- Brown, B. R. & MacBride, J. A. H. (1963). *Chem. & Ind.* p. 1037.
- Clark-Lewis, J. W. & Jackman, L. M. (1961). *Proc. chem. Soc.* p. 165.
- Clark-Lewis, J. W., Jackman, L. M. & Williams, L. R. (1962). *J. chem. Soc.* p. 3858.
- Clark-Lewis, J. W. & Katekar, G. F. (1962). *J. chem. Soc.* p. 4502.
- Clark-Lewis, J. W. & Mortimer, P. I. (1960). *J. chem. Soc.* p. 4106.
- Conroy, H. (1960). *Advanc. org. Chem.* **2**, 308.
- Corey, E. J., Philbin, E. M. & Wheeler, T. S. (1961). *Tetrahedron Lett.* p. 429.
- Dean, H. F. & Nierenstein, M. (1925). *J. Amer. chem. Soc.* **47**, 1676.
- Drewes, S. E. & Roux, D. G. (1963). *Chem. & Ind.* p. 532.
- Drewes, S. E. & Roux, D. G. (1964). *Biochem. J.* **90**, 343.
- Fujise, S., Hishida, S., Onuma, T., Adachi, K., Fujise, Y. & Munekata, T. (1962). *Bull. chem. Soc. Japan*, **35**, 1245.
- Grassman, W. & Hannig, K. (1952). *Hoppe-Seyl. Z.* **290**, 1.
- Karplus, M. (1959). *J. chem. Phys.* **30**, 15.
- Kashikar, M. D., Kulkarni, R. S., Borkar, A. M. & Kulkarni, A. B. (1961). *Chem. & Ind.* p. 1872.
- King, F. E. & Bottomley, W. (1954). *J. chem. Soc.* p. 1399.
- Kostanecki, S. & Nitkowski, S. (1905). *Ber. dtsh. chem. Ges.* **38**, 3587.
- Lillya, P., Drewes, S. E. & Roux, D. G. (1963). *Chem. & Ind.* p. 783.
- Roux, D. G. (1957). *Nature, Lond.*, **180**, 973.
- Roux, D. G. & de Bruyn, G. C. (1963). *Biochem. J.* **87**, 439.
- Roux, D. G. & Evelyn, S. R. (1958). *Biochem. J.* **70**, 344.
- Roux, D. G. & Maihs, A. E. (1960). *Biochem. J.* **74**, 44.
- Roux, D. G. & Paulus, E. (1960). *Biochem. J.* **77**, 315.
- Shah, V. R. & Kulkarni, A. B. (1958). *J. sci. industr. Res.* **17 B**, 420.
- Swain, T. (1953). *Biochem. J.* **53**, 200.

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Condensed Tannins

20. CHROMOPHORIC PROPERTIES OF FLAVAN-4-OLS, FLAVAN-3,4-DIOLS AND CONDENSED TANNINS

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Structural features involved in the commonly observed reddening that develops in certain condensed tannins when these are exposed to sunlight have been examined by comparing the characteristics of the free phenolic forms of flavan, flavan-3-ol, flavan-4-ol and flavan-3,4-diol analogues with 3',4',5',7-, 3',4',7- and 4',7-patterns of phenolic substituents (Roux & Paulus, 1961, 1962; Roux, 1963*a*; Roux & de Bruyn, 1963).

The benzylic 4-hydroxyl position, in conjunction with free phenolic hydroxyl groups in at least the 4'- and 7-positions, has been shown to be contributory to redness induced by indirect sunlight and heat (Roux, 1963*a*; Roux & de Bruyn, 1963). These preliminary conclusions have been summarized (Roux, 1963*b*).

Synthesis of the free phenolic form of two new flavan-4 β -ols now enables the correlation of some of the main structural factors involved in reddening.

EXPERIMENTAL AND RESULTS

C, H, methoxyl and acetyl estimations are by K. Jones, Micro-analytical Laboratory, C.S.I.R., Pretoria. Some of the nuclear-magnetic-resonance spectra are by Dr K. Pachler, Chemical Physics Group, C.S.I.R., Pretoria. All

nuclear-magnetic-resonance spectra were recorded on a Varian A-60 spectrometer with deuterochloroform as solvent and tetramethylsilane as internal standard. Band positions are expressed as p.p.m. downfield with the standard as origin (Table 1). Coupling constants are measured with an accuracy of ± 0.2 cyc./sec. Two-dimensional chromatograms were run with water-satd. butan-1-ol or butan-2-ol for the first direction, and 2% (v/v) acetic acid for the second direction.

Synthesis of flavan-4 β -ols

Synthesis of ($\pm$)-7-hydroxyflavanone and ($\pm$)-7-hydroxyflavan-4 β -ol. 2',4'-Dihydroxychalcone was prepared by the acid condensation method of Goel, Jain & Seshadri (1958) from resacetophenone dibenzoate (m.p. 81°) and freshly distilled benzaldehyde, as yellow needles, m.p. 147°, in 55% yield. Direct condensation of resacetophenone with benzaldehyde under alkaline conditions (Saiyad, Nadkarni & Wheeler, 1937) gave a lower yield (20%), m.p. 146–147°.

($\pm$)-7-Hydroxyflavanone. The flavanone was prepared by the following modified method. 2',4'-Dihydroxychalcone (600 mg.) dissolved in aqueous 1% sodium acetate (43 ml.) and ethanol (20 ml.) was refluxed for 7.5 hr. and left overnight. Buff crystals, m.p. 176–185° (453 mg., 75% yield), separated. These were recrystallized from ethanol in slightly buff plates, m.p. 190–191° (300 mg.). Saiyad *et al.* (1937) cite m.p. 189–190° and a 60% yield from the alkaline method of isomerization.

($\pm$)-7-Hydroxyflavan-4 β -ol. 7-Hydroxyflavanone (437 mg.) in methanol (50 ml.) over Adams catalyst (325 mg.) absorbed 83.5 ml. of hydrogen (twice the theoretical) and gave the 7-hydroxyflavan-4-ol as an amorphous white powder, m.p. 42–46° (285 mg.), on removal of the methanol. The compound failed to crystallize from ethanol, ethanol–water or acetone (Found: C, 74.1; H, 6.6. $C_{15}H_{14}O_3$ requires C, 74.4; H, 5.8%). The compound was located as a single spot on two-dimensional paper chromatograms, R_F 0.78 (butan-2-ol), 0.85 (butan-1-ol) and 0.39 (2%, v/v, acetic acid), with toluene-*p*-sulphonic acid (orange–yellow) and 2,6-dichloroquinonechloroimide (blue). Colour reactions to heat, sunlight and mineral acid are given in Table 1.

($\pm$)-4,7-Diacetoxyflavan. 7-Hydroxyflavan-4-ol (50 mg.) was acetylated with pyridine (0.4 ml.) and acetic anhydride (0.4 ml.) at room temperature for 3 hr. The sticky product recovered from water crystallized from ethanol in clusters of needles, m.p. 128–130° (26 mg.) (Found: C, 70.0; H, 5.8; CO $\cdot$ CH $_3$, 26.5. $C_{19}H_{18}O_5$ requires C, 69.9; H, 5.5; CO $\cdot$ CH $_3$, 26.4%). The compound was examined by nuclear-magnetic-resonance spectrometry (Table 2).

($\pm$)-4,7-Dibenzoyloxyflavan. 7-Hydroxyflavan-4-ol (50 mg.), dissolved in a minimum of pyridine (0.2 ml.) and cooled to 0°, was treated with benzoyl chloride (0.15 ml.) and the solution left overnight. An oily solid, resulting when the mixture was poured on to crushed ice, was dissolved in ethanol to give white prisms, m.p. 151–152° (60 mg.) (Found: C, 76.9; H, 5.2. $C_{29}H_{22}O_5$ requires C, 77.3; H, 4.9%).

The methyl ether of 7-hydroxyflavan-4-ol, obtained on methylation with diazomethane, was an oil which refused to crystallize. The compound did not redden with heat or in indirect sunlight.

Synthesis of ($\pm$)-4'-hydroxyflavanone and ($\pm$)-4'-hydroxyflavan-4 β -ol. 2',4-Dihydroxychalcone was obtained in 75% yield from *p*-hydroxybenzaldehyde and *o*-hydroxyacetophenone under alkaline conditions, as yellow needles from benzene, m.p. 159–160°. Geissman & Clinton (1946*a*) cite m.p. 162°.

($\pm$)-4'-Hydroxyflavanone. 2',4-Dihydroxychalcone (1 g.) was converted into the flavanone with sodium acetate under the same modified conditions as those used for 7-hydroxyflavanone. Long light-yellow needles formed overnight (922 mg., 92% yield). These were recrystallized from ethanol–water (1:1, v/v) to give white needles, m.p.

186°. Hattori (1925) and Geissman & Clinton (1946*a*) quote m.p. 186° and 186–187° respectively.

($\pm$)-4'-Hydroxyflavan-4 β -ol. 4'-Hydroxyflavanone (500 mg.) in methanol (50 ml.) over Adams catalyst (250 mg.) absorbed 90.2 ml. of hydrogen (twice theoretical). From the solution 4'-hydroxyflavan-4-ol (254 mg., 51% yield) was readily obtained, and recrystallized from ethanol, m.p. 181° (Found: C, 73.9; H, 5.9. $C_{15}H_{14}O_3$ requires C, 74.4; H, 5.8%). The compound was located as a single spot on two-dimensional paper chromatograms, R_F 0.89 (butan-2-ol), 0.83 (butan-1-ol) and 0.41 (2%, v/v, acetic acid), with toluene-*p*-sulphonic acid (yellow) and 2,6-dichloroquinonechloroimide (blue fading to brown). Colour reactions to heat, light and mineral acid are shown in Table 1.

($\pm$)-4-O-Methylflavan-4 β -ol. 4'-Hydroxyflavan-4 β -ol (50 mg.) in methanol (50 ml.) was methylated with excess of ethereal diazomethane to give the methoxyl derivative from ethanol in large needle clusters, m.p. 150° (34 mg.) (Found: C, 74.3; H, 6.5; OCH $_3$, 12.1. Calc. for $C_{16}H_{16}O_3$: C, 75.0; H, 6.3; OCH $_3$, 12.1%). Bogner, Rakosi & Wheeler (1962), and Bokadia, Brown & Cummings (1960) give m.p. 150–151° and 152–153° respectively for the same compound synthesized from 4'-O-methylflavanone. The compound did not redden to heat or indirect sunlight.

($\pm$)-4,4'-Diacetoxyflavan. 4-Hydroxyflavan-4 β -ol (30 mg.) was acetylated with pyridine (0.2 ml.) and acetic anhydride (0.2 ml.) at room temperature for 2 hr. The diacetate crystallized from ethanol in needles, m.p. 117° (31 mg.) (Found: C, 70.0; H, 5.7; CO $\cdot$ CH $_3$, 26.6. $C_{19}H_{18}O_5$ requires C, 69.9; H, 5.5; CO $\cdot$ CH $_3$, 26.4%). This compound was examined by nuclear-magnetic-resonance spectrometry (Table 2).

Synthesis of ($\pm$)-4',7-dihydroxyflavan-4 β -ol and ($\pm$)-4',7-dihydroxyflavanone. 2',4,4'-Trihydroxychalcone (960 mg.), m.p. 202° (Nadkarni & Wheeler, 1938), was dissolved in aqueous 1% sodium acetate (95 ml.) and ethanol (30 ml.) and the solution was refluxed for 11.5 hr. The solution was left overnight and extracted with ethyl acetate–ether (1:3, v/v). The extracts were dried with CaCl $_2$, concentrated to dryness and the product crystallized from a water–ethanol mixture, m.p. 204° (582 mg., 62% yield). This yield contrasts with the best obtainable (8–12%) under acid isomerization conditions (cf. Roux & de Bruyn, 1963).

($\pm$)-4',7-Dihydroxyflavan-4 β -ol. The compound was prepared under conditions used before (Roux & de Bruyn,

Table 1. Colour reactions of the free phenolic forms of flavan-4 β -ols

Flavan-4 β -ol	Colour developed		
	On exposure to sunlight*	On heating under vacuum†	On heating with HCl‡
4'-Hydroxy	White	White	Red§
7-Hydroxy	Orange-red	Red	Orange–yellow precipitate
4',7-Dihydroxy	Pink	Red	Purple-red§
3',4',7-Trihydroxy	Red	Red	Purple–blue§
3',4',5',7-Tetrahydroxy	Mauve-red	Blue	Purple–blue§

* Solid exposed for 20 days.

† Solid heated at 100° for 2–3 hr. over P $_2$ O $_5$.

‡ Reaction in methanolic solution. Similar colours are given on paper chromatograms with toluene-*p*-sulphonic acid spray when heated at 80°.

§ Transient colours, fading to yellows or usually to faint pinks.

Table 2. *Coupling constants and τ values from nuclear-magnetic-resonance spectra of flavan-4 β -ol acetates*

Flavan	τ (p.p.m.)				
	Acetyl Me		C ₍₃₎ -methylene protons	2H	4H
	4-	3',4',5',7-			
4,4'-Diacetyl	7.91	7.72	7.1-7.6	4.78	3.79
4,7-Diacetyl	7.91	7.72	7.1-7.5	4.78	3.82
3',4,4',7-Tetra-acetyl†	7.93	7.73	7.1-7.6	4.75	3.88
3',4,4',5',7-Penta-acetyl†	7.97	7.74	7.1-7.6	4.77	3.92
			$J_{2,3a} + J_{2,3b}^*$	$J_{3a,4} + J_{3b,4}^\dagger$	
4,4'-Diacetyl			13.1	16.5	
4,7-Diacetyl			13.4	16.5	
3',4,4',7-Tetra-acetyl†			13.0	15.2	
3',4,4',5',7-Penta-acetyl†			12.8	14.8	

* From 2-proton.

† From 4-proton.

‡ These compounds have been shown to be 2,4-*cis*-flavan-4-ols (Lillya, Drewes & Roux, 1963).

1963), 400 mg. of flavanone absorbing 73 ml. of hydrogen. The white amorphous solid obtained consisted of two compounds, R_F 0.45 and 0.67 in 2% (v/v) acetic acid. The latter gave a blue colour with 2,6-dichloroquinonechloroimide, compared with a purple of the former flavan-4-ol. The flavan-4-ol gave a purple with toluene-*p*-sulphonic acid. The flavan-4-ol was separated by preparative paper chromatography in 2% (v/v) acetic acid, but failed to crystallize from water (white sludge or ethanol-water as before; cf. Roux & de Bruyn, 1963). The colour reactions of the compound to heat, light and mineral acid are listed in Table 1.

Aging (1 month) of the compound in solid form in indirect sunlight gave a pink product that still gave a purple-red with toluene-*p*-sulphonic acid, but which had polymerized (low R_F streak in 2%, v/v, acetic acid). The acetate of the polymer was examined by nuclear-magnetic-resonance spectrometry, showing the complete absence of the methyl proton signal due to the 4-acetyl group at τ 7.91 (cf. Table 2).

Generation of butinidin chloride from

($\pm$)-3',4',7-trihydroxyflavan-4 β -ol

($\pm$)-3',4',7-Trihydroxyflavan-4 β -ol (Roux & Paulus, 1961) (30 mg.), was dissolved in 1.2 ml. of propan-2-ol and 0.3 ml. of 3N-HCl in the cold, in a sealed pressure tube. The solution was heated at 60° for 1-3 min., when a deep purple-blue characteristic of flavan-4-ols (when substituted in at least the 4'- and 7-positions) developed. The tube was cooled in ice immediately and the solution examined by paper chromatography on Whatman no. 1 paper with synthetic butinidin chloride (3',4',7-trihydroxyflavylium chloride) (Freudenberg & Maitland, 1934) and robinetinidin chloride (3,3',4',5',7-pentahydroxyflavylium chloride) (Roux & Freudenberg, 1958) as reference compounds. The chromatogram was developed in the highly selective 3N-HCl-90% formic acid (1:1, v/v) solvent system (Roux, 1957). Butinidin chloride, R_F 0.48 (robinetinidin chloride, R_F 0.26), separated as a significant component from the purple-blue products. The synthetic and generated butinidin chlorides had the same orange, $\lambda_{\max}$ 490 m μ , and, when fumed with ammonia, the same mauves, $\lambda_{\max}$ 540 m μ . Both formed complexes readily with potassium alum, forming mauve lakes, $\lambda_{\max}$ 525 m μ .

However, the predominant component generated from the flavan-4-ol separated as a mauve streak centred mainly at R_F 0.34. The mauve component gave no typical anthocyanidin reactions and appeared to be stable under these conditions of chromatography.

DISCUSSION

Pronounced redness develops in 2,4-*cis*-flavan-4-ol (I) and 2,3-*trans*-flavan-3,4-*trans*-diol analogues (II) with 3',4',7-trihydroxy and 3',4',5',7-tetrahydroxy patterns of phenolic substitution by their exposure to indirect sunlight and by heating at 100° under vacuum over phosphorus pentoxide. The further observation that this phenomenon is absent in the corresponding flavan (III) and 2,3-*trans*-flavan-3-ol analogues (IV) suggests that in flavan nuclei the 4 β -hydroxyl group may be correlated with reddening under neutral conditions (Roux & Paulus, 1961, 1962; Roux, 1963a). However, the methyl ethers of these flavan-4 β -ols and flavan-3,4-diols show no chromogenic properties under similar conditions (Roux, 1963b), and from this may be inferred that free phenolic hydroxyl groups are contributory to redness in the presence of the 4 β -hydroxyl group.

Synthetic 4',7-dihydroxy-2,4-*cis*-flavan-4-ol (V, R = H) and 4',7-dihydroxy-2,3-*trans*-flavan-3,4-*trans*-diol (V, R = OH) (their stereochemistry is inferred from the method of synthesis) also redden rapidly at 80° under vacuum over phosphorus pentoxide, at 130-140° in air, and when exposed to indirect sunlight (Roux & de Bruyn, 1963), showing that either the 4'- or 7-hydroxyl group or their simultaneous presence is required. Decision between these alternatives is now possible through synthesis of the free phenolic forms of 7-hydroxy-2,4-*cis*-flavan-4-ol (VI, R = OH, R' = H) and 4'-hydroxy-2,4-*cis*-flavan-4-ol (VI, R = H, R' = OH). Study of their chromogenic properties (Table 1)

shows that the simultaneous presence of 7- and 4-hydroxyl groups is alone responsible for redness induced under neutral conditions. Nevertheless, although 4'-hydroxy-2,4-*cis*-flavan-4-ol does not redden, it is evident from comparisons of more highly substituted analogues (Table 1) that the substitution pattern on the 2-phenyl group affects the shade of colour generated in indirect sunlight. The colour gradation varies from mauve-red or blue (3',4',5'-trihydroxy) to red (3',4'-dihydroxy), pink (4'-hydroxy) and orange-red (unsubstituted) in the presence of both 4- and 7-hydroxyl groups. A similar but less sharply defined trend is obtained on heating (Table 1).

The above suggests that a benzylic 4-hydroxyl group, activated through the presence of the 7-hydroxyl group, is required for redness resulting from both sunlight and heat. Reddening of a flavan-4-ol is probably the consequence of the elimination of the elements of water from the 3- and 4-positions, followed by oxidation of the 2-proton of the resulting flav-3-ene to form traces of the anthocyanidin. In the middle pH range the anthocyanidin will most likely exist in the preferred (cf. Arora, Jain & Seshadri, 1962; Jurd & Geissman, 1963) 7-keto form of the anhydro-base (VII), and its colour will be affected by the pattern of substitution in the 2-phenyl ring, as observed. Less

Scheme 1

likely is the alternative of the elimination of the elements of water from the 7- and 4-position to form quinonemethene structures (VIII). Here the substituent hydroxyl groups on the 2-phenyl ring are isolated from the chromophoric group.

Partial reactions, similar to the former, are probably responsible for the pronounced but not intense redness or pink induced in wattle-bark and wood tannins (*Acacia mearnsii*), and quebracho tannins (*Schnopsis* spp.), which are composed mainly of both monomeric and polymeric forms of leucorobinetinidins (II, R = OH) and leuco-fisetinidins (II, R = H). Mangrove-bark tannins (*Rhizophora* spp.) consisting of polymeric leuco-cyanidins (3',4',5,7-tetrahydroxyflavan-3,4-diol) develop a far more intense red than any of the above condensed tannins.

Apart from the above structural factors, certain stereochemical factors will also affect the degree of reddening. Thus the leuco-anthocyanidin, peltogynol B, with a presumed 4-*axial*-hydroxyl group, unlike peltogynol (presumed 4-*equatorial*-hydroxyl), yields peltogynidin when heated in air. Elimination of the 4-*axial*-hydroxyl group will occur most readily when the 3-H, the 4-OH and the 3- and 4-carbon atoms are coplanar (cf. Chan, Forsyth & Hassall, 1958).

For the free phenolic forms of flavan-3,4-diols and flavan-4-ols, the generation of redness through sunlight or heat under neutral conditions parallels the development of red and red or purple respectively with hydrochloric acid (ethanolic solution) or toluene-*p*-sulphonic acid (on paper chromatograms) (cf. Table 1 and Roux, 1963c; Roux, Maihs & Paulus, 1961). However, the methylated flavan-4 β -ols (also methylated 2,3-*trans*-3,4-*trans*-, 2,3-*trans*-3,4-*cis*- and 2,3-*cis*-3,4-*cis*-flavan-3,4-diols; Drewes & Roux, 1964a, b), which do not develop redness in sunlight, produce red and mainly purple transient colours with these acids. This apparent anomaly is due to the stronger dehydrating action of mineral acids.

The nature of the blue colorations formed from acid treatment of the reduction products of flavanones is unknown, but Geissman & Clinton (1946a, b) have shown from examination of their visible-range spectra that they are not flavylum salts. The generation of butinidin chloride (3',4',7-trihydroxyflavylium chloride) from synthetic ($\pm$)-3',4',7-trihydroxyflavan-4 β -ol, however, indicates that during acid treatment the expected reaction, similar to the well-known conversion of flavan-3,4-diols into 3-oxyflavylium salts, and with mechanism similar to that described above, occurs. Paper chromatography of the reaction products shows that the orange-red of butinidin chloride is masked by the more intense blue due to a conversion product of unknown constitution. Formation

of the latter from flavan-4-ols is undoubtedly associated with the instability of 3-deoxypyranols (Hill, 1936; Arora *et al.* 1962), as only anthocyanidins (23–30 % yield) and flavonols (traces) are formed as recognizable products from flavan-3,4-diols, blue components being absent.

In the synthesis of flavan-4 β -ols, the conversion of chalcone into flavanone may be effected in good yield with the sodium acetate method (cf. Bognar, Farkas & Rakosi, 1962), whereas for 5-deoxyflavanones the isomerization is normally reversible under acid or alkaline conditions. By means of nuclear-magnetic-resonance spectrometry synthetic 4'-hydroxyflavan-4 β -ol and 7-hydroxyflavan-4 β -ol are shown to have a 2,4-*cis* arrangement of substituents, the coupling constants of their 2-, 3- and 4-protons correlating with those of other flavan-4-ols of known 2,4-*cis* arrangement (Table 2), but differing from those of known 2,4-*trans* arrangement (Lillya, Drewes & Roux, 1963). 4',7-Dihydroxyflavan-4 β -ol undergoes remarkably easy self-condensation on aging in the solid form. The conversion is accompanied by reddening and apparent loss of a free 4-hydroxyl group.

SUMMARY

1. The light-red or blue generated in 2,4-*cis*-flavan-4-ols and 2,3-*trans*-flavan-3,4-*trans*-diols by sunlight and heat under neutral conditions is shown to be due to the simultaneous presence of 4- and 7-hydroxyl groups. This conclusion is based on previous work, and on the synthesis and properties of 7-hydroxy- and 4'-hydroxy-flavan-4 β -ols. The shade of colour developed is affected by substitution in the 2-phenyl group.

2. Butinidin chloride, in addition to a deep-blue product of unknown structure, is generated from ($\pm$)-3',4',7-trihydroxyflavan-4 β -ol under acid conditions.

3. The mechanism of colour formation in flavan-4-ols, flavan-3,4-diols and condensed tannins under neutral and acid conditions is discussed.

4. The above synthetic flavan-4 β -ols are shown to have the 2,4-*cis* configuration by nuclear-magnetic-resonance spectrometry.

The nuclear-magnetic-resonance spectra of some of the flavan-4 β -ols were recorded during the tenure of the Charles Bullard Fellowship (1962–1963) at Harvard University by D.G.R. This work is supported by the annual grant of the African Territories Wattle Industry Fund to the Leather Industries Research Institute.

REFERENCES

- Arora, S. K., Jain, A. C. & Seshadri, T. R. (1962). *J. Indian chem. Soc.* **39**, 285.
Bognar, R., Farkas, I. & Rakosi, M. (1962). *Acta chim. Acad. Sci. hung.* **30**, 87.

- Bognar, R., Rakosi, M. & Wheeler, T. S. (1962). *Tetrahedron*, **18**, 135.
- Bokadia, M. M., Brown, B. R. & Cummings, W. (1960). *J. chem. Soc.* p. 3308.
- Chan, W. R., Forsyth, W. G. C. & Hassall, C. H. (1958). *J. chem. Soc.* p. 3174.
- Drewes, S. E. & Roux, D. G. (1964a). *Biochem. J.* **90**, 343.
- Drewes, S. E. & Roux, D. G. (1964b). *Biochem. J.* **92**, 555.
- Freudenberg, K. & Maitland, P. (1934). *Liebigs Ann.* **510**, 193.
- Geissman, T. A. & Clinton, R. O. (1946a). *J. Amer. chem. Soc.* **68**, 697.
- Geissman, T. A. & Clinton, R. O. (1946b). *J. Amer. chem. Soc.* **68**, 706.
- Goel, R. N., Jain, A. C. & Seshadri, T. R. (1958). *Proc. Indian Acad. Sci.* **48**, 180.
- Hattori, S. (1925). *Acta phytochim.* **2**, 99.
- Hill, D. W. (1936). *Chem. Rev.* **19**, 23.
- Jurd, L. & Geissman, T. (1963). *J. Amer. chem. Soc.* **28**, 2394.
- Lillya, C. P., Drewes, S. E. & Roux, D. G. (1963). *Chem. & Ind.* p. 783.
- Nadkarni, D. R. & Wheeler, T. S. (1938). *J. chem. Soc.* p. 1320.
- Roux, D. G. (1957). *Nature, Lond.*, **179**, 305.
- Roux, D. G. (1963a). *Biochem. J.* **87**, 435.
- Roux, D. G. (1963b). *Leder*, **14**, 179.
- Roux, D. G. (1963c). *J. Chromat.* **10**, 473.
- Roux, D. G. & de Bruyn, G. C. (1963). *Biochem. J.* **87**, 439.
- Roux, D. G. & Freudenberg, K. (1958). *Liebigs Ann.* **613**, 56.
- Roux, D. G., Maihs, A. E. & Paulus, E. (1961). *J. Chromat.* **5**, 9.
- Roux, D. G. & Paulus, E. (1961). *Biochem. J.* **80**, 476.
- Roux, D. G. & Paulus, E. (1962). *Biochem. J.* **84**, 416.
- Saiyad, I. Z., Nadkarni, D. R. & Wheeler, T. S. (1937). *J. chem. Soc.* p. 1737.

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Corticosteroids and Ascorbic Acid Transport in Adrenal Cortex *in vitro*

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We have previously reported (Sharma, Johnstone & Quastel, 1963) that the uptake of [1-¹⁴C]-ascorbic acid in guinea-pig adrenal-cortex slices is a process dependent on metabolic energy and inhibited by dinitrophenol and anaerobiosis. The concentration of [1-¹⁴C]ascorbic acid in the tissue may rise to five times that of the medium. Extraction of the tissue with 85% (v/v) ethanol, followed by chromatography and radioautography, showed the presence of a single component whose R_F corresponded to that of ascorbic acid. ACTH,* which depletes the adrenal gland of ascorbic acid *in vivo* (Sayers, Sayers, Liang & Long, 1945, 1946; Long & Fry, 1945), inhibits the active uptake of [1-¹⁴C]-ascorbic acid in guinea-pig adrenal-cortex slices *in vitro*. Moreover, the inhibition was obtained only under conditions leading to adrenal-steroid synthesis. In the absence of glucose, ACTH had no effect on ascorbic acid uptake. It is already well established that added glucose is required to obtain enhanced steroid synthesis by adrenal-cortical tissue *in vitro* in the presence of ACTH (Schonbaum, Birmingham & Saffran, 1956). Adenosine 3',5'-monophosphate, which stimulates adrenal steroid synthesis *in vitro* (Haynes, Koritz & Peron, 1959; Birmingham, Kurlents, Lane, Muhlstock & Traikov, 1960), also inhibited the uptake of [1-¹⁴C]-

ascorbic acid (Sharma *et al.* 1963). As corticosteroids inhibit ascorbic acid uptake (Sharma *et al.* 1963), we investigated further the nature and the specificity of the effects of steroids on ascorbic acid uptake.

MATERIALS AND METHODS

Adrenal-cortex slices. The preparation of guinea-pig adrenal-cortex slices and the incubation procedure that we have used are fully described by Sharma *et al.* (1963). The incubations were carried out in Krebs-Ringer phosphate containing NaCl (134 mM), KCl (5.2 mM), KH_2PO_4 (1.3 mM), MgSO_4 (1.3 mM), CaCl_2 (2.8 mM) and sodium phosphate buffer, pH 7.4 (10 mM). Glucose (10 mM) and thiourea (1 mM) were also added. All experiments were run in duplicate.

Adrenal-cortex homogenate. Adrenal-cortex slices were homogenized in ice-cold Krebs-Ringer phosphate solution (400 mg. wet wt. of tissue in 20 ml. of Krebs-Ringer phosphate) with a Teflon-pestle homogenizer. A 0.5 ml. sample of the homogenate was added to each Warburg manometric vessel. The total volume used was 4.0 ml.

Influx experiments. The influx of [1-¹⁴C]ascorbic acid was measured as described by Sharma *et al.* (1963). When measuring uptake of [1-¹⁴C]ascorbic acid it is necessary to add thiourea (1 mM) to the incubation medium to prevent oxidation of ascorbic acid in the adrenal tissue. This is not necessary for brain slices.

Efflux experiments. The adrenal-cortex slices, after pre-incubation as described by Sharma *et al.* (1963), were

* Abbreviation: ACTH, adrenocorticotrophic hormone.