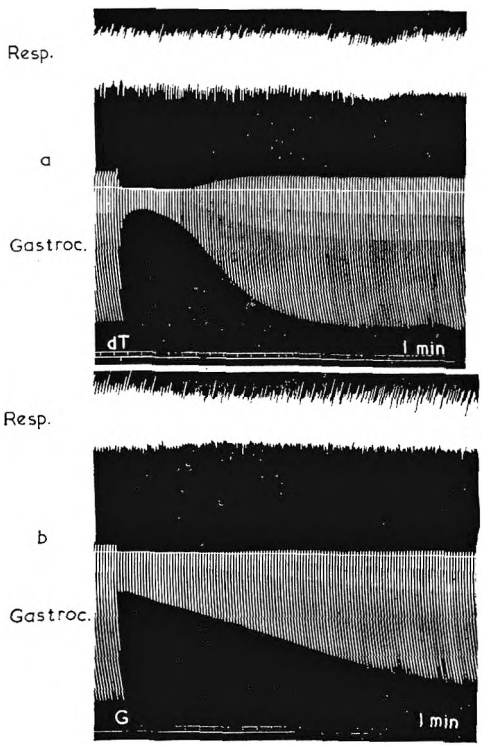



FIG. 6. The relative effects on respiration and gastrocnemius twitch of tubocurarine and gonioma.
Vervet monkey, 5·3 kg., sodium phenobarbitone.
(a) At dT, 400 $\mu$g. tubocurarine.
(b) At G, 14 mg. gonioma.

pressure in response to acetylcholine was unchanged. Little recovery from this blocking action took place until $3\frac{1}{2}$ hr. had elapsed. Larger doses did not influence the response to acetylcholine. Gonioma has therefore a prolonged blocking action on cardiac parasympathetic ganglia, but no appreciable atropine-like effect on the circulatory mechanism.

*Effect on the blood pressure.* Small doses of gonioma produce a fall in blood pressure (Fig. 7), and the fall is proportional to the dose until a level of about 40 mm. Hg is reached. Further doses produce no further fall. Recovery is rather prolonged; doses sufficient to produce maximal fall require an hour for recovery. The fall was not modified by previously

administered large doses of antihistamines. Experiments on the cat nictitating membrane showed that no block of the sympathetic ganglion occurred with doses of gonioma up to those producing complete block of the gastrocnemius. No further investigation of the mechanism of this effect was made during this series of experiments.

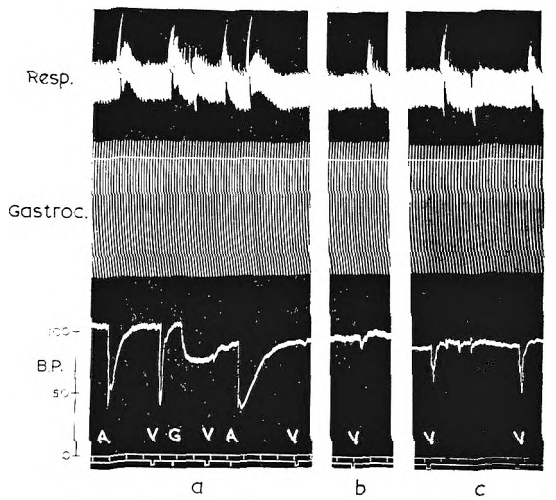



FIG. 7. The effect of gonioma on the response of blood pressure to acetylcholine and vagal stimulation. Vervet monkey, 1·6 kg., sodium phenobarbitone; respiration, gastrocnemius twitch, blood pressure in mm. Hg. At A, 30 $\mu$g. acetylcholine intravenously; at V, stimulation of the peripheral end of the cut vagus, at G, 3 mg. gonioma intravenously, b, after 40 min., c, after 210 min.

## DISCUSSION

The paucity of curarising alkaloids so far reported from Africa is in marked contrast to the large number of different species yielding these from South America. *Gonioma kamassi* is, however, a notable example, but appears to have attracted little attention from either makers of arrow poisons or pharmacologists. There appear to be no reports of its use as an arrow poison in spite of the reasonably high toxicity of crude extracts by injection and negligible toxicity by mouth.

Of marked interest is that gonioma, unlike the other naturally occurring curarising alkaloids such as tubocurarine, the toxiferines and the erythrine-alkaloids, shows many properties of a depolarising blocking drug. While absolute proof must await the chemical separation of the curarising principle or principles, the combination of enhancement of the block by neostigmine, the increased response to nervous stimulation of the gastrocnemius muscle, and the characteristic opisthotonic attitude of the young chick after intravenous injection, present strong presumptive evidence that this is so.

The blocking effect resembles the toxiferines in its relatively prolonged action compared with tubocurarine; it does not show the relatively powerful action on the respiratory muscles, Paton and Perry (1951).

Of note also is the long acting block of the cardiac parasympathetic ganglia, and the less prolonged but appreciable hypotensive effect. These two properties cannot as yet be ascribed with certainty to the curarising activity, and it is felt that further pharmacological analysis should await separation of the chemical entities.

The potency of the curarising activity is also unknown. The most active fraction so far obtained during this series of experiments has shown a potency of one third that of tubocurarine; at least six components were shown to be present by electrophoresis.

*Acknowledgements.* I express my gratitude to the Wellcome Foundation Ltd., for a grant in aid, and for hospitality in their Chemical Research Laboratories during June and July, 1960; also to Dr. S. Wilkinson of those laboratories for helpful discussion, and to the Forest Department, Pretoria, for supplying samples of gonioma.

## REFERENCES

Bülbring, E., and Burn, J. H. (1942). *J. Physiol.*, **101**, 224–235.
Buttle, G. A. H., and Zaimis, E. J. (1949). *J. Pharm. Pharmacol.*, **1**, 991–992.
Chou, T. C. (1947). *J. Pharmacol.*, **2**, 1–7.
Dixon, W. E. (1911). *Proc. Roy. Soc., B.*, **83**, 287.
Gaddum, J. H. (1941). *J. Physiol.*, **99**, 257–264.
Gellért, E., and Witkop, B. (1952). *Helv. chim. Acta*, **35**, 114–120.
Kebrle, J., Schmid, H., Waser, P., and Karrer, P. (1953). *Ibid.*, **36**, 102–121.
Lederer, M. (1959). *Chromatographic Rev.*, **1**, 79, The Netherlands: Elsevier.
Lock, J. A. (1961). *J. Pharm. Pharmacol.*, **13**. In the press.
Paton, W., and Perry, W. (1951). *Brit. J. Pharmacol.*, **6**, 299–310.
Schittler, E., and Gellért, E. (1951). *Helv. chim. Acta*, **34**, 920–923.
Schmid, H., and Karrer, P. (1950). *Ibid.*, **33**, 512–515.
Schmid, H., Kebrle, J., and Karrer, P. (1952). *Ibid.*, **35**, 1864–1879.
West, A. (1947). *Quart. J. Pharm. Pharmacol.*, **20**, 518–527.

# THE ISOLATION OF CYMARIN AND PERIPLOCYMARIN FROM THE SEEDS OF *CASTILLOA ELASTICA* CERV.

BY (MISS) GILLIAN R. ADAMS AND S. WILKINSON

*From the Chemical Research Laboratory, The Wellcome Research Laboratories, Beckenham, Kent*

Received December 13, 1960

Two known cardiac glycosides, cymarin and periplocymarin have been isolated from the seeds of the South American rubber tree, *Castilloa elastica*.

CARDIAC glycosides have been found in a limited number of plants of the botanical family *Moraceae*. Thus, the latex of *Antiaropsis* K. Schum and *Ogcodeia ternstroemiiflora* (Bisset, 1958) and *Antiaris toxicaria* Lesch (Upas tree) (Dolder, Tamm and Reichstein, 1955), which are native arrow poisons, contain a complex series of glycosides including α- and β-antiarin. The presence of cymarin in the roots of *Cannabis indica*, Lam has been reported (Soldatova, 1957).

In the course of examining a number of botanical species and *materia medica* collected by the late Dr. David Hooper, and now housed in the Wellcome Historical Medical Museum, a small sample of the seeds of *Castilloa elastica* Cerv. (*syn. Castilla elastica* Cerv.)* was found to give the characteristic colour reactions of the cardenolides. Raffauf and Morris (1960) have recently drawn attention to the persistence of alkaloids in plant tissue after prolonged storage. Although the precise date when the seeds were collected is not known, they are at least 50 years old and this illustrates the stability of the cardenolides in the seeds over this period of time. Similar results have been found with old seeds of *Strophanthus kombé*, *sarmentosus*, *gratus* and *dichotomus* from the same collection.

As a result of this preliminary chemical screening, fresh seeds and latex of *C. elastica* were obtained. Although glycosides could not be detected in the latex, they were readily extracted from the defatted seeds with chloroform. Examination by paper chromatography (Whatman No. 1 paper impregnated with formamide (25 per cent) in acetone; solvent methylisobutylketone: diisopropyl ether 100:25, saturated with formamide) revealed a major component ($R_F$ 0·45), a minor component ($R_F$ 0·66) and a trace of a third glycoside ($R_F$ 0·25).

The glycosides could be crystallised directly from methanol as colourless mixed crystals but it was found more convenient to fractionate the crude mixture on acetic acid (10 per cent)-deactivated alumina, with benzene: chloroform (1:2 v/v) as the solvent. The first runnings, which contained only a yellow pigment, were followed by the glycoside ($R_F$ 0·66) and then by the major component.

The main glycoside (0·76 per cent of the seed) was identified as cymarin from its physical constants and by hydrolysis to strophanthidin and

* A brief history of *C. elastica* is given by Loomis, *Agriculture of the Americas*, September, 1942.

cymarose. The second glycoside (0·14 per cent) gave periplogenin and cymarose on hydrolysis and was found to be periplocymarin. The remaining glycoside could not be isolated in quantities sufficient for identification.

## EXPERIMENTAL

The seeds (3,550 g.), from a mixture of kernels and seeds (9 kg.) were finely ground and percolated with light petroleum (b.p. 60–80°). Evaporation of the extract gave a thick viscous oil (1,010 g.). The marc was dried in a current of air and percolated with chloroform until no further blue colour was obtained with alkaline *m*-dinitrobenzene (Raymond's reagent). The percolate was evaporated under reduced pressure and the gummy residue triturated with dry ether (750 ml.). The pale yellow solid was filtered, washed with ether and dried (67·5 g; 1·9 per cent). Percolation of the residual seed powder with methanol gave a thick gum (200 g.) which contained only a trace of cardenolides.

The crude glycosides (20 g.) in benzene:chloroform (1:2 v/v; 50 ml.) were absorbed on a column of deactivated alumina (7 × 32 cm.) and the chromatogram was developed with the same solvent mixture. The following fractions were detected by paper chromatographic analysis.

*Column I: Fraction* (25 ml.):

| | | |
|---|---|---|
| (*a*) | 1–54: pigment | |
| (*b*) | 55–77: $R_F$ 0·66 | (0·685 g.) |
| (*c*) | 78–136: trace $R_F$ 0·66 + $R_F$ 0·45 | (3·5 g.) |
| (*d*) | 137–269: $R_F$ 0·45 | (6 g.) |

Fraction (*c*) was concentrated and the solid fractionated further on a second alumina column (4 × 30 cm.), using the same solvent mixture.

*Column II: Fraction* (10 ml.)

| | | |
|---|---|---|
| (*a*) | 1–30: $R_F$ 0·66 | (0·8 g.) |
| (*b*) | 31–71: trace $R_F$ 0·66 | (0·4 g.) |
| (*c*) | 71–90: $R_F$ 0·45 | (2·0 g.) |

*Cymarin.* Fractions (*d*) (Column I) and (*c*) (Column II) were combined and evaporated. The residue (8·0 g.) crystallised from methanol and ether in needles, m.p. 141–143° (after sintering at 138°) (Found: C, 64·1; H, 8·3 per cent. Calculated for $C_{30}H_{44}O_9$,MeOH: C, 64·1; H, 8·3 per cent). Recrystallisation from dilute ethanol gave hexagonal plates of the sesquihydrate, m.p. 184–185° (Found: C, 62·5; H, 8·2 per cent. Calculated for $C_{30}H_{44}O_9$,$1\frac{1}{2}H_2O$: C, 62·6; H, 8·2 per cent) which lost water on drying at 120°/0·01 mm. to give anhydrous cymarin $[\alpha]_D^{22} = +39{\cdot}0°$ ($c = 1{\cdot}7$ in $CHCl_3$) (Found: C, 65·8; H, 8·0; OMe, 5·75 per cent. Calculated for $C_{30}H_{44}O_9$: C, 65·7; H, 8·0; OMe, 5·7 per cent) (Authentic cymarin: m.p. 184°, $[\alpha]_D^{20} = +39{\cdot}3°$ (MeOH)) (Stoll, Renz and Kreis, 1937). Acetylcymarin crystallised from aqueous methanol in long needles, m.p. 175–176°, $[\alpha]_D^{22} = +45{\cdot}1°$ ($c = 1{\cdot}0$ in EtOH) (Found: C, 64·6; H, 7·4; OMe, 4·8 per cent. Calculated for C, 65·0; H, 7·85; OMe, 5·3 per cent).

Hydrolysis of the cymarin with hydrochloric acid (0·1N) in aqueous methanol (50 per cent) gave strophanthidin, m.p. 230–233° $[\alpha]_D^{22} = +43{\cdot}5°$ ($c = 0{\cdot}4$ in MeOH) (Found: C, 67·6; H, 8·1 per cent. Calculated for $C_{23}H_{32}O_6$: C, 68·3; H, 7·95 per cent). The $R_F$ (0·48) with the system described previously was identical with that of authentic strophanthidin prepared from k-strophanthin. Further proof of identity was obtained by preparing the oxime, m.p. 270° (decomp.) (Found: C, 66·3; H, 8·1; N, 3·0 per cent. Calculated for $C_{23}H_{33}NO_6$: C, 65·9; H, 7·9; N, 3·3 per cent) and the 3-acetyl derivative, m.p. 243–244° $[\alpha]_D^{22} = +57{\cdot}7$ ($c = 0{\cdot}45$ in $CHCl_3$) (Found: C, 67·0; H, 7·65 per cent. Calculated for $C_{25}H_{34}O_7$: 67·3; H, 7·6 per cent).

*Cymarose* was isolated and purified by molecular distillation (120°/0·0001 mm.). Crystallisation from ether and light petroleum gave prisms, m.p. 93–94°, $[\alpha]_D^{20} = +54{\cdot}7°$ ($c = 3{\cdot}2$ in $H_2O$ after 24 hr.). Cymaronic acid phenylhydrazide, prepared according to the procedure of Shoppee and Reichstein (1940), crystallised from methanol and ether in needles, m.p. 152–152·5°, $[\alpha]_D^{20} = +0{\cdot}25°$ ($c = 4{\cdot}3$ in MeOH) (Found: C, 57·8; H, 7·6; N, 10·5; OMe, 12·3 per cent. Calculated for $C_{13}H_{20}N_2O_4$ requires C, 58·2; H, 7·5; N, 10·4; OMe, 11·6 per cent) (Authentic cymaronic acid phenylhydrazide, m.p. 151–152°, $[\alpha]_D^{19} = +0{\cdot}5 \pm 3°$ (MeOH) (Hunger and Reichstein, 1950).

*Periplocymarin.* Concentration of fraction (*b*) (Column I) and fraction (*a*) (Column II) gave a solid (1·48 g.) which crystallised from methanol and ether in prisms, m.p. 146–147° (after sintering at 140°) and which, after drying at 120°/0·001 mm., had m.p. 138–140°, $[\alpha]_D^{22} = +27{\cdot}8°$ ($c = 1{\cdot}1$ in MeOH) (Found: C, 66·9; H, 8·5; OMe, 6·3 per cent. Calculated for $C_{30}H_{46}O_8$: C, 67·4; H, 8·6; OMe, 5·8 per cent) (Authentic periplocymarin, m.p. 138–139·5°, $[\alpha]_D^{20} = +28{\cdot}3 \pm 2°$ ($c = 1{\cdot}2$ in MeOH) (Ruppol and Trukovic, 1955). Acetylperiplocymarin crystallised from aqueous ethanol in hexagonal plates, m.p. 129–130°, after drying at 60°/0·01 mm., $[\alpha]_D^{22} = +45{\cdot}25°$ ($c = 0{\cdot}44$ in $CHCl_3$) (Found: C, 64·6; H, 8·4 per cent. Calculated for $C_{32}H_{48}O_9,H_2O$: C, 64·6; H, 8·45 per cent).

Hydrolysis of periplocymarin gave cymarose ($R_F$ 0·79; Whatman No. 1, butanol: pyridine: water, 3:2:1·5; identical with that of authentic cymarose) and periplogenin, which crystallised from aqueous ethanol in prisms which sintered at *ca.* 140° and then melted at 220–222°. Recrystallisation from aqueous methanol gave prisms, m.p. 233–235° (Found: C, 67·2; H, 8·7; $H_2O$, 4·5 per cent. Calculated for $C_{23}H_{34}O_5,H_2O$: C, 67·6; H, 8·9; $H_2O$, 4·4 per cent). After drying at 120°/0·001 mm., $[\alpha]_D^{22} = +31{\cdot}1°$, $[\alpha]_{5461}^{22} = +38{\cdot}8°$ ($c = 0{\cdot}39$ in EtOH) (Authentic periplogenin, m.p. 142–150° and then 238–245°, from chloroform and ether, $[\alpha]_D^{23} = +29{\cdot}48°$ (MeOH), $[\alpha]_D^{19} = +30{\cdot}0°$ ($CHCl_3$) (Ruppol and Trukovic, 1955), $[\alpha]_D^{27} = +31{\cdot}5°$ ($c = 1{\cdot}04$ in EtOH) (Lehmann, 1897).

For further identification, 3-acetylperiplogenin, m.p. 242–244°, $[\alpha]_D^{22} = +46{\cdot}9°$ ($c = 0{\cdot}32$ in $CHCl_3$) was prepared (Found: C, 68·9; H, 8·2 per cent. Calculated for $C_{25}H_{36}O_6$: C, 69·4; H, 8·35 per cent).

*Acknowledgements.* We wish to thank Dr. E. A. Underwood, Director of the Wellcome Historical Medical Museum for granting permission to

examine Dr. Hooper's collection and Dr. Margaret E. Rowbottom for her assistance in sorting the samples.

## REFERENCES

Bisset, N. G. (1957). *Ann. bogor* (Indonesia), **2**, 211–217; (1958), *Chem. Abstr.* **52**, 12090.
Dolder, F., Tamm, Ch., and Reichstein, T. (1955). *Helv. chim. Acta*, **38**, 1364–1396.
Hunger, A., and Reichstein, T. (1950). *Ibid.*, **33**, 76–99.
Lehmann, E. (1897). *Arch. Pharm.*, **235**, 157.
Raffauf, R. F., and Morris, E. A. (1960). *Science*, **131**, 1047.
Ruppol, E., and Trukovic, I. (1955). *J. pharm. Belg.*, **10**, 221–238.
Shoppee, C. W., and Reichstein, T. (1940). *Helv. chim. Acta*, **23**, 975–978.
Soldatova, T. G. (1957). *Pharmacology and Toxicology* (U.S.S.R.), **20**, 96.
Stoll, A., Renz, J., and Kreis, W. (1937). *Helv. chim. Acta*, **20**, 1484–1510.

# THE CHEMICAL ASSAY OF DIGITALIS

By J. G. Bhatt,* H. Brindle and A. D. Macdonald

*From the Department of Pharmacology, University of Manchester*

Received December 16, 1960

A method suggested by Williams (1958) for the separation of "digitoxin" and "primary glycosides" from digitalis leaf extract, using a column of cellulose powder, has been re-investigated. Two digitalis leaf samples were assayed chemically for their content of total glycosides, "digitoxin" and "primary glycosides", using a solution of 3,5-dinitrobenzoic acid as the reagent. The total activity and the activity of the two fractions, namely "digitoxin" and "primary glycosides", were also determined biologically using rabbit isolated auricles and pigeons (U.S.P. XV method). From the results of the chemical assays it was concluded that in some assays some substance, less biologically active than digitoxin must react with the reagent used, and in others the full biological activity was not indicated chemically. Biological estimations indicated that the two fractions were almost equal in potency, and that they accounted for the total activity. Comparisons of the activities of "pure" digitoxin and desacetyldigilanid A by biological methods confirmed that the primary glycoside was almost three times as active as digitoxin.

Active glycosides of digitalis give colour reactions by which they can be determined quantitatively. Unfortunately, although they give similar colour reactions, their biological activities vary considerably. Tests in which the glycosides have been determined colorimetrically as a group have, therefore, always given misleading results. Paper partition chromatography has been used by a number of workers to separate the constituents of *Digitalis purpurea* (Brindle, Rigby and Sharma, 1954; Heftman and Levant, 1952; Jensen, 1953; Rigby and Bellis, 1956). Although it is possible by this technique to separate quantitatively, practically all the known active glycosides of digitalis from an extract of the leaf and to estimate the quantities present colorimetrically, the process is very time consuming and the results hitherto have usually been considerably below the figures given by biological methods of determining the activity.

If a chemical assay process is to be satisfactory, it must be reasonably easy to carry out. It appeared possible that a partial separation of the glycosides into two or three groups, each of which could be determined colorimetrically, and each containing glycosides of similar activity, might ultimately lead to a chemical assay which would be satisfactory. Williams (1958) used a system of cellulose powder: formamide as the stationary phase and chloroform as eluant to separate "digitoxin" or secondary glycosides and the primary glycosides. He found that the presence of formamide in the fractions gave rise to moist residues and to interferences in colour development. Furthermore, he found that reversed phase partition chromatography on silane-treated kieselguhr could not be used as the glycosides were insoluble in cyclohexane or n-hexane. However, he found that adsorption chromatography on cellulose powder gave

* Present address: Haffkine Institute, Bombay 12, India.

qualitative and quantitative results similar to those obtained in the presence of formamide, but with the advantage of clean and dry residues. Williams examined by means of paper chromatography the two fractions thus separated, and his results indicated that the "digitoxin" or secondary glycoside fraction contained no primary glycoside and that the primary glycoside fraction contained no digitoxin. Williams also reported that the two fractions together represented the total activity of the drug in so far as this could be determined by chemical methods. The process appeared to offer promise towards separating active glycosidal fractions from digitalis and, as it had not been published, it was decided to examine its possibilities further.

## Experimental

Permission was obtained from Dr. Williams to use his process and to publish details of the original method and any modifications which might be introduced. Dr. Williams also gave details of his method to the Digitalis Panel of the Joint Committee of the Pharmaceutical Society and the Society for Analytical Chemistry on the Methods of Assay of Crude Drugs and permission has been obtained from this committee to publish the method and to use for investigation two samples of powdered digitalis leaf which they had issued.

It was considered desirable first to establish the validity of the method by using it to separate quantitatively the constituent glycosides from a mixture of commercially pure digitoxin and desacetyldigilanid A.

*Separation of Digitoxin and Desacetyldigilanid A from a known Mixture*

*Reagents. Cellulose powder.* Whatman's standard grade powder for chromatography.

*Chloroform* (ethanol free). This was prepared by washing chloroform B.P. with four separate portions of water. Each portion of water was equal to the volume of chloroform being washed. The washed chloroform was dried overnight over anhydrous sodium sulphate, filtered and then distilled. The first few millilitres of the distillate were rejected.

*Ethanol.* 10 per cent v/v in chloroform.

*Solution of digitoxin.* 40 mg. of "pure" crystalline digitoxin in 100 ml. of ethanol A.R.

*Solution of desacetyldigilanid A.* 50 mg. of "pure" desacetyldigilanid A in 100 ml. of ethanol A.R.

## Method

*Preparation of the column.* A cotton-wool plug was placed at the bottom of a glass tube about 35 cm. long and 1 cm. internal diameter, of which one end was drawn out to form a small funnel-shaped opening. A tap was fitted at the other end. About 3·0 g. of the cellulose powder was mixed with ethanol-free chloroform into a slurry and poured into the prepared glass tube. After allowing to stand for a short time, the chloroform was run off until the level fell to the surface of the cellulose powder. The column was washed with 15·0 ml. of ethanol-free chloroform to remove any soluble matter from the column.

Accurately measured volumes of the solutions of digitoxin and desacetyldigilanid A were mixed in an evaporating dish and evaporated to dryness on a boiling water bath, taking care not to overheat the residue. 3·0 ml. of ethanol-free chloroform was added to the residue obtained, warmed gently and mixed well. The mixture was allowed to cool and transferred to the prepared column of cellulose powder with a pipette. Chloroform was run off till the solution fell to the surface of the column. The residue was extracted with another 3·0 ml. of ethanol-free chloroform and the extract was transferred to the column in a similar way. The eluate was collected in an evaporating dish. 2–3 drops/sec. emerged from the column. The residue was further extracted with two 5·0 ml. portions

TABLE I

CHEMICAL ASSAY OF GLYCOSIDES GIVING THE AMOUNT OF EACH GLYCOSIDE AND TOTAL *GLYCOSIDES APPLIED TO THE COLUMN AND THE AMOUNT OF EACH GLYCOSIDE RECOVERED*, ESTIMATED AS DIGITOXIN, USING 3,5-DINITROBENZOIC ACID AS THE REAGENT WITH LIMITS OF ERROR P = 0·95

| Weight of each glycoside applied to the column | | Amount of total glycosides calculated as digitoxin, applied to the column as estimated colorimetrically | Estimated amount of separated glycosides recovered from the column | |
|---|---|---|---|---|
| Digitoxin µg. | Desacetyl-digilanid A µg. | µg. | Digitoxin µg. | Desacetyl-digilanid A calculated as digitoxin µg. |
| 800 | 500 | 1,482* (1,478–1,486) | 660 (635–690) | 456† (426–484) |
| 600 | 750 | 1,518* (1,516–1,519) | 542 (536–548) | 654‡ (632–678) |

* Two assays. † Four assays. ‡ Three assays.

of ethanol-free chloroform, the extracts transferred to the column and the eluate collected as before. Elution of the column was continued with further quantities of ethanol-free chloroform till about 40 ml. of the eluate had been collected. The chloroform was removed on a boiling water bath and the digitoxin estimated colorimetrically in the residue by means of an alkaline solution of 3,5-dinitrobenzoic acid (Rowson, 1952).

Desacetyldigilanid A was eluted from the column with ethanol 10 per cent v/v in chloroform, keeping the rate of fall of the drops at 2–3/sec. The eluate was collected directly in the evaporating dish which was to contain the glycoside. The column was eluted until a total of about 40 ml. was collected. The eluate obtained was evaporated to dryness and desacetyldigilanid A was estimated in the residue as above. In each case after 40 ml. of the eluate had been collected no more glycoside could be extracted from the column. The results are given in Table I.

It will be noted that in the assay of the first mixture the recovered glycosides totalled 76 per cent, and in the case of the second mixture 79 per cent.

*Assays of Digitalis Leaf Samples*

The digitalis leaf extracts were prepared and extracted according to the procedure described by Rowson (1955). Definite volumes of the chloroform extracts were measured and the total glycosides estimated by the

method described above. "Digitoxin" and primary glycosides were separated from the residue obtained from a definite volume of chloroform extract using a cellulose powder column. The method followed was that described above. The results are given in Table II.

TABLE II

CHEMICAL ASSAY OF DIGITALIS LEAF. THE ESTIMATION OF "DIGITOXIN" AND "PRIMARY GLYCOSIDES" AND OF TOTAL GLYCOSIDES ALL CALCULATED AS DIGITOXIN WITH LIMITS OF ERROR (P = 0·95)

| Digitalis leaf | *Total glycosides* calculated as digitoxin per cent w/w | "Digitoxin" per cent w/w | *Primary glycosides* calculated as digitoxin per cent w/w |
|---|---|---|---|
| A | 0·38* (0·37–0·43) | 0·19† (0·176–0·20₁) | 0·19† (0·182–0·210) |
| B | 0·33‡ (0·325–0·328) | 0·16§ (0·156–0·161) | 0·13§ (0·128–0·137) |

* Mean of fourteen assays. ‡ Mean of four assays.
† Mean of six assays. § Mean of three assays.
With limits for each.

It was considered that valuable information would result from a biological assay of the separated fractions. Such information is not usually available since it is generally difficult to obtain sufficient material by chromatographic methods for the established methods of biological assay. The isolated auricles method investigated and reported by Bhatt and Macdonald (1960), requires very small amounts of glycosides and enables

TABLE III

BIOLOGICAL ASSAY OF SEPARATED FRACTIONS. ESTIMATED POTENCY IN TERMS OF INTERNATIONAL UNITS OF TOTAL GLYCOSIDES, "DIGITOXIN" AND "PRIMARY GLYCOSIDES" AURICLES AND THE U.S.P. XV METHODS AS DETERMINED BY THE ISOLATED RABBIT WITH LIMITS OF ERROR (P = 0·95)

| Digitalis leaf | *Isolated rabbit auricles method* | | | *U.S.P. XV method* | | |
|---|---|---|---|---|---|---|
| | Total glycosides I.U./g. | "Digitoxin" I.U./g. | Primary glycosides I.U./g. | Total glycosides I.U./g. | "Digitoxin" I.U./g. | Primary glycosides I.U./g. |
| A | 9·25* (8·41–10·1) | 4·53 | 4·71* (4·68–4·74) | 7·02 | 3·10 | 4·26 |
| B | — | — | 1·94* (1·90–1·98) | — | — | — |

* Mean of two assays and figures found.

biological assays to be made on the "digitoxin" and "primary glycosides" fractions separated from digitalis leaf.

*Biological Assays of Total Glycosides, "Digitoxin" and "Primary Glycosides"*

The total glycosides, "digitoxin" and "primary glycosides" obtained by the procedure described above were assayed by the rabbit isolated auricles method (Bhatt and Macdonald, 1960) and the U.S.P. XV methods. Digitalis leaf extract B was decolorised by passing it through a column of alumina as described by Brindle, Rigby and Sharma (1955), since the total glycosides could not be estimated by the isolated rabbit auricles method after lead acetate treatment. The results are given in Table III.

The potency of the "digitoxin" fraction from leaf B could not be determined directly by the isolated auricle method. It would appear that

some material is extracted from this poor quality specimen which interfered with the beat of the auricle. It was later discovered by microscopical examination that leaf B was adulterated with mullein.

It was not possible to obtain sufficient glycosides from leaf B by the cellulose column method to allow an assay to be carried out by the U.S.P. XV method.

As with the chemical assays of the leaf extracts (Table II), and as distinct from the chemical determinations of the mixed pure glycosides (Table I) the recovery of the glycoside fractions from the leaf extracts would appear to be about complete. It would be reasonable to assume that the "digitoxin" fraction of leaf B would have an activity of about 1·66 I.U./g., the

TABLE IV

POTENCY OF DIGITOXIN AND DESACETYLDIGILANID A OBTAINED BY ISOLATED RABBIT AURICLES AND U.S.P. XV METHODS

| Glycoside | Isolated rabbit auricles method I.U./mg. | U.S.P. XV method I.U./mg. |
|---|---|---|
| Digitoxin .. .. .. .. | 1·35* (1·35–1·35) | 1·38 |
| Desacetyldigilanid A .. .. .. | 3·96* (3·92–4·00) | 3·55 |

* Mean of two assays, and figures found.

difference between the total activity and the activity of the primary glycosides. It will be observed that the U.S.P. XV method gave a much lower figure than that given by the isolated auricles method for the total glycosides and "digitoxin" fraction in leaf A.

*Biological Comparison of "Pure" Digitoxin and Desacetyldigilanid A*

For the isolated rabbit auricles method (Bhatt and Macdonald, 1960), measured volumes of alcoholic solutions of digitoxin and desacetyldigilanid A were diluted to definite volumes with 50 per cent v/v propylene glycol in distilled water. The concentration of alcohol was kept below 10 per cent v/v in the final test dilution, as it was observed that a higher amount was harmful to isolated rabbit auricles. If the dilution of the alcoholic solution was made with water, digitoxin was precipitated, but the 50 per cent propylene glycol mixture was found to be satisfactory; 50 per cent propylene glycol in itself had no perceptible effect on the beat of the auricles. Dispersing agents, like Tween 80 and Crill S.6, were found to be unsuitable. Both digitoxin and desacetyldigilanid A were also assayed for their biological activity by the U.S.P. XV method, and the results are summarised in Table IV.

## DISCUSSION

The separation of the "pure" glycosides, digitoxin and desacetyldigilanid A, using a cellulose powder column was found to be satisfactory. Biological estimations of the "digitoxin" and "primary glycoside" fractions from the digitalis leaf extracts showed that they were almost equal in activity and that they accounted for the total biological activity of the extracts. Biological assays of "pure" digitoxin and desacetyldigilanid A showed that desacetyldigilanid A was about three times more

active than digitoxin. This confirmed the previous findings of Brindle, Rigby and Sharma (1955).

*Correlation of the Results of the Chemical and Biological Assays*

Since "pure" digitoxin and desacetyldigilanid A were found to have activities of about 1·36 and 3·75 I.U./mg. respectively (mean of two biological methods in each case), it is possible to calculate if any relation could exist between the results of the chemical and biological assays of the two samples of digitalis. The "digitoxin" fraction is almost certainly a mixture of secondary glycosides, of which the main portion is digitoxin. If the "digitoxin" fractions had consisted of pure digitoxin, their potencies, calculated by multiplying the biological activities of digitoxin (I.U./mg. rabbit auricle method) by the amount of "digitoxin" estimated chemically (mg./ml.) would be as follows:

Sample A. 1·9 × 1·35 = 2·56 "I.U."/g.
Sample B. 1·6 × 1·35 = 2·10 "I.U."/g.

The actual figures obtained biologically were:

Sample A. 4·53 I.U./g. (auricle method)
3·10 I.U./g. (U.S.P. method)
Sample B. 1·66 I.U./g. (by difference)

It would appear that this fraction of Sample A contained material which was considerably more active than digitoxin, but in Sample B some less active material was present.

In the case of the primary glycoside fractions, the main constituents are probably desacetyldigilanids A and B. The latter has been stated to have about the same biological activity as digitoxin. Since desacetyldigilanid A has about three times this activity, it is necessary to know the proportions of these two which are present before any attempt at the correlation of the chemical and biological assays can be made.

If these fractions were pure glycoside A, the results would be as follows:

Sample A. 1·9 × 3·96 = 7·52 I.U./g.
Sample B. 1·3 × 3·96 = 5·15 I.U./g.

If pure glycoside B, the figures would be about:

Sample A. 1·9 × 1·3 = 2·47 I.U./g.
Sample B. 1·3 × 1·3 = 1·69 I.U./g.

Since the actual figures obtained by the auricle method were:

Sample A. 4·71 I.U./g.
Sample B. 1·94 I.U./g.

the possible activities calculated from the results of the chemical assays are consistent with the primary glycoside fractions being, in the main, a variable mixture of desacetyldigilanids A and B. Actually, if the fraction from Sample A consisted of equal proportions of desacetyldigilanids A and B, the chemical result would agree with the biological figure. In the case of Sample B, the results are consistent with the fraction containing about 10 per cent of desactyldigilanid A and 90 per cent of B.

*Acknowledgements.* Use has been made of material and information available from the Joint Committee of the Pharmaceutical Society and the Society for Analytical Chemistry on the Methods of Assay of Crude Drugs and this assistance is gratefully acknowledged. We wish to thank Messrs. Croda Ltd., Messrs. Honeymill and Stein Ltd., and Messrs. Sandoz Ltd. for gifts of Crill S.6, Tween 80 and desacetyldigilanid A, respectively.

## REFERENCES

Bhatt, J. G., and Macdonald, A. D. (1960). *J. Pharm. Pharmacol.*, **12**, 733–738.
Brindle, H., Rigby, G., and Sharma, S. N. (1954). *Ibid.*, **6**, 887–893.
Brindle, H., Rigby, G., and Sharma, S. N. (1955). *Ibid.*, **7**, 942–951.
Heftmann, E., and Levant, A. J. (1952). *J. biol. Chem.*, **194**, 703–709.
Jensen, K. B. (1953). *Acta pharm. tox., Kbh.*, **9**, 99–108.
Rigby, G., and Bellis, D. M. (1956). *Nature, London.*, **178**, 415.
Rowson, J. M. (1952). *J. Pharm. Pharmacol.*, **4**, 814–830.
Rowson, J. M. (1955). *Ibid.*, **7**, 924–931.
Williams, W. D. (1958). Communication to Committee on Methods of Assay of Crude Drugs.

# A PHARMACOLOGIC STUDY OF SOME NUCLEOSIDES AND NUCLEOTIDES

By Paul V. Buday*, C. Jelleff Carr† and Tom S. Miya

*From the Department of Pharmacology, Purdue University, School of Pharmacy, Lafayette, Indiana*

Received January 17, 1961

Adenosine, adenosine-5′-phosphoric acid (AMP), adenosine-5′-triphosphate (ATP), guanosine-5′-triphosphate (GTP), uridine, uridine-5′-triphosphate (UTP), and cytidine diphosphate-5′-choline (CDPCh) were studied *in vitro* and *in vivo*. The intraventricular injection of high doses of adenosine, ATP, and methacholine into rats elicited clonic-tonic convulsions or an akinetic state, or both. Perfusion of frog hearts *in situ* with equimolar concentrations of the "high-energy" compounds, with the exception of ATP, failed to exert any positive inotropic effects. ATP did not potentiate the inotropic actions of (−)-adrenaline or (−)-noradrenaline. The cholinergic-like properties of adenosine and ATP were partially antagonised by concomitant perfusion with methylene blue. ATP, but not adenosine, obliterated transitorily, the cardio-toxicity of pilocarpine. The inhibition of the isolated clam heart by AMP and ATP qualitatively resembled the effects of acetylcholine (Ach). Antagonism of the cardio-depressant properties of AMP and ATP by benzoquinonium and 5-hydroxytryptamine (5-HT) also supports previous evidence that the adenyl compounds act similarly to the cholinergic mediator. ATP produced marked contractions and a tonotropic effect on the oestrogenised, isolated, quiescent rat uterus; these effects were destroyed by low concentrations of (−)-adrenaline and papaverine. Adenosine, AMP, and CDPCh in equimolarities elicited no response from this test preparation.

The energy-rich nucleotides have been found to catalyse and serve as substrates for numerous biotransformations which use energy liberated by catabolism. Some, in addition, have been implicated in the mode of action of drugs. Their pharmacodynamic effects and their ability to modify the action of other pharmacologic agents was studied *in vivo* and *in vitro* to confirm previous reports, and to compare these actions with those of some recently available nucleotides.

The nucleosides and nucleotides used were adenosine, adenosine-5′-phosphoric acid, adenosine-5′-triphosphate, disodium, guanosine, guanosine-5′-triphosphate, disodium, uridine, uridine-5′-triphosphate, trisodium, and cytidine diphosphate-5′-choline, monosodium.

## Experimental Methods

### *Cerebral Intraventricular Injections*

Intraventricular injections of adenosine, ATP, and methacholine chloride were made into conscious albino rats (55–140 g.) after the method of

* Present Address: Department of Pharmacology, University of Rhode Island, Kingston, R.I., U.S.A.

† Present Address: Psychopharmacology Service Center, National Institute of Mental Health, Bethesda 14, Md., U.S.A.

Haley and McCormick (1957), employing a 26 G–3/8 in. hypodermic needle. The injection site was determined by preliminary injections of diluted India ink. Gross observations of the ink particle localisation showed that the injections were being made into the right lateral ventricle of the brain. All drugs were dissolved in normal physiological saline solution and the volume injected was 0·05 ml. Gentle heating was required to dissolve the nucleoside and the ATP at the higher doses. Both adenyl compounds were preserved either in the crystalline form, or in fresh solution frozen, at − 15°.

*Perfusion Studies*

*In situ* frog heart perfusions were performed from December to May employing *Rana pipiens* (L.) measuring 7·5–8·8 cm. in length, following the technique of Sollmann and Barlow (1926). The hearts were perfused with a modified Howell-Ringer solution (pH 7·2) at ambient room temperature (24–27°). All drugs were dissolved in the perfusate to obtain the final anhydrous molarities.

*Isolated Clam Heart*

Isolated hearts (ventricles) of the lamellibranch clam, *Venus mercenaria* (L.), were suspended in a 11 ml. tissue chamber. The techniques employed generally followed the recommendations of Wait (1943), Welsh and Taub (1948), and Tower and McEachern (1948). The mollusca were satisfactorily preserved for 6 to 8 days by storage at 9°. An artificial "sea water" containing the following salts in g./l. was employed: NaCl 30·00, KCl 0·90, $CaCl_2$ (anhydrous) 1·10, $MgSO_4.7H_2O$ 4·95, $NaHCO_3$ 0·03, and dextrose (anhydrous) 0·25. The bathing fluid (pH 7·5) was aerated and maintained between 17 and 20°. All drugs, with the exception of guanosine which was dissolved in bicarbonate-free fluid, were dissolved in double glass-distilled water, and added to the muscle chamber in 0·05 ml. volumes. Isotonic contractions were recorded kymographically. This investigation was restricted to early June to mid-July to minimise possible seasonal variations in drug responses (Prosser, 1940).

*Isolated Rat Uterus*

Uteri were excised from albino rats (120–235 g.) previously oestrogen-primed by subcutaneous injection of diethylstilboestrol in cottonseed oil 50 μg./100 g. at 2–24 hr. intervals before killing on the 72nd hr. The isolated horns were suspended in a muscle chamber (14 ml. capacity) containing de Jalon solution (1945), and the system was maintained at a temperature of 28–29°. Isotonic contractions were recorded by means of a writing lever and smoked kymograph. All drugs were introduced into the bath in 0·05 ml. volumes.

## RESULTS

*Intraventricular Injections*

The administration of high doses (1,000 and 1,500 μg.) of ATP into rats elicited severe, generally unilateral, clonic-tonic convulsions. These

seizures, at times, were characterised by a spiral body movement ("rolling pin" motion) and persisted for from 15 to 90 sec. A condition of anergia or akinesis with a concomitant asthenia of the limbs then appeared. This latter state persisted for 4 to 26 min. Lower concentrations (25–500 $\mu$g.) of ATP and adenosine (6 rats at each dosage level) elicited solely the akinetic state and weakness. Methacholine chloride in 125 $\mu$g. doses (5 rats) evoked, in all but one instance where convulsions occurred, a catatonic or akinetic reaction. These animals appeared completely

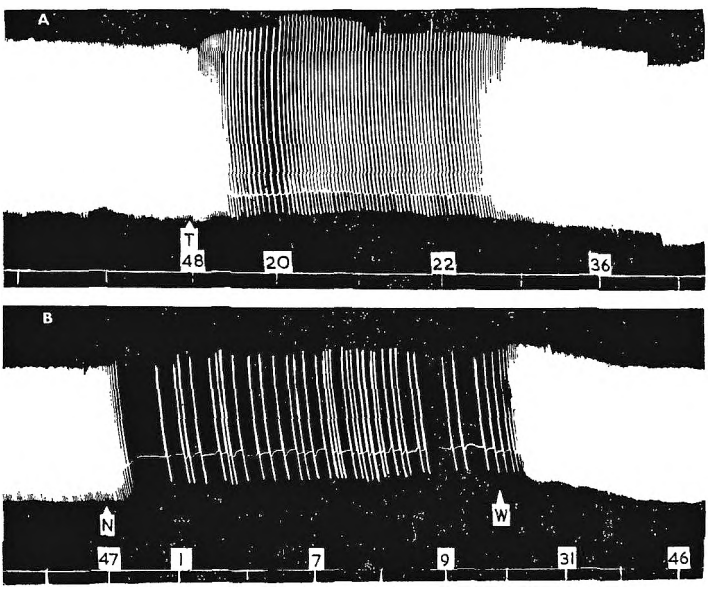



FIG. 1. Perfusion of *in situ* frog hearts with ATP and adenosine.
A. At T perfusion with ATP ($8{\cdot}26 \times 10^{-6}$M) started. Numbers above time line indicate heart rate in beats/min. Time marker, 1 min.
B. At N perfusion with adenosine $8{\cdot}26 \times 10^{-6}$M begun. At W perfusion of nucleoside stopped and washout with Howell-Ringer solution begun.

composed within $5\frac{1}{2}$ min. Control saline injections produced no comparable effects although the animals exhibited transient clockwise circling. This latter effect also supervened in the adenyl-treated animals.

*Frog Heart Perfusions*

ATP as low as $8{\cdot}26 \times 10^{-7}$ M produced an increased systolic force with a concurrent bradycardia. High concentrations ($8{\cdot}26 \times 10^{-6} - 3{\cdot}31 \times 10^{-5}$ M) elicited a profound, but transitory, heart block. Auricular, followed by ventricular diastolic stoppage occurred, but a spontaneous recovery of the myocardium was often noted, an action resembling "vagal escape". Perfusion with fresh Howell-Ringer solution readily reversed these effects. In general the reactions observed confirm the isolated amphibian and mammalian auricular and intact animal studies of Wedd and Fenn (1933), Drury (1936), and the frog heart perfusion investigations of Ostern and Parnas (1932), Gillespie (1934), Meyer (1951),

and Porro (1952). GTP and UTP in $3{\cdot}31 \times 10^{-5}$ and $1{\cdot}32 \times 10^{-4}$ M effected only slight augmentations in rate and little or no positive inotropism. CDPCh in $3{\cdot}31 \times 10^{-5}$ and $6{\cdot}62 \times 10^{-5}$ M, and sodium pyrophosphate in $8{\cdot}26 \times 10^{-6}$ and $3{\cdot}31 \times 10^{-5}$ M elicited no demonstrable responses. Adenosine in $8{\cdot}26 \times 10^{-7}$ — $3{\cdot}31 \times 10^{-5}$ M manifested a bradycardia and a diastolic stoppage in the higher concentrations, with only minor enhancement of cardiac excursions. These results with adenosine parallel the mammalian heart effects previously reported (Drury and

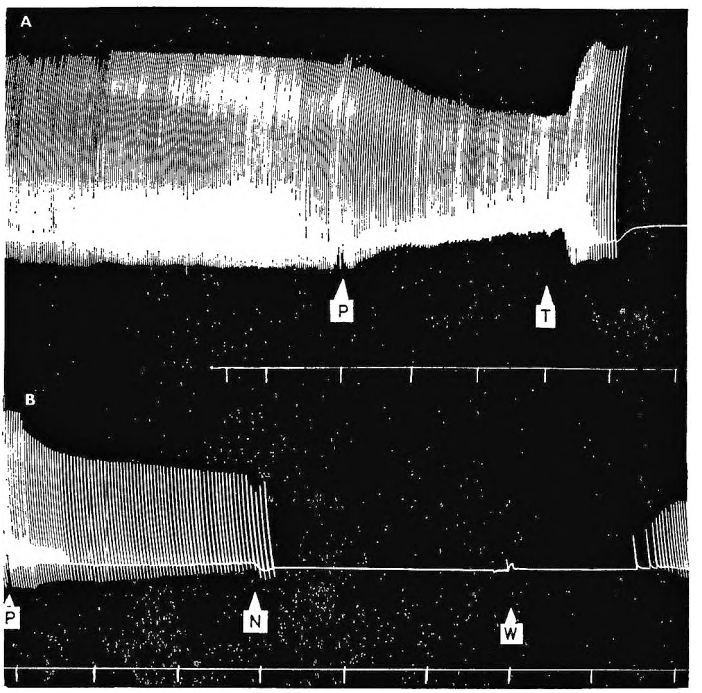



FIG. 2. Cardio-toxicity of pilocarpine on the *in situ* frog heart modified by ATP and adenosine.
A. At P perfusion with pilocarpine nitrate ($7{\cdot}37 \times 10^{-4}$M). At T perfusion begun with ATP in $3{\cdot}31 \times 10^{-5}$M. Time marker, 1 min.
B. At P perfusion with pilocarpine nitrate ($7{\cdot}37 \times 10^{-4}$M) begun. At N perfusion with adenosine $3{\cdot}31 \times 10^{-5}$M started. At W perfusion of nucleoside terminated and washout with Howell-Ringer solution begun.

Szent-Gyorgyi, 1929; Chevillard and Guerin, 1955; and Rand and others, 1955). Fig. 1 shows typical responses of the *in situ* frog heart to adenosine and ATP.

A 4 min. concomitant perfusion of methylene blue ($3{\cdot}0 \times 10^{-5}$ M) through 7 amphibian hearts inhibited (2–61 per cent) the bradycardia characteristically induced by $8{\cdot}26 \times 10^{-6}$ M concentrations of the adenyl derivatives when contrasted with the subsequent perfusion of the latter agents through the thoroughly washed, but blue stained heart. The phenazothionium dye when perfused concomitantly with ATP did not suppress the normal inotropic effect.

The cardio-accelerations induced by (−)-adrenaline HCl ($5{\cdot}46 \times 10^{-9}$ M) and (−)-noradrenaline bitartrate ($6{\cdot}26 \times 10^{-9}$ M) were readily inhibited (7–100 per cent) by the concomitant perfusion of each of the amines with $3{\cdot}37 \times 10^{-5}$ M adenosine. The perfusion of a just subthreshold concentration ($1{\cdot}09 \times 10^{-12}$ M) of (−)-adrenaline or (−)-noradrenaline ($1{\cdot}25 \times 10^{-11}$ M) with a threshold level ($4{\cdot}13 \times 10^{-7}$ M) of ATP failed to show, with 6 hearts, any demonstrable potentiation of amplitude.

The cardio-depressant properties of pilocarpine nitrate ($7{\cdot}37 \times 10^{-4}$ M) were sharply and readily, but only temporarily, reversed by ATP ($8{\cdot}26 \times 10^{-6}$ or $3{\cdot}31 \times 10^{-5}$ M), but not by equimolarities of the adenyl nucleoside. Fig. 2 graphically compares their antagonistic properties towards pilocarpine.

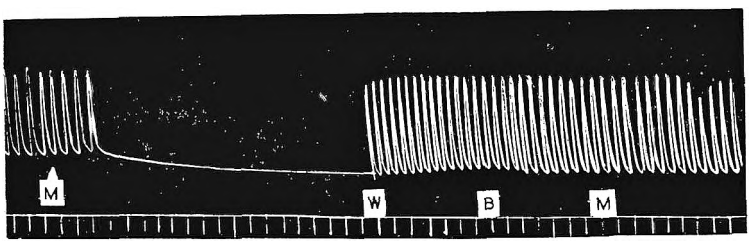



FIG. 3. Blocking action of benzoquinonium on the isolated clam heart. At M addition of AMP to bath to give $4{\cdot}48 \times 10^{-4}$M. At W washing out of the bath. At B addition of benzoquinonium chloride to give $4{\cdot}48 \times 10^{-5}$M. Time marker, 10 sec.

*Isolated Clam Heart Experiments*

ATP, $2{\cdot}24 \times 10^{-4} - 1{\cdot}34 \times 10^{-3}$ M, after a short latency, produced a gradual inhibition of amplitude and a diastolic arrest. Depression of rate was small. Occasionally, the ventricular stoppage was abrupt and was not preceded by progressive depression. In certain instances a transitory and erratic, partial or complete, escape occurred. AMP in equimolarities caused a similar effect. Uridine and guanosine, even in high concentrations ($4{\cdot}48 \times 10^{-4} - 1{\cdot}79 \times 10^{-3}$ M), produced only feeble inhibitions of rate and amplitude. No tachyphylaxis was noted with any of these compounds.

The addition of benzoquinonium chloride, an Ach-blocking agent on the clam heart (Luduena and Brown, 1952), to the muscle bath to make a final concentration of $4{\cdot}84 \times 10^{-5}$ M (1 min. before addition of either AMP or ATP in $4{\cdot}48 \times 10^{-4}$ M) either increased the latency period preceding depression or wholly prevented this quasi-muscarinic action of the nucleotides Fig. 3 shows a typical kymogram of this complete protection.

5-HT (creatinine sulphate complex) in $1{\cdot}18 \times 10^{-6}$ M was found, as in earlier studies (Welsh, 1953, 1954, 1957), to be a potent excitant to the clam heart, the stimulation consisting of a singular increase in cardiac

excursion and, in some instances, of a pronounced tonotropic effect. Four of 5 hearts when partially or completely inhibited by ATP ($4{\cdot}48$–$8{\cdot}96 \times 10^{-4}$ M) were subsequently and completely protected from a second depressant concentration by 5-HT.

Using 6 clam ventricles a semi-quantitative comparison of the inhibitory properties of Ach and ATP showed that a mean concentration of $9{\cdot}37 \times 10^{-11}$ and $6{\cdot}72 \times 10^{-4}$ M respectively was required to educe complete inhibition of the isolated ventricles within a 1 min. period (Fig. 4).

*Rat Uterus Experiments*

ATP in serial molarities ($2{\cdot}24 \times 10^{-5}$ — $2{\cdot}24 \times 10^{-4}$) elicited an almost immediate spasm of the isolated, quiescent uterus, which was followed by contractions of irregular magnitude. These contractile responses

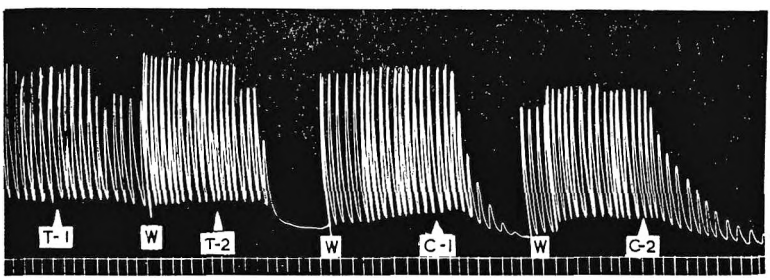



FIG. 4. Comparative actions of ATP and Ach on the isolated clam heart. At T-1 addition of ATP to bath to give $4{\cdot}48 \times 10^{-4}$M. At W washing out of the bath. At T-2 addition of ATP to bath to give $8{\cdot}96 \times 10^{-4}$M. At C-1 addition of Ach to bath to give $2 \times 10^{-10}$M. At C-2 addition of Ach to bath to give $1 \times 10^{-10}$M. Time marker, 10 sec.

were only partially obliterated by thorough washings with de Jalon solution thus suggesting that the phosphoriboside enhanced the irritability of the smooth muscle tissue. In contrast, GTP ($7{\cdot}48 \times 10^{-5}$ M) usually produced only a minor, single contraction with no display of a tonotropism. CDPCh ($7{\cdot}48 \times 10^{-5}$ M), adenosine, and AMP ($7{\cdot}48 \times 10^{-5}$ and $2{\cdot}24 \times 10^{-4}$ M), even after 12 min. of contact, demonstrated no uteromotor effects.

Ten uteri under an 8 min. stimulation by ATP ($7{\cdot}48 \times 10^{-5}$ M) were readily and completely inhibited by minute concentrations ($4{\cdot}05$ and $8{\cdot}10 \times 10^{-11}$ M) of (−)-adrenaline; papaverine HCl ($2{\cdot}65 \times 10^{-5}$ and $6{\cdot}63 \times 10^{-6}$ M) showed a similar, but slower, effect on another series of *in vitro* preparations. Fig. 5 shows the former antagonism.

## DISCUSSION

Although some cerebral injury was inevitable with concurrent, but transitory effects, due to the intraventricular injection method, the akinetic condition induced by adenosine and ATP confirms the studies of Feldberg and Sherwood (1954) in the cat. This reaction and the asthenia after convulsive doses of the adenyl compounds might be interpreted as

fatigue, but this seems untenable, in part, in light of the fact that these effects supervened in response to the lower, non-convulsive doses.

The direct central actions of Ach were first described by Dikshit (1934), and later by Schnedorf and Ivy (1939) and Haley and McCormick (1957). These reports indicated the production of a sleep-like or akinetic state. It would appear most inferential from the literature and from the results reported here that the direct central actions of the adenyl compounds resemble those elicited by some cholinergic agents.

UTP, GTP, and CDPCh, although all possessing "high-energy" bonds, were incapable of significantly affecting either the rate or amplitude of the

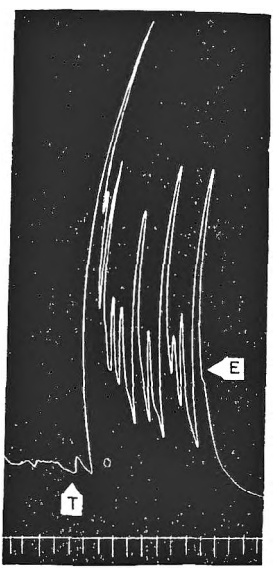



FIG. 5. The effects of ATP on the isolated, quiescent, oestrogenised rat uterus antagonised by adrenaline. At T addition of ATP to bath to give $7{\cdot}48 \times 10^{-5}$M. At E addition of (—)-adrenaline bitartrate to bath to give $4{\cdot}05 \times 10^{-11}$M. Time marker, 1 min.

frog heart. In contrast, ATP was noted, as other amphibian and mammalian studies have shown (Chevillard and Guerin, 1955; Green and Stoner, 1950; Kanda and others, 1954; Meyer, 1951; Sekiya, 1953) to initiate a positive inotropic effect soon succeeded by a bradycardia or diastolic arrest. The lack of biologic activity of the non-adenyl compounds implies a stringent biochemical-myokinetic specificity of the myocardium. The vagomimetic properties of adenosine, without significant attendant inotropic effects, were first described by Drury and Szent-Gyorgyi (1929) and are well documented (Drury, 1932; Drury, 1936; Green and Stoner, 1950; Wedd and Fenn, 1933). The cardiovascular actions of the adenyl series are due apparently to the ribose residue (Sydow and Ahlquist, 1954) and to the presence of a 6-$NH_2$ group on the purine moiety (Clarke and others, 1952; Green and Stoner, 1950), with phosphate esterification enhancing and modifying the activity (Green and Stoner, 1950).

Cook (1926) demonstrated, on the isolated frog heart, that the vagomimetic characteristics of Ach were antagonised by simultaneous perfusion with methylene blue. This finding suggested that the neurohormone's site of action was restricted to the cell membrane or to a surface receptor. Thus the partial antagonism of the myocardial actions of adenosine and ATP by this same dye suggests that the adenyl derivatives also exert their cholinergic-like effects, at least in part, through a reversible alteration of the plasmatic membrane. This adds substance to the unproven speculation (Gillespie, 1934) that the rapidity of action of the adenyl agents might be explained by their ability to alter cellular colloids. Adenosine's antagonism of the cardio-acceleratory properties of certain sympathomimetic amines further adds to the evidence that the active members of the adenyl family possess cholinergic actions.

The inability of ATP to enhance the positive inotropic actions of (−)-adrenaline and (−)-noradrenaline was a rather surprising finding since both amines affect intermediary metabolism in muscle. Our results do not agree with those of Edwards and Booker (1959) who found ATP to enhance the myocardial action of adrenaline on the guinea-pig isolated heart. Perhaps species and perfusion differences underlie this incongruity. The ephemeral, but conspicuous, antagonism of ATP towards pilocarpine induced cardio-toxicity indicates that the former substance possesses the ability to exert its typical inotropic effects independent of intracellularly fixed alkaloid, perhaps, in contrast to adenosine, by antagonising the decreased myocardial oxygen consumption as engendered by the parasympathomimetic agent (David, 1930).

The Ach-like actions of AMP and ATP were clearly demonstrated on the isolated clam heart inferring the latters' ability to produce a cardioplegic action on the diffuse myogenic pacemaker or conduction mechanisms of this organism. The spontaneous and irregular recovery of the heart at various times while under the depressant influence of ATP seems to add credence to this hypothesis. Although the inhibitory actions of ATP differed from preparation to preparation, and on the same specimen, its generally rapid action, and ready removal by washing suggests again that ATP, like Ach, is acting upon or near the surface of the cell membrane. The blocking action of benzoquinonium towards the purine nucleotides also lends support to this conclusion, since the depolarising activity of the bis-onium salts may be explained by the adsorption of the polymethylene chain via Van der Waal's forces to the cellular surface (Barlow, 1955). However, it is most unlikely that a specific competitive antagonism between the adenyl compounds and benzoquinonium exists.

The fact that 5-HT application to the molluscan heart before the addition of ATP prevents the resultant cholinergic-like effects indicates that these two compounds are pharmacodynamically antagonistic. The inertness of uridine and guanosine implies that these nucleosides play little, if any, role in molluscan myokinetics.

In contrast to the reported uterine stimulatory properties of adenosine (Bennet and Drury, 1931; Moulton and others, 1957) and AMP (Deuticke, 1932) on the guinea-pig, adenosine and AMP were found to be biologically

inactive on the quiescent, isolated rat uterus. ATP on the other hand, as in the guinea-pig (Deuticke, 1932), caused pronounced contractile and tonic effects. These observations seem to implicate the high-energy linkages as responsible for the pharmacologic differences between the energy-rich and non-energy-rich derivatives. Previous investigators (Jurascheck, 1932; Flossner, 1934) reported that guanylic acid and guanosine were stimulatory to the guinea-pig uterus, while others claim the nucleotide (von Euler, 1932) and nucleoside (Moulton and others, 1957) to be inert. Drury (1932) found the former compound to be relaxant. The feeble and ephemeral action of GTP compared to ATP again seems to indicate the proper position of the $NH_2$ group on the purine ring is fundamental to activity and dominates the possession of high-energy bonds by a nucleotide. The inertness of CDPCh was conspicuous and puzzling, for cytidylic acid has been reported (Jono, 1936) to be briefly stimulatory to the rat isolated uterus.

Csapo (1950) has shown with *in vitro* mammalian actomyosin threads that contraction occurs slowly in the presence of ATP. Because of the antagonism of ATP's uterotonic effects by low concentrations of papaverine and (−)-adrenaline it is conceivable that these spasmolytics directly interfere with the contractile protein-ATP reaction, although the possibility still exists that the antagonism is non-specific and may involve, at least for adrenaline, an action on an independent structure which counteracts contractile stimuli (Jensen and Lund, 1960).

*Acknowledgements.* The authors acknowledge the generosity of the Sigma Chemical Company, St. Louis, Missouri, for graciously supplying certain of the nucleotides employed in this study. Thanks are also given to Dr. K. E. Hamlin of Abbott Laboratories, Chicago, Illinois, for the gift of 5-HT. This investigation was supported, in part, by a Graduate Fellowship from the American Foundation for Pharmaceutical Education.

## REFERENCES

Barlow, R. B. (1955). *Introduction to Chemical Pharmacology*, p. 186, New York, John Wiley and Sons, Inc.
Bennet, D. W. and Drury, A. N. (1931). *J. Physiol.*, **72**, 288–320.
Chevillard, L. and Guerin, J. (1955). *C. R. Soc. Biol., Paris*, **149**, 235–238.
Clarke, D. A., Davoll, J., Philips, F. S., and Brown, G. B. (1952). *J. Pharmacol.*, **106**, 291–302.
Cook, R. B. (1926). *J. Physiol.*, **62**, 160–165.
Csapo, A. (1950). *Amer. J. Physiol.*, **160**, 46–52.
David, J. C. (1930). *J. Pharmacol.*, **40**, 229–234.
de Jalon, P. G., Bayo, S. B., and de Jalon, M. G. (1945). *Farmacoter. act.*, **2**, 313.
Deuticke, H. J. (1932). *Pflug. Arch. ges. Physiol.*, **230**, 537–555.
Dikshit, B. B. (1934). *J. Physiol.*, **83**, 42P.
Drury, A. N. (1932). *Ibid.*, **74**, 147–155.
Drury, A. N. (1936). *Physiol. Rev.*, **16**, 292–325.
Drury, A. N. and Szent-Gyorgyi, A. (1929). *J. Physiol.*, **68**, 213–237.
Edwards, D. R. and Booker, W. M. (1959). *Fed. Proc.*, **18**, 41.
Feldberg, W. and Sherwood, S. L. (1954). *J. Physiol.*, **123**, 148–167.
Flossner, O. (1934). *Arch. exp. Path. Pharmak.*, **174**, 245–254.
Gillespie, J. H. (1934). *J. Physiol.*, **80**, 345–359.
Green, H. N. and Stoner, H. B. (1950). *Biological Actions of the Adenine Nucleotides*, pp. 65–103, London, H. K. Lewis and Co., Ltd.
Haley, T. J. and McCormick, W. G. (1957). *Brit. J. Pharmacol.*, **12**, 12–15.
Jensen, K. B. and Lund, R. B. (1960). *Acta pharm. tox., Kbh.*, **17**, 173–181.

Jono, Y. (1936). *J. orient. Med.*, **24**, 1185–1191.
Jurascheck, H. (1932). *Arch. exp. Path. Pharmak.*, **167**, 451–458.
Kanda, Z., Sekiya, A., and Inoue, K. (1954). *Nagoya J. med. Sci.*, **17**, 85–90.
Luduena, F. P. and Brown, T. G., Jr. (1952). *J. Pharmacol.*, **105**, 232–239.
Meyer, A. E. (1951). *Arch. exp. Path. Pharmak.*, **213**, 154–161.
Moulton, R., Spector, W. G., and Willoughby, D. A. (1957). *Brit. J. Pharmacol.*, **12**, 365–370.
Ostern, P. and Parnas, J. K. (1932). *Biochem. Z.*, **248**, 389–397.
Porro, A. (1952). *Boll. Soc. Biol. sper.*, **28**, 1560–1562.
Prosser, C. L. (1940). *Biol. Bull., Wood's Hole*, **78**, 92–102.
Rand, M. J., Stafford, A., and Thorp, R. H. (1955). *Aust. J. exp. Biol. med. Sci.*, **33**, 663–670.
Schnedorf, J. G. and Ivy, A. C. (1939). *Amer. J. Physiol.*, **125**, 491–505.
Sekiya, A. (1953). *Folia pharm. jap.*, **49**, 268–281.
Sollmann, T. and Barlow, O. W. (1926). *J. Pharmacol.*, **29**, 233–255.
Sydow, V. and Ahlquist, R. P. (1954). *J. Amer. pharm. Ass., Sci. Ed.*, **43**, 166–170.
Tower, D. B. and McEachern, D. (1948). *Canad. J. Res.*, **26**, (Sect. E) 183–187.
von Euler, U. S. (1932). *Arch. exp. Path. Pharmak.*, **167**, 171–176.
Wait, R. B. (1943). *Biol. Bull., Wood's Hole*, **85**, 79–85.
Wedd, A. M. and Fenn, W. O. (1933). *J. Pharmacol.*, **47**, 365–375.
Welsh, J. H. (1953). *Arch. exp. Path. Pharmak.*, **219**, 23–29.
Welsh, J. H. (1954). *Fed. Proc.*, **13**, 162.
Welsh, J. H. (1957). *Ann. N.Y. Acad. Sci.*, **66**, 618–630.
Welsh, J. H. and Taub, R. (1948). *Biol. Bull., Wood's Hole*, **95**, 346–353.

# PHARMACOGNOSY OF INDIAN SUBSTITUTES OF MALE FERN

## I. *DRYOPTERIS RAMOSA* (HOPE) C. CHR. AND *D. CHRYSOCOMA* (CHRIST) C. CHR.

BY P. N. MEHRA AND T. C. MITTAL

*From the Department of Pharmacognosy, Panjab University, Chandigarh, India*

Received December 19, 1960

A taxonomic description, and the macroscopy and microscopy of the rhizome and leaf bases of *Dryopteris ramosa* (Hope) C. Chr. and *D. chrysocoma* (Christ) C. Chr. are given. Both the species compare very favourably in oleoresinous content and "crude filicin" with the official male fern. Because of their abundance in nature they could be commercially exploited. Like the official male fern, both the species possess schizogenous cavities in the ground tissue with internal glands. *D. ramosa* is devoid of sclerotic nests while a few of these are present in the other species both in the rhizome as well as in the leaf bases.

CONSIDERABLE quantities of the expensive male fern extract are imported into India for medicinal purposes. Search for an Indian substitute for this important drug has shown that there are substitutes that grow wild in the Himalayas, some abundantly enough to be exploited commercially (Mittal and Mehra, 1960). The present communication concerns the pharmacognostic investigation of two of these, namely, *Dryopteris ramosa* (Hope) C. Chr. and D. *chrysocoma* (Christ) C. Chr. Whereas the official B.P. requirements of the crude filicin is not less than 1·5 per cent, the two species contain a much higher percentage of oleoresin and crude "filicin." The amount of oleoresin in *D. ramosa* varies from 12–15 per cent and in *D. chrysocoma* 14–17 per cent. The crude "filicin" content of the former is approximately 3·8 per cent and in the latter 4·3 per cent as calculated by the B.P. (1953) method.

### MATERIAL AND METHODS

The material for *D. ramosa* was gathered from various localities at Pahlgam and Gulmarg, Kashmir (North-western Himalayas) while *D. chrysocoma* was sampled from Darjeeling (Eastern Himalayas).

The plants were uprooted so as not to dislodge the stele within the rhizome. These were cut into thin slices for microtomy, fixed in formalin, acetic acid and ethanol (5 : 5 : 90) and passed through the usual grades of ethanol and xylol, and embedded in paraffin. Because of the abundant starch and resin they were kept in each grade for 24–48 hr. and 2–4 days on the bath in paraffin. Sections were stained with safranin-fast green. A saturated solution of Sudan III in equal parts of glycerol and rectified spirit was used for detecting the presence of oleoresin in the intercellular cavities and glands. The latter are normally hyaline but with this reagent stain bright red and can be easily located. Schultze's and Jeffrey's fluids (Sauss, 1958) were used as macerating agents for the study of individual cells. For detailed study of glands a 5 per cent sodium hydroxide solution was found to be a better maceration fluid as with this reagent the glands

do not undergo any distortion in shape. Whole mounts of scales were prepared in 50 per cent glycerol after clearing.

*Dryopteris ramosa* (Hope) C. Chr.

*General description.* This is one of the commonest species growing in Kashmir (North-western Himalayas) and is plentiful in Pahlgam and Gulmarg as an undergrowth in forests at an altitude of about 7,000 ft. The species is characterised by an ascending rhizome which is densely clothed, as are the bases of the fronds, with large, broad, pale brown ramenta each ending in a hair. The stipe is up to 45 cm. long, stout, pale brown or straw coloured, sometimes mottled. The fronds (Fig. 1A)

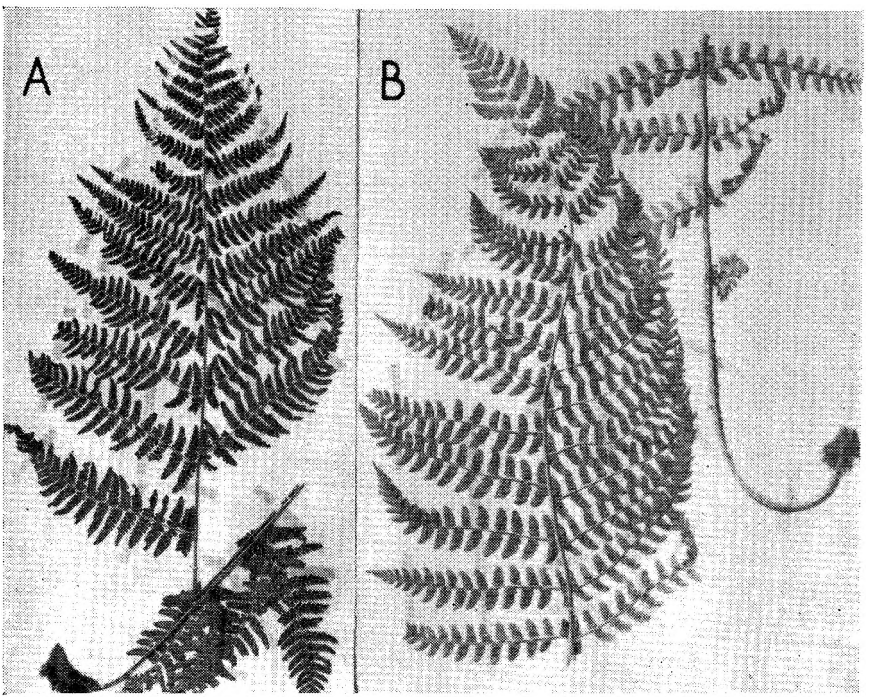



FIG. 1. A, *Dryopteris ramosa*, frond × 1/5; B, *D. chrysocoma* frond × 1/5.

are 30–60 cm. long, 20–35 cm. broad, oblong or ovate-lanceolate, tripinnatisect. The rachises are straw coloured, sparsely covered with pale coloured, linear ramenta and fibrils, but sometimes glabrous; the pinnae are in 16–30 pairs besides the deeply pinnatifid apex, the middle pinnae being generally the largest, the upper and lower ones are gradually shortened. Pinnae near the base of fronds are distantly placed; the lowest pinnae are 13–25 cm. in length and 5–13 cm. broad, with 12–20 pairs of pinnules. Pinnules in the lower pinnae are 5 cm. or more long and 1 cm. broad at base, lanceolate, gradually acuminate from a deltoid base, pinnatisect to a narrow wing along the costule, the ultimate segments possess sharply acuminate deltoid teeth. The texture is herbaceous,

the colour pale green but on drying sometimes pale brown. The veins are pinnate in segments and forked in large lobes. The lower third of the frond is sterile; sori are uniseriate along the costule of the pinnules; the indusium is thick, reniform, moderate sized, persistent brown, and glabrous.

This species is very much like *D. marginata* (Wall.) Christ but differs in rather slender habit and smaller size of all parts excepting the rhizome. The pinnules in the lower pinnae of *D. marginata* are generally much longer and 2–3 cm. broad, oblong-ovate, short acuminate or blunt, and the sori are larger, not so close to the costules of pinnules or the

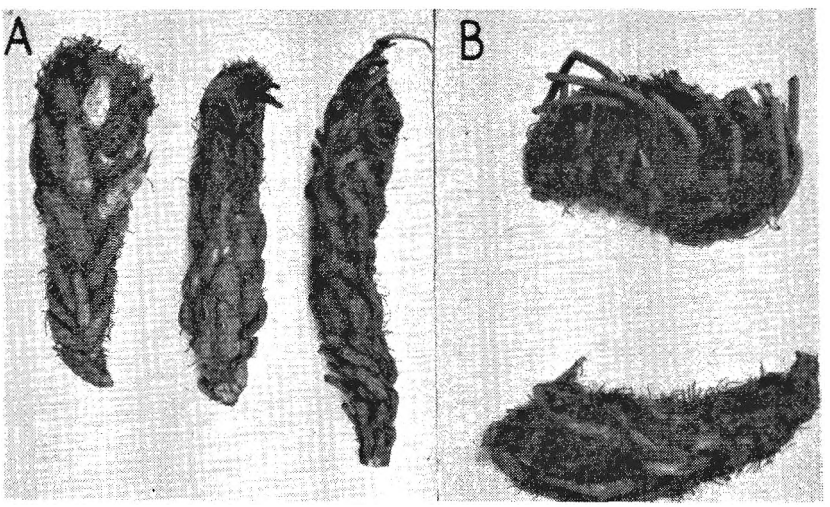



FIG. 2. A, *Dryopteris ramosa* rhizome × 1/3; B, *D. chrysocoma* rhizome × 1/3.

midrib of segments in the lower pinnae. *D. ramosa* also resembles *D. blanfordii* (Hope) C. Chr. which may be distinguished by the dark ramenta and short frond base.

*Macroscopy of the rhizome.* The rhizome (Fig. 2A) is woody, stout, cylindrical and curved or nearly straight, tapering towards one end, 10–25 cm. long, 2–5 cm. thick (the thickness of the rhizome without leaf bases 1–3 cm.), externally reddish brown, covered over by hard, persistent, curved, flato-convex, reddish brown frond bases, the latter up to 1 cm. in thickness and somewhat wrinkled longitudinally or smooth showing transverse scars of fallen ramenta. The upper one-third to one-half of the rhizome and the frond bases are furnished with linear-lanceolate golden brown ramenta. The fracture is short. The transversely cut surface of the fresh rhizome is pale green, later turning pale brown, spongy, marked by whitish patches of 4–6 cauline meristeles and a few groups of 7–11 smaller vascular strands, each group is arranged in the form of closed "C" and constitutes a leaf trace.

The ramenta (Fig. 5A) are lanceolate to ovate, hair pointed, pale brown to golden brown, and measure 1–2 cm. × 2–6 mm. The texture is thin,

the surface is more or less corrugated, and the margin wavy with a few inconspicuous marginal peg-like projections, each consisting of two parallel and contiguous cells (Fig. 5Bb).

*Histology of the rhizome.* The rhizome in transverse section presents the appearance shown in Fig. 3, and its cortical region on an enlarged scale is represented in Fig. 5E.

The epidermis is composed of a single layer of oblong to rectangular brownish cells which contain occasional tannin-resin masses. Many of the cells bear ramenta.

The hypodermis consists of a zone of 3–8 layers of thick walled lignified fibres which appear polygonal in transverse section. This region is compact and lacks intercellular spaces. In unstained preparations the

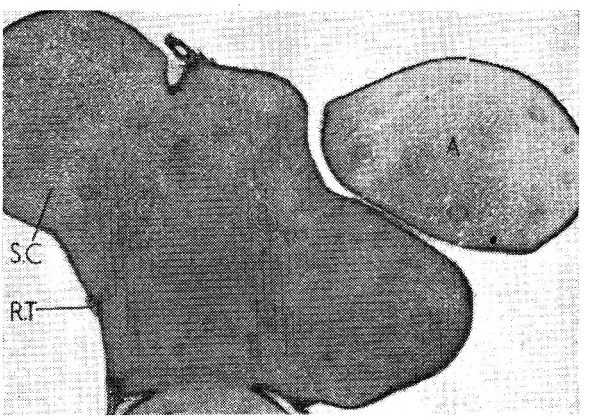



FIG. 3. *Dryopteris ramosa*, T. S. rhizome × 3½; A, T.S. stipe. R.T., root trace; S.C., schizogenous cavity.

walls of the fibres appear yellowish brown due to the deposition of a tannin like substance. In maceration they possess blunt ends and a few rounded pits on their walls.

The cortex consists of many layers of large parenchymatous cells with slightly thick cellulosic or suberised walls. They are often filled with starch grains which are roundish to oval in outline, 3–21 $\mu$ in diameter, and do not exhibit any marked striations. Irregular aggregates of tanoid masses are commonly present in the cells surrounding the meristeles and in the outermost layers of the cortex adjoining the hypodermis. In maceration and longitudinal sections the cells of the cortex appear predominantly rectangular in shape, each measuring 100–300 × 35–65 $\mu$. In transverse section they number about 500/mm.$^2$ Within the cortex as well as the pith are observed numerous small intercellular spaces and large schizogenous cavities (Fig. 5E). Into the latter project characteristic unicellular glandular hairs each of which possesses a short stalk and round head which under natural conditions is surrounded by orange yellow secretion. When stained with Sudan III these become very conspicuous (Fig. 6A). However, when the secretion is dissolved by suitable reagents

the glands appear hyaline (Fig. 6B). The schizogenous cavities are distributed all over the ground tissue but are more abundant around the meristeles and the outer zone of the cortex. The cavities number 50–70/mm.$^2$ in the area of maximum distribution. In longitudinal section they measure 500–1,400 × 50–112 $\mu$, and each contains 3–10 unicellular glands. Often more than one gland is found attached to a single subtending cell. Each gland measures 50–80 $\mu$ in length, and consists of a distinct stalk, 20–30 $\mu$ long with a globular or rounded head, 25–55 $\mu$ in diameter. Since the glands do not arise with any regular orientation within the cavities they are commonly cut in various planes in transverse sections and consequently their true shape and structure is seldom revealed.

As a general feature the sclerotic nests are absent in the species, but very rarely isolated and imperfectly developed sclerosed cells may be observed (Fig. 5E).

Pith is well developed and is continuous with the cortex in the regions intervening the bundles. It is composed of parenchymatous tissue and resembles the cortex in all respects.

Cortex and pith are separated from each other by a ring of 4–6 cauline meristeles. Numerous smaller vascular strands are present in the cortex

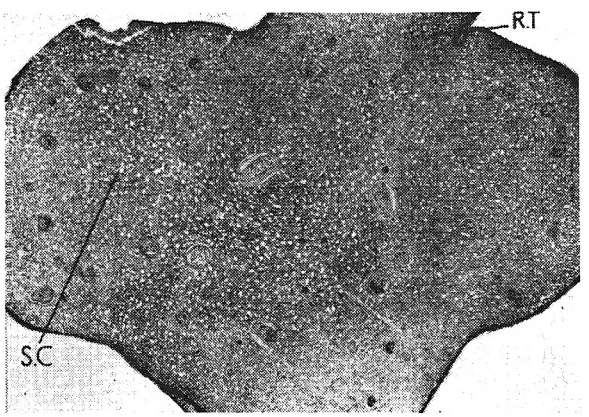



FIG. 4. *Dryopteris chrysocoma*, T.S. rhizome × 4 R.T., root trace; S.C., schizogenous cavity.

in the region of leaf gaps constituting the leaf traces. Each leaf trace is in the form of a semicircle comprising of 7–12 small vascular bundles (Fig. 3). Occasionally a root trace is abstricted from the outer limits of the cauline meristele and is observed passing out through the cortex.

*Structure of a meristele.* The structure of a meristele is similar to that generally found in ferns.

*Histology of the stipe.* Fig. 3 at A shows transverse section of the stipe. It is oval in outline. The epidermis has dark brown outer walls. Attached to it are many ramenta, cut variously. The hypodermis is composed of 6–10 layers of thick walled, lignified sclerenchymatous

fibres. The fibrous zone extends around the petiole except on the two lateral sides where the cells are broader and loosely arranged, forming a ventilating system for the petiole.

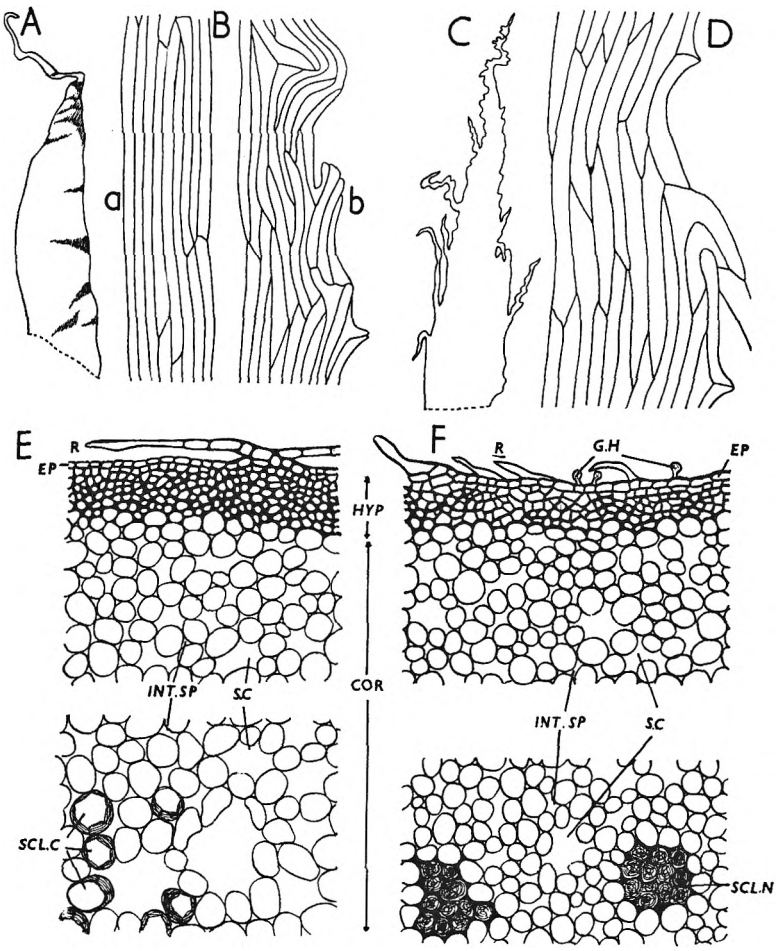



FIG. 5. A, *Dryopteris ramosa*, ramentum from rhizome × 5. B, centre and margin of ramentum × 80. C, *D.chrysocoma*, ramentum from rhizome × 5. D, Margin of ramentum × 80. E, *D. ramosa*, T.S. rhizome, a section magnified × 80. F, *D. chrysocoma*, T.S. rhizome, a section magnified × 80. COR, cortex; EP, epidermis; G.H, glandular hair; HYP, hypodermis; INT.SP, intercellular space; R, ramentum; S.C, schizogenous cavity; SCL.C, sclerosed cell; SCL.N, sclerotic nest.

The cortex is composed of many layers of parenchymatous cells similar to those of the rhizome excepting that they are much broader and shorter. They number only about 200/mm.$^2$ in contrast to 500 in the rhizome. The marked decrease in this number in the frond base is due to the cells being predominantly of larger diameter and the oleoresin cavities being much broader than in the rhizome.

The pith is similar to the cortex. Sometimes the free surface of the cells of the cortex and pith lining the schizogenous cavities are pressed inwardly and are transformed into crescent shaped cells. In between the cortex and the pith is a ring of 7–12 vascular strands. The number of true bundles in the petiole varies from 7–10 and the rest are smaller accessory strands connecting the bundles in their longitudinal course.

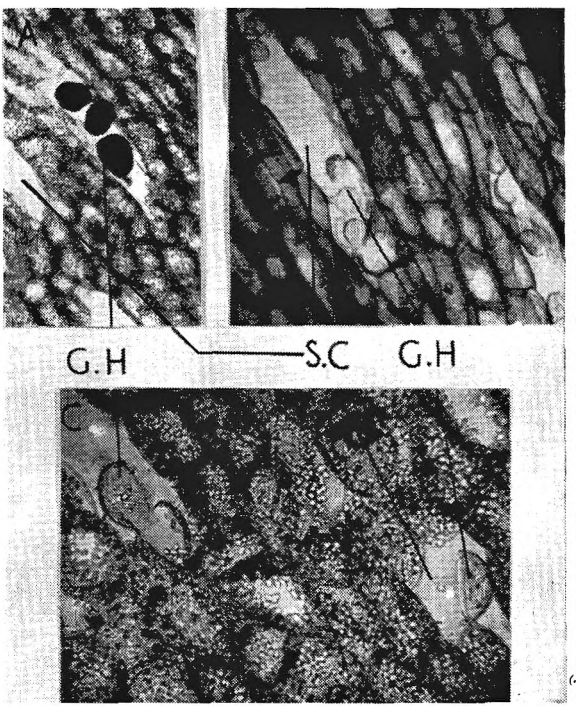



FIG. 6. A, *Dryopteris ramosa*, L.S. rhizome showing schizogenous cavities containing glands enveloped in oleoresin secretion, stained with sudan III × 40. B, Unstained glands in the cavities × 100. C, *D. chrysocoma*, unstained glands × 166.

The two adaxial bundles are larger and conspicuous because of the inward notching of the xylem which forms prominent hooks. The structure of the individual bundle is on the same pattern as in the rhizome.

*D. chrysocoma* (Christ) C. Chr.

The species occurs in abundance around Darjeeling between the altitudes of 5,000–8,000 ft. On the way to Sandakphu from Tonglu (Dist. Darjeeling), the hillocks are almost covered with the species as if cultivated. It is also common along the Mussoorie-Tehri Road, between 6,000–8,000 ft.

The species is characterised by a short, ascending, thick and tufted rhizome. The stipe is up to 20 cm. long, stramineous, and densely scaly or muricated due to the persistent bases of fallen ramenta. The

laminae are lanceolate, less pointed, bipinnate, acute, 30–60 cm. long and 8–20 cm. broad at the middle, the lower pinnae gradually shorten (Fig. 1B). The pinnae are alternate or sub-opposite in the lower part of the frond, they are subsessile, lanceolate to ovate, sub-arcuate and the middle pinnae are up to 13 cm. long, while the basal ones are up to 6 cm. long. The ultimate pinnules are up to 20 in number, 2 cm. long and 5 mm. broad, sessile, with a broad base opposite or sub-opposite, oblong, crenate or lobato-incised. The venation is pinnate, veinlets bifurcating into the teeth, the sori are in two rows, one on either side of the midrib of the pinnae, dorsal on veinlets, distinctly kidney shaped, in 2–6 pairs. The indusium is large and light brown, crustaceous and convex.

*Macroscopy of the rhizome.* The rhizome (Fig. 2B) is cylindrical to conical, up to 15 cm. long and 2–5 cm. broad (without leaf bases only 1–3 cm. in diameter), densely covered with shining reddish brown chaffy ramenta which form a felt like covering at the apex. The stipe bases are hard, persistent, curved, spirally arranged; dark coloured wiry roots emerging in between the bases. The petiole is stramineous, 2–5 mm. thick, furnished at the base with cushion like mass of reddish ramenta and showing transverse scars of fallen ramenta on the upper part. The fracture is short. The transversely cut surface is yellowish green or greenish brown even on drying, and is marked by 5–8 cauline meristeles. In the peripheral region are present 3–4 leaf traces each comprising of 6–10 relatively smaller bundles arranged in the form of a closed "C." Dark spots which represent the position of sclerotic nests are observed in the rhizome (Fig. 4) as well as in the petiole. The taste at first sweetish, later becoming acrid and nauseous.

The ramenta (Fig. 5C) are lanceolate to ovate-lanceolate with a hair pointed apex, pale brown to chestnut brown, up to 3 cm. long and 6 mm. broad. The margin of the mature scale is markedly wavy bearing a few laciniae, and with twin celled peg-like projections common along the margin (Fig. 5D). Unicellular glands may occasionally be observed on the surface of the ramenta. Young ramenta at the apex of the rhizome are characterised by fimbriated margin.

*Histology of the rhizome.* A transverse section of the rhizome is represented in Fig. 4. The histological pattern is the same as described in the previous species except that a few dark patches of sclerenchyma or sclerotic nests can be observed in the ground tissue. The salient features are as follows.

The epidermis is formed of rectangular to oblong, brownish cells, and bears numerous cut ramenta and single celled epidermal glands (Fig. 5F). The hypodermis consists of 3–7 layers of sclerenchymatous fibres whose position is variable. This zone may be next to the epidermis, or it may be separated from the epidermis by 1–3 layers of cellulosic fibres. The cortex consists of many layers of isodiametric parenchymatous cells. The cells of the outermost region of the cortex and a few layers around the meristeles possess aggregate granules of tannoid material, otherwise the cells are packed with starch grains. The latter are oval to

oblong in outline and measure 2–16 $\mu$. A few cells may occasionally contain globules of fat. In maceration the cortical cells appear round to rectangular, each measuring 60–140 $\times$ 35–70 $\mu$. In transverse section such cells number about 500/mm.$^2$.

Numerous intercellular spaces and schizogenous cavities are present all over the cortex and pith. The frequency of the latter varies from 40–60/mm.$^2$ in transverse section. In longitudinal section the cavities measure 200–480 $\times$ 48–96 $\mu$, and into each project 2–4 unicellular glandular hairs (Fig. 6C). The diameter of the head is up to 40 $\mu$. Pith is similar to cortex.

As stated above a few sclerotic nests are scattered in the ground tissue. Each nest is composed of a few sclereids. The individual sclereid is polygonal in transverse section and on maceration appears as an elongated cell with pointed or blunt ends measuring 200–450 $\times$ 30–50 $\mu$. Its wall is markedly pitted and the lumen is almost occluded due to the thickening on the walls. The latter are impregnated with a colouring matter because of which the nests appear dark in colour.

There is a diffused circle of 5–8 cauline meristeles. About 6–10 smaller strands, arranged in the form of closed "C," constitutes a leaf trace and there are observed 3–4 such leaf traces in a transverse section of the rhizome (Fig. 4). The structure of the meristele is the same as the ferns generally. Occasionally root traces are observed passing out of the cortex.

*Histology of the stipe.* The structure is similar to the previous species except for the presence of a few, dark coloured sclerotic nests in the ground tissue, and a number of unicellular glands on the epidermis. The number of vascular strands varies from 6–10. The cortical cells are relatively broader than the corresponding ones in the rhizome, and in transverse section number about 350/mm.$^2$.

Apart from the presence or otherwise of sclerotic nests, the general pattern of the internal structure in both the species is essentially the same.

The epidermis bears two types of appendages, single celled glandular hairs and ramenta. Below is the hypodermis composed of fibrous cells which lack intercellular spaces. The cortex is parenchymatous. The important feature is the occurrence of varying number of schizogenously developed cavities in the cortex and the pith in both the species. Into each cavity project numerous, unicellular, glandular hairs which are enveloped in oleoresinous secretion. In *D. ramosa* scattered sclerosed cells occur in the ground tissue while in *D. chrysocoma* sclerotic nests are present. The stele is dissected and composed of 4–6 cauline meristeles in the former and 5–8 meristeles in the latter.

## References

Mittal, T. C., and Mehra, P. N. (1960). *J. Pharm. Pharmacol.*, 1960, **12**, 317–319.
Sauss, J. E. (1958). *Botanical Microtechnique*, London: Constable.

# A NEW ANALEPTIC: 5,5-DIETHYL-1,3-OXAZIN-2,4-DIONE (DIOXONE)

## II. Antagonism to some C.N.S. Depressants

By G. Maffii and M. G. Serralunga

*From the Department of Pharmacology, Lepetit S.p.A. Research Laboratories Milan, Italy*

Received December 5, 1960

Dioxone (5,5-diethyl-1,3-oxazine-2,4-dione), has been found to exhibit a strong antagonistic effect to barbiturates and chlorpromazine. Given subcutaneously, dioxone significantly reduces the mortality in mice from phenobarbitone and allows an increase in the *LD50 of quinalbarbitone and hexobarbitone in mice, and* pentobarbitone in rats and mice, thus showing its barbiturate antagonist action both at lethal and depressant dose levels. As with leptazol and bemegride, dioxone does not show any significant effect on the depressant activity of ethanol. It has no effect on lethal doses of chlorpromazine but it has an awakening effect on mice sedated with the drug. The pharmacological actions of dioxone suggest that its mode of action is functional, rather than at a cellular competition level.

In a previous paper (Maffii, Dezulian and Silvestrini, 1961) the convulsant activity and toxicity of dioxone (5,5-diethyl-1,3-oxazin-2,4-dione) a new compound selected among a series of substituted oxazindiones (Maffii and Silvestrini, 1961) and synthetised by Testa, Fontanella, Cristiani and Gallo (1959) has been reported.

The convulsant activity of dioxone, studied in different species and under various experimental conditions resembles that of leptazol and bemegride. However dioxone appeared more active than leptazol and its margin of safety, as determined in mice, is greater than that of bemegride.

It was also shown that the convulsant activity of dioxone could be antagonised by pretreatment with other substituted oxazindiones which possess anticonvulsant activity, as well as by trimethadione and meprobamate.

It seemed of obvious interest to investigate whether or not dioxone could antagonise the depressant effect of barbiturates and other CNS depressants such as ethanol and chlorpromazine and to compare its effects with those of leptazol and bemegride.

### Materials and Methods

CF 1 mice and CF-Wistar rats of both sexes were used. The average weight, respectively was 18–22 and 180–200 g. To ascertain the effect of the three compounds upon the toxicity of several depressant agents, two methods were used. In some experiments the *LD50* of a given agent was determined in a control group and then the LD50 was retested in animals also receiving the supposed antagonist. In other experiments a dose of depressant of approximately 90 per cent of the lethal one was

taken as basis and the mortality checked in control animals and in animals also receiving the test agents. Finally in some experiments the changes in the most characteristic symptoms of depressant-poisoning, produced by the given stimulant were observed.

The tested drugs were injected either intraperitoneally (i.p.) or subcutaneously (s.c.) as solutions (sodium salt of phenobarbitone, pentobarbitone, hexobarbitone and quinalbarbitone and hydrochloride of chlorpromazine). For calculating the LD50 and ED50 the method of Litchfield and Wilcoxon (1949) was used. Dioxone, leptazol and bemegride were usually injected in doses that were a multiple of their respective LD50,

TABLE I

PROTECTION SHOWN BY DIOXONE, BEMEGRIDE AND LEPTAZOL IN MICE INJECTED WITH TOXIC DOSE OF PHENOBARBITONE (250 MG./KG.)

| Compound | Dose mg./kg. s.c. | *LD50* s.c. | Died/ treated | Survivors per cent | Death per cent within | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | 0–12 hr. | 12–36 hr. | 36–84 hr. | 84–108 hr. |
| Phenobarbitone alone | | 250 mg./kg. i.p. | 59/70 | 25·8 | 54·2 | 28·8 | 17 | 0 |
| Dioxone | | 60·5 (54·0–67·7) | | | | | | |
| | 15 | | 7/10 | 30 | 42·8 | 57·2 | 0 | 0 |
| | 30 | | 12/20 | 40 | 66·6 | 8·3 | 16·6 | 8·3 |
| | 60 | | 12/20 | 40 | 66·6 | 16·6 | 8·3 | 16·3 |
| | 120 | | 16/30 | 46·7 | 12·5 | 37·5 | 50·0 | 0 |
| | 240 | | 4/20 | 80 | 25·0 | 25·0 | 50·0 | 0 |
| Leptazol | | 101·0 (93·0–109·5) | | | | | | |
| | 60 | | 11/20 | 45 | 81·8 | 18·2 | 0 | 0 |
| | 120 | | 9/20 | 55 | 44·5 | 33·3 | 22·2 | 0 |
| | 240 | | 14/30 | 53·4 | 7·1 | 28·5 | 64·3 | 0 |
| | 480 | | 4/20 | 80 | 25·0 | 25·0 | 50·0 | 0 |
| Bemegride | | 40·5 (36·1–45·3) | | | | | | |
| | 20 | | 16/20 | 20 | 56·3 | 31·2 | 12·5 | 0 |
| | 40 | | 14/20 | 30 | 50·0 | 42·8 | 7·2 | 0 |
| | 80 | | 14/30 | 53·4 | 28·6 | 35·7 | 35·7 | 0 |
| | 160 | | 3/20 | 85 | 33·3 | 66·6 | 0 | 0 |

as determined previously (Maffi, Desulian and Silvestrini, 1961). Determinations of the LD50 with the depressant agent alone and with an antagonist were always made on the same day.

To study the awakening action in chlorpromazine treated mice, the righting reflex was used. During the experiments the room temperature was kept between 23 and 24°.

## RESULTS

### *Effects of Dioxone on the Action of Barbiturates*

*Phenobarbitone.* In mice, this drug was given i.p. at 250 mg./kg., a dose found to correspond approximately to the LD90. The solution was 1·25 per cent in distilled water and the volume injected 0·4 ml./20 g. After 1 hr., animals received different doses of dioxone, bemegride and leptazol subcutaneously. The mortality was checked for 108 hr.

The results of this experiment are in Table I from which it appears that the potency of the three compounds, all effective in protecting mice

from death due to phenobarbitone, is inversely proportional to their LD50 determined by the same route.

Plotting the percentages of survivors against the doses expressed as multiples of the respective LD50, the plots obtained with bemegride, leptazol and dioxone may be considered similar (Fig. 1).

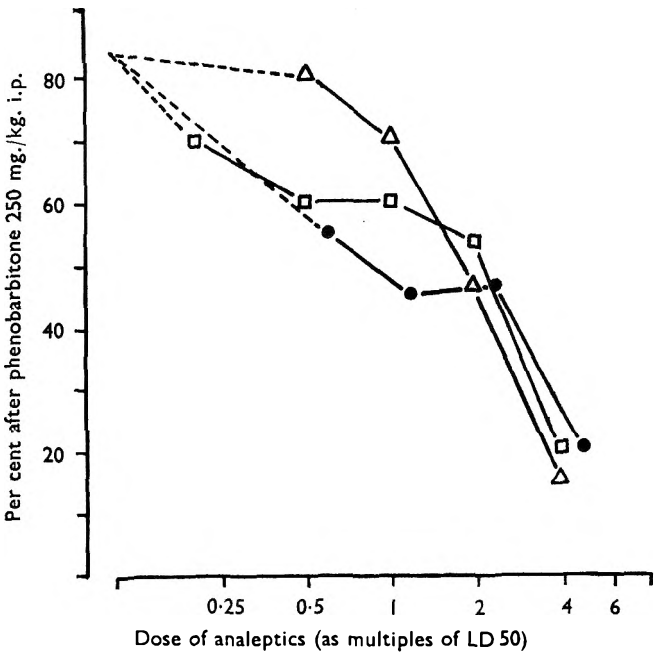



FIG. 1. Reduction in number of deaths due to phenobarbitone 250 mg./kg. i.p. in mice receiving □dioxone, △ bemegride and ● leptazol.

Since the three analeptics were always given in a single dose 1 hr. after the phenobarbitone, the effect of the compounds on mortality can be compared (see Table I). It appears that dioxone gives a significant protection even at half the LD50. The same does not seem to be true

TABLE II

EFFECTS OF DIOXONE, BEMEGRIDE AND LEPTAZOL ON THE TOXICITY OF PENTOBARBITONE IN MICE

| Analeptic Agent | Doses mg./kg. s.c. | Number of doses of pentobarbitone | Animals/dose | LD50 mg./kg. and [Fiducial Limits (P = 0·05)] |
|---|---|---|---|---|
| | (Pentobarbitone alone i.p.) | 4 | 17 | 124 [117–131] |
| Dioxone s.c. | 15 | 4 | 20 | 138 [133–143] |
| | 30 | 5 | 10 | 169 [155–184] |
| | 120 | 4 | 10 | 167 [160–174] |
| | 240 | 3 | 10 | 152 [144–160] |
| Bemegride | 20 | 3 | 10 | 141 [135–147] |
| | 80 | 3 | 10 | 179 [165–194] |
| | 160 | 4 | 10 | 168 [158–178] |
| Leptazol | 60 | 4 | 5 | 140 [134–146] |

with bemegride and leptazol when the doses needed are at least twice the respective LD50.

Though the three analeptics were administered in much higher doses than the convulsant ones, no convulsions were observed.

*Pentobarbitone.* The antagonism of dioxone towards pentobarbitone toxicity was studied through the changes in the i.p. LD50 of the barbiturate in animals treated simultaneously with standard s.c. doses of dioxone. Similar experiments were made with bemegride and leptazol. In Tables II and III the results obtained in rats and mice are shown. They demonstrate that dioxone resembles bemegride quantitatively in the ability to lower the acute toxicity of pentobarbitone.

Both dioxone and bemegride appear much more active than leptazol on the basis of weight : weight or toxicity : activity ratios. Moreover the

TABLE III

EFFECTS OF DIOXONE, BEMEGRIDE AND LEPTAZOL ON THE TOXICITY OF PENTOBARBITONE IN THE RAT

| Analeptic agent | Doses mg./kg. s.c. | Number of doses | Rats/dose | LD50 of pentobarbitone (mg./kg. i.p.) and Fiducial Limits (P = 0·05) |
|---|---|---|---|---|
| | Pentobarbitone (alone) i.p. | 4 | 15 | 104 [100–108] |
| Dioxone | 20 | 3 | 10 | 112 [107–118] |
| | 40 | 5 | 14 | 127 [120–135] |
| | 80 | 3 | 10 | 117 [112–122] |
| | 160 | 4 | 12 | 145 [134–157] |
| Bemegride | 20 | 3 | 10 | 113 [109–117] |
| | 40 | 5 | 14 | 127 [118–137] |
| | 80 | 3 | 10 | 132 [122–142] |
| | 160 | 3 | 10 | 119 [103–137] |
| Leptazol | 40 | 3 | 10 | 99 [ 95–103] |
| | 160 | 3 | 10 | 124 [114–134] |

relationship between doses and protective effect both with bemegride (the more active in the mouse) and with dioxone (the more active on the rat) shows the limitations of this antagonism. It is clear that for both dioxone and bemegride, there are dose levels over which no further amelioration of effect could be obtained and perhaps a deterioration could be expected. This end point is difficult to establish and, obviously, it largely depends upon experimental conditions. However, in the comparison of dioxone and bemegride, the evidence excluded any significant difference between their anti-pentobarbitone activity (see Fig. 2). Since subcutaneously dioxone has a toxicity significantly lower than bemegride in mice and in rats, it may be considered superior in reducing the probability of death in animals given toxic doses of pentobarbitone.

Antagonism of dioxone towards sub-lethal effects of pentobarbitone was also investigated in a preliminary experiment where the influence of s.c. dioxone was observed on the sleeping time of rats pretreated with 50 mg./kg. i.p. of pentobarbitone 10 min. before. The results, shown in Table IV, demonstrate that dioxone significantly reduces the sleeping time in the rat and in this respect it is at least twice as active as leptazol.

*Quinalbarbitone and hexobarbitone.* The protective effect of dioxone against quinalbarbitone and hexobarbitone was also compared with that of bemegride and leptazol. As in the previous experiment, the effects were evaluated on changes in the LD50. The barbiturates were administered i.p. immediately after the subcutaneous injection of the analeptic

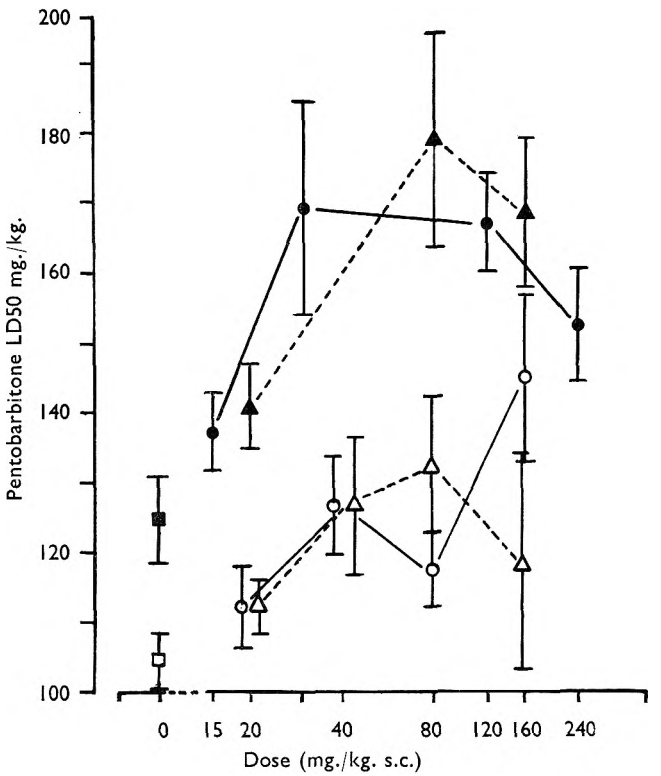



FIG. 2. LD50 of pentobarbitone i.p. alone and with the analeptic agents, in rats and mice.

■ Pentobarbitone alone in mice.
● Pentobarbitone + dioxone in mice.
▲ Pentobarbitone + bemegride in mice.
□ Pentobarbitone alone in rats.
○ Pentobarbitone + dioxone in rats.
△ Pentobarbitone + bemegride in rats.
⊢——⊣ = fiducial limits (P = 0·05).

agents. The results in Table V show the effectiveness of dioxone against these two short-acting barbiturates. Bemegride gave comparable results while leptazol had lower activity.

*Interaction of dioxone with ethanol.* The LD50 of ethanol was found to be in mice 7·10 ml./kg. In animals receiving dioxone in doses of 30 mg./kg. and bemegride 20 mg./kg. 15 min. after the ethanol, the frequency and rate of deaths was the same as in the controls (see Table VI). With 90 mg./kg. of dioxone a limited increase in the LD50 value was obtained

but this was not significant. As reported by May (1957) bemegride even at 100 mg./kg. has no effect on the mortality of mice poisoned with ethanol 6–7 mg./kg.

*Interaction between dioxone and chlorpromazine.* Dioxone in doses of 10–25 mg./kg. s.c. every hr. for 5 hr., and of 50 mg./kg. in a single dose.

TABLE IV

AVERAGE SLEEPING TIMES OF RATS TREATED WITH 50 MG./KG. I.P. OF PENTOBARBITONE, AND WITH VARIOUS DOSES OF DIOXONE AND LEPTAZOL

| Treatment and dosage mg./kg. | Average awakening time (min.) |
|---|---|
| Pentobarbitone 50 i.p. | 95 ± 12 |
| ,, ,, + leptazol 50 s.c. . . | 70 ± 10 |
| ,, ,, + ,, 100 s.c. | 45 ± 7 |
| ,, ,, + dioxone 25 s.c. . . | 65 ± 6 |
| ,, ,, + ,, 50 s.c. . . | 45 ± 5 |

TABLE V

EFFECTS OF DIOXONE, BEMEGRIDE AND LEPTAZOL ON THE TOXICITY OF QUINALBARBITONE AND HEXOBARBITONE IN MICE

| Antagonist and dose mg./kg. s.c. | Barbiturate | Dose No. | Mice/dose | LD50 mg./kg. | [Fiducial Limits] (P = 0·05) |
|---|---|---|---|---|---|
| | Quinalbarbitone | 4 | 10 | 100 | [ 94–107] |
| Dioxone 15 | ,, | 3 | 10 | 106 | [ 91–123] |
| ,, 30 | ,, | 3 | 10 | 135 | [117–155] |
| Leptazol 30 | ,, | 3 | 10 | 108 | [100–117] |
| ,, 60 | ,, | 3 | 10 | 121 | [114–128] |
| ,, 120 | ,, | 3 | 10 | 128 | [121–136] |
| Bemegride 20 | ,, | 4 | 15 | 119 | [112–126] |
| ,, 40 | ,, | 4 | 10 | 123 | [115–132] |
| | Hexobarbitone | 4 | 12 | 285 | [271–299] |
| Dioxone 30 | ,, | 3 | 13 | 318 | [301–335] |
| Leptazol 60 | ,, | 3 | 13 | 304 | [290–318] |

TABLE VI

INFLUENCE OF DIOXONE AND BEMEGRIDE UPON THE TOXICITY OF ETHANOL

| Analeptic agent | Doses mg./kg. s.c. | Number of doses | Mice/dose | LD50 of ethanol ml./kg. and Fiducial Limits (P = 0·05) |
|---|---|---|---|---|
| | Ethanol i.p. (alone) | 3 | 20 | 7·10 [5·96–8·44] |
| Dioxone .. | 30 | 3 | 10 | 6·35 [5·20–7·74] |
| | 90 | 5 | 12 | 7·50 [6·84–8·21] |
| Bemegride .. | 20 | 3 | 10 | 6·80 [5·61–8·22] |

failed to modify the mortality of mice due to chlorpromazine 40 mg./kg. i.p. Leptazol and bemegride tested in the same dose range also did not affect the frequency of death.

However, in mice given chlorpromazine, 20 mg./kg., the awakening activity of dioxone was easily demonstrated. In fact under our conditions no animal given chlorpromazine alone, was able to right itself during the 2 hr. after treatment. Of mice receiving dioxone s.c. 1 hr. after chlorpromazine, some righted themselves within 30 min. after the analeptic. Fig. 3 shows the relationships between doses of dioxone and this wakening action and compares it with the results after leptazol. The ratio of the ED50 leptazol : dioxone is 2·41, a value that corresponds

approximately to the activity ratios of the two compounds as shown in other experiments by Maffi, Dezulian and Silvestrini (1961).

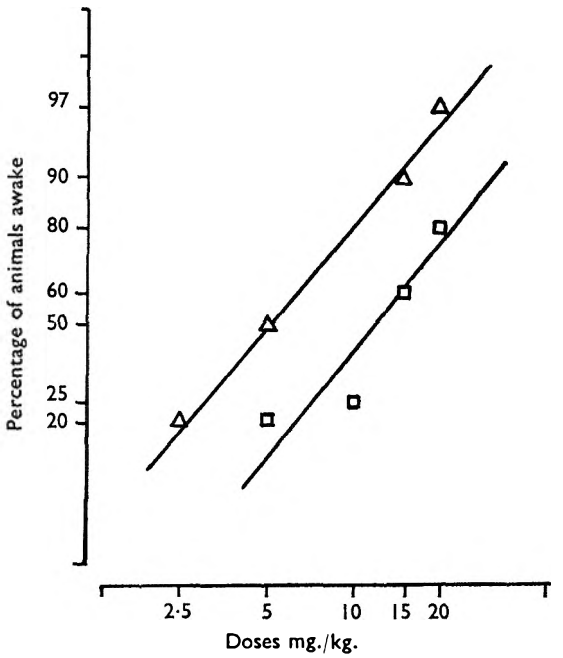



FIG. 3. Awakening effect of △ dioxone and □ leptazol in mice given chlorpromazine 20 mg./kg.

ED50 mg./kg. dioxone = 5·1 (8·0 – 3·2).
leptazol = 12·3 (15·9 – 9·4).

The convulsant doses of dioxone, leptazol and bemegride produce convulsions in the animals treated with chlorpromazine, however the seizures are only clonic and rarely followed by the tonic phase like the occurrence in animals receiving the analeptics alone.

## DISCUSSION

In the study of the barbiturate-antagonist properties of dioxone we have shown its ability to reduce the mortality of a long acting barbiturate (phenobarbitone) a medium-acting one (pentobarbitone), a short acting derivative (quinalbarbitone) and a very short acting barbiturate (hexobarbitone). In most of our experiments the effect of dioxone was evaluated on the basis of the LD50 because of the obvious statistical weight of this kind of data and also for practical purposes.

By comparison with bemegride and leptazol, dioxone may be considered to be more active than leptazol and is comparable in potency with bemegride. However, some minor differences in the action of dioxone and bemegride are worth considering; they are quantitative and concern both the depressing agents and the species. Dioxone is more active against short and very short acting barbiturates than against phenobarbitone and

perhaps more effective in rats than in mice, and the opposite being true for bemegride. However, if the two analeptics are evaluated on the basis of their respective toxicity: activity ratios, the difference that has been found in the protective potency against phenobarbitone, tends to disappear and also leptazol may hardly be defined as less active (see Fig. 1). These results probably depend upon the experimental conditions used, that is a single administration of the analeptic 1 hr. after injection of phenobarbitone, but this hypothesis cannot affect the evidence and the value of the comparison. With hexobarbitone and quinalbarbitone, the toxicity: activity ratios allow a favourable comparison of dioxone with both bemegride and leptazol.

However, the pharmacological properties of dioxone strongly suggest that this drug counteracts the barbiturate action by its proper functional activity and not through a competition at the level of common cell receptors. This results from: (*a*) the nature of dioxone as a convulsant agent (which it shares with all other analeptics and bemegride); (*b*) the antagonism between dioxone and anticonvulsant and depressive agents that are also effective against other convulsant agents in a degree that is proportional to potency of the convulsant agent; (*c*) the primary pharmacological properties of dioxone that are specific to it such as the activation of cerebral electrical activity, the stimulation and the increase in excitability of bulbar centre (Maffii, Bianchi, Schiatti and Silvestrini, 1961); (*d*) the restoring action in other types of CNS depression. We thus consider the protection against mortality by barbiturates, as well as the wakening action, as expressions of a general exciting action of dioxone which extends over functions of the central nervous system other than the motor ones. This view is supported by the evidence that in laboratory animals, larger doses of dioxone—as well as of other analeptics—are often necessary to reduce barbiturate narcoses than are required for the production of convulsions. Then the structural relationship between dioxone and anticonvulsant oxazindiones (Maffii and Silvestrini, 1961) as well as that between dioxone and barbiturates, has probably no particular significance in view of the explanation of pharmacological antagonism, and we may only accept the fact that the exciting and depressing actions on the CNS are thus sometimes closely interrelated from the point of view of chemical structure.

Some practical implication may be drawn also from our results obtained in animals given chlorpromazine. It is true that no significant protection was produced by dioxone towards the lethal doses of this drug; but an obvious effect was found in animals which received paralyzing doses. The potency of dioxone in awakening the mice from paralysis by chlorpromazine is demonstrated by the low level of the ED50–6·1 mg./kg. s.c. Thus dioxone may be considered of some use in reducing the depression and hypnotic effect produced by excessive doses of chlorpromazine, and may also be of some help in severe intoxication, in addition to other drugs.

On the basis of experimental evidence it may be concluded that the new analeptic agent, dioxone, has a strong antagonistic action against lethal effects of barbiturates and depressive effects of other CNS depressants.

*Acknowledgement.* The technical assistance of Mr. Silvano Banfi is gratefully acknowledged.

## REFERENCES

Litchfield, J. T. and Wilcoxon, F. (1949). *J. Pharmacol.*, **96**, 99–113.
Maffii, G., Dezulian, V. M. and Silvestrini, B. (1961). *J. Pharm. Pharmacol.*, **13**, 244–253.
Maffii, G. and Silvestrini, B. (1961). *Il Farmaco* (Ed. Scient.), **16** (in the press).
Maffii, G., Bianchi, G., Schiatti, P., and Silvestrini, B. (1961). *Brit. J. Pharmacol.*, **16.** (In the press.)
May, J. (1957). *Dissertationes Pharm.*, **9**, 131–142.
Testa, E., Fontanella, L., Cristiani, G., and Gallo, G. (1959). *J. org. Chem.*, **24**, 1928–1936.

# LETTERS TO THE EDITOR

## Banana and Gastric Secretion

SIR,—It has been reported previously that bananas have a high 5-hydroxytryptamine (5-HT) content (Waalkes, Sjoerdsma, Creveling, Weisebach Udenfriend, 1958; West, 1958). In view of the fact that 5-HT may be concerned in peristaltic reflexes (Bulbring and Lin, 1958) and also in inhibiting acid gastric secretion (Black, Fisher and Smith, 1956), it was considered of importance to make a systematic study of this amine in vegetables and fruits commonly employed as food, and further note, if such food has any effect on acid gastric secretion.

5-HT was extracted with acetone and assayed on rat oestrous uterus and rat colon (Parratt and West, 1957), and further identified chromatographically.

It was confirmed that high amounts of 5-HT are contained in bananas. Moderate amounts were detected in tomatoes, which also contain tryptamine. Trace amounts were detected in vegetables belonging to the natural order cucurbitacae.

The effect of feeding bananas on gastric secretion was studied by cannulating the pyloric and the cardiac ends of the stomach in guinea-pigs of either sex, anaesthetised with urethane (1·6 g./kg.); secretion was induced by intramuscular injections of histamine (1 mg./kg.) in animals pretreated with mepyramine (10 mg./kg.). The entire amount of secretion for 30 min. was washed out by distilled water introduced through the cardiac cannula and collected from the duodenal one. The total amount of acidity was determined in terms of N/100 NaOH with phenolphthalein as indicator.

In control animals, when the stomach contained either distilled water or starch solution at the time of histamine injection, the average acidity in six observations was found to be 15·3 ml. whereas average acidity in seven test observations was only 2·4 ml. when the stomach contained 3–4 ml. of neutralised banana extracts. The results on statistical analysis yielded a confidence limit greater than 99/100. This experiment has been repeated in different strains of guinea-pigs, at different times of the year and has always yielded similar results.

It is possible to induce a chronic state of hyperchlorhydria in guinea-pigs by repeated injections of histamine in increasing doses under antihistamine cover. When animals were maintained on the usual diet, such treatment produced ulceration and fatal perforation of the stomach usually by the 5th day. However, in animals fed only with bananas such histamine treatment did not produce any acute symptom and there was no evidence of ulceration in the stomach when the animals were killed.

Thus it was seen that banana emulsions introduced directly into the stomach reduce acid gastric secretion, and also prevent chronic ulceration and perforation induced by repeated injections of histamine. Whether this activity is due to its 5-HT content, or may prove of clinical use, is now under investigation.

Department of Pharmacology,
Maulana Azad Medical College,
New-Delhi. R. K. SANYAL.

Lady Hardinge Medical College,
New-Delhi. P. K. DAS.

Darbhanga Medical College, S. SINHA.
Bihar. Y. K. SINHA.
March, 17, 1961.

REFERENCES

Waalkes, T. P., Sjoerdsma, A., Creveling, C. R., Weissbach, H., and Udenfriend, S., (1958). *Science*, **127**, 648–650.
West, G. B. (1958). *J. Pharm. Pharmacol.*, **10**, 589–590.
Bulbring, E., and Lin, R. C. Y. (1958). *J. Physiol*, **140**, 381–407.
Black, J. W., Fisher, E. W. and Smith, A. N. (1956). *Proc. 20th International Physiology Congress*, Page 102.
Parratt, J. R. and West, G. B. (1957). *J. Physiol.* **137**, 169–178.

## Modification of Quantitative Colorimetric Estimation of Glutethimide in Toxicology

SIR,—In the course of an investigation of the vomitus of a patient containing glutethimide, certain snags were encountered in its quantitative estimation.

The measurement of the ultra-violet absorption spectrum and characteristic "half-life" in ethanolic potassium hydroxide (Goldbaum and Williams, 1960) works satisfactorily with urine and blood. Applied to the vomitus, there was enough fatty matter present in the chloroform extract to cause a turbid solution in the final ethanolic potassium hydroxide mixture, thus making the spectrophotometric reading impossible. Dissolving the extract in 10 per cent ethanol removed most of the fat and gave a qualitative identification of the glutethimide. However, the recovery of glutethimide was not quantitative.

The colorimetric method of (Sheppard, D'Asaro and Plummer, 1956) also gave a turbid solution and treatment with aluminium hydroxide column chromatography recommended by these authors did not remove the fatty impurities. We have now found that by extracting the purple colour into isobutanol the fatty impurities does not interfere. We also found the choice of 0·5 ml. of the ferric chloride reagent to be more satisfactory in imparting a less strong yellow colour to the isobutanol layer. Accurate and reproducible results were obtained as long as the readings were taken within half an hour.

*Reagents.* (*a*) Hydroxylamine hydrochloride 2M (store in refrigerator) (*b*) Sodium hydroxide 3·5N (*c*) Hydrochloric acid 3·5N (*d*) Ferric chloride 0·37M in 0·1N hydrochloric acid. (*e*) Isobutanol A.R. (*f*) Methanol A.R. (*g*) Chloroform A.R.

*Method.* Extract the specimen with chloroform. Filter and evaporate the solvent at a low temperature to 2–3 ml. and then at room temperature (30°) to dryness. Dissolve the residue in a small amount of methanol, so that it contains not more than 1 mg. of glutethimide per ml., for colour development.

*Standard Graph.* Introduce 1 ml. of methanol solution of glutethimide, containing respectively 0·25, 0·50, 0·75 and 1·00 mg. into 4 × 10 ml. glass stoppered cylinders. Add 1 ml. of hydroxylamine hydrochloride reagent and 1 ml. 3·5N of sodium hydroxide. Allow to stand for 30 min. Add 1·5 ml. 3·5N hydrochloric acid, 5 ml. isobutanol and then 0·5 ml. of ferric chloride reagent. Shake vigorously for 30 sec. and allow to separate. As soon as separation is complete, pipette off the isobutanol and filter through a 5 cm. No. 1 Whatman filter paper. Measure its optical extinction, without delay, at 510 mμ using the reagents as blanks.

*Recovery experiments.* We have investigated the recovery of glutethimide in vomitus by adding 1 mg. of pure glutethimide to four specimens of vomitus,

and obtained an average recovery of 90 per cent. Barbiturates and brominated ureides were found not to interfere.

S. E. PHANG.
M. C. DUTT.
THNG SOON TEE.

Government Department of Chemistry,
Outram Road,
Singapore.
March 14, 1961.

## REFERENCES

Goldbaum, L. R. and Williams, M.A. (1960). *Analyt. Chem.*, **32**, 81–84.
Sheppard, H., D'Asaro, B.S. and Plummer, A. J. (1956). *J. Amer. pharm. Ass., Sci. Ed.*; **45**, 681–684.