Howe, P. E. [1921]. *J. biol. Chem.* **49**, 109.
Jackson, C. M. [1908]. *J. exp. Zool.* **19**, 99.
—— [1932]. *Amer. J. Anat.* **51**, 347.
Lafon, M. [1939]. *Ann. Physiol. Physicochim. biol.* **15**, 1.
Osborne, T. B. & Mendel, L. B. [1912]. *J. biol. Chem.* **12**, 473.
—— —— [1914]. *J. biol. Chem.* **17**, 325.
—— —— [1916]. *J. biol. Chem.* **25**, 1.
—— van Slyke, D. D., Leavensworth, S. & Vinograd, M. [1915]. *J. biol. Chem.* **22**, 259.
Pearson, P. B. [1937]. *Amer. J. Physiol.* **118**, 788.
Pearson, P. B., Elvehjem, C. A. & Hart, E. B. [1937]. *J. biol. Chem.* **119**, 749.
Rimington, C. [1942]. *Brit. med. J.* **1**, 177.
Rose, W. C. [1938]. *Physiol. Rev.* **18**, 109.
—— & Eppstein, S. H. [1939]. *J. biol. Chem.* **127**, 677.
Schoenheimer, R. & Rittenberg, D. [1940]. *Physiol. Rev.* **20**, 218.
Vickery, H. B. [1938]. *C.R. Lab. Carlsberg (sér. chim.)*, **22**, 519.
Winnick, T. [1942]. *J. biol. Chem.* **142**, 461.
Winters, J. C., Smith, A. H. & Mendel, L. B. [1927]. *Amer. J. Physiol.* **80**, 577.

The Mechanism of Coprosterol Formation *in vivo*

1. CHOLESTENONE AS AN INTERMEDIATE

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In view of the inherent improbability that the organism is able to convert cholesterol by direct hydrogenation into its *cis*-decalin derivative coprosterol, it has been suggested that the reaction proceeds in two stages [Rosenheim & Starling, 1933]. According to this view cholestenone, the intermediate oxidation product of cholesterol, is subsequently reduced in the intestine to coprosterol and partly to *epi*-coprosterol.

Although feeding experiments with cholestenone have since provided considerable experimental evidence for the correctness of this view [Rosenheim & Webster, 1935; Schoenheimer, Rittenberg & Graff, 1935; Rosenheim & Starling, 1937; Anchel & Schoenheimer, 1938], the final proof of the occurrence of cholestenone itself in the animal organism or in its excreta was still lacking. The difficulties of isolating such small amounts of cholestenone as escape complete reduction from the faeces of animals kept on an ordinary mixed diet, which is poor in cholesterol, are obvious. In our search for cholestenone we have, therefore, made use of the fact that brain contains a substance, the administration of which enables the organism to convert large amounts of cholesterol into coprosterol [Rosenheim & Webster, 1941]. By employing Girard and Sandulesco's ketone reagent, we have, under such conditions, been able to isolate cholestenone from the faeces of a dog and of rats fed on brain, and to characterize it both chemically and physically.

The isolation of cholestenone from faeces after feeding on brain necessitated a search for its possible occurrence in brain itself. The ultra-violet absorption spectra of cholesterol fractions obtained from various organs had been examined by Page & Menschick [1930 *a*, *b*; 1931], who were unable to find the characteristic absorption band of cholestenone at 2400 A, in cholesterol prepared from normal brain. Their results do not, however, exclude the presence of traces of cholestenone which would have been removed by the solvents used in the preparation of the various cholesterol fractions. We therefore subjected brain to the treatment which led to the isolation of cholestenone from faeces. Although we obtained evidence that traces of a substance absorbing at 2380 A occur in brain, we were unable to characterize it as cholestenone. In view of its small amount, the possibility that this unidentified substance may be the source of the relatively large amount of cholestenone isolated from faeces after the administration of brain must be considered as remote.

Whilst this work was in progress, Marker, Wittbecker, Wagner & Turner [1942] succeeded in isolating *epi*-coprosterol from the faeces of a dog on a normal diet, thus bringing further support to the view that cholestenone is an intermediate in the formation of coprosterol in the organism.

EXPERIMENTAL

Cholestenone from rats' faeces. Rats of our Institute strain of Wistar rats and of the average weight of 200 g. were used. Six rats were kept on a diet of steamed sheep's brain and bone meal for 4 days, each rat consuming *ca.* 30 g./day of the diet. The housing of the animals, the preparation of the diet and the collection of the faeces have been described previously [Rosenheim & Webster, 1941].

The faeces of the last 3 days (140 g. moist) were collected, dried *in vacuo* over conc. H_2SO_4 and exhaustively extracted with ether in a Soxhlet. The lipids (23·5 g.) yielded on saponification 15·2 g. of unsaponifiable matter, which was dried in a high vacuum. 14 g. of the unsaponifiable fraction in acetic acid-ethanol solution were treated with 5 g. of the

'T' reagent of Girard & Sandulesco [1936]. The ketonic fraction, a yellowish oil weighing 0·4596 g., was dissolved in 25 ml. ethanol and its ultra-violet absorption examined. The absorption curve plotted from a 1:1000 dilution showed the characteristic absorption band of cholestenone with a well-defined maximum at 2400 A, the intensity of which, on comparison with a standard curve, indicated 0·144 g. cholestenone. The ketonic fraction, therefore, contained 31% and the unsaponifiable matter 1% cholestenone. In several similar experiments spectroscopic examination of the unsaponifiable fractions indicated variations of from 1 to 1·9% cholestenone.

The identity of the absorbing substance with cholestenone was established by the preparation of its well-crystallized *o*-tolylsemicarbazone [Rosenheim & Starling, 1937]. The solution of the ketonic fraction was mixed with a solution of 200 mg. of *o*-tolylsemicarbazide in ethanol containing 5 drops of acetic acid. After 24 hr. the semicarbazone had separated in hard, slightly yellowish aggregates of needles, weighing 0·2054 g., m.p. 238–239°, corresponding to 0·148 g. cholestenone. The m.p. rose to 240–241° after one recrystallization from butanol. A further recrystallization from dioxan gave colourless clusters of needles of cholestenone-*o*-tolylsemicarbazone, m.p. 242–243°, not depressed in admixture with an authentic specimen. Found: N, 7·9%. Calc. for $C_{35}H_{53}ON_3$: N, 8·0%.

Cholestenone from dogs' faeces. Our previous failure to induce coprosterol excretion in dogs fed on raw brain, whilst a diet of cooked brain, given to rats, raised the excretion of coprosterol to 80% of the faecal sterols [Rosenheim & Webster, 1941], led us to repeat the experiment with dogs fed on cooked, instead of on raw, brain. A male Airedale terrier, weighing 16 kg., under the care of our colleague, Dr G. L. Brown, received steamed sheep's brain mixed with 4% bone meal as his sole food for 4 days, and consumed *ca.* 350 g. brain/day. The faeces (310 g. moist) of the last 3 days, dried *in vacuo* and extracted with ether, yielded on saponification of the ether residue 25·2 g. of unsaponifiable matter as a semi-crystalline mass. On analysis by the gravimetric digitonin method, it was found to contain 16·4 g. sterols, of which 80·5% consisted of coprosterol. The cause of the strikingly different results obtained on feeding raw and cooked brain remains unexplained.

A solution of 20 g. of the unsaponifiable fraction was treated with the Girard-Sandulesco reagent as above described and yielded 0·810 g. of a ketonic fraction. The ultra-violet absorption curve of the latter in alcoholic solution showed a sharply defined maximum at 2400 A, indicating 0·203 g. cholestenone on comparison with a standard curve. The cholestenone content of the unsaponifiable fraction was, therefore, 1% and of the same order as in the case of the rats. The *o*-tolylsemicarbazone was prepared by the method above described and crystallized in colourless needles, m.p. 242–243°. Found: N, 8·2%. Calc. for $C_{35}H_{53}ON_3$: N, 8·0%.

Coprosterol from dogs' faeces. The non-ketonic fraction yielded 9·6 g. coprosterol, m.p. 98–100° on a single recrystallization from acetone-methanol solution. Coprosterol may be conveniently prepared directly from the faeces of dogs fed on cooked brain by mixing them with anhyd. $CaSO_4$ and extracting the dry product several times at room temperature, with acetone (cf. preparation of cholesterol from brain [Rosenheim, 1906]). The extracts are passed through a column of norit, freed from solvent and the colourless residue recrystallized from a mixture of acetone and methanol (2:1). This procedure, which is based on the fact that coprosterol occurs unesterified in faeces, yields a cleaner product than the usual method in which the lipidal impurities, introduced by the saponification of the primary ether extracts, complicate the purification of coprosterol.

Search for cholestenone in brain. 500 g. of minced ox brain were mixed with 1500 g. anhyd. $CaSO_4$. The hardened small lumps were extracted with 1200 ml. acetone at room temperature for 24 hr. and the extraction repeated 6 times. The extracts were taken to dryness *in vacuo* and the crystalline residue saponified in ethereal solution with an ethanolic solution of sodium ethoxide at room temperature. The ethereal solution, freed from soaps, was washed with dilute H_2SO_4 and H_2O. The crystalline residue (11·4 g.), obtained after removal of ether, was dried in a high vacuum and treated with the Girard-Sandulesco reagent as above described. Only traces of an oily ketonic fraction, weighing 28 mg., were obtained. Spectroscopic examination revealed a sharply defined absorption band with a maximum at 2380 A, the intensity of which, calculated as cholestenone, would indicate 7 mg. The identity of the absorbing substance with cholestenone, however, is doubtful since its characteristically insoluble *o*-tolylsemicarbazone could not be obtained. The suggestion, that the unidentified substance present in the calcified wall of the aorta and absorbing at the same wave-length [Page & Menschick, 1930*a*] may also occur in brain, may have to be considered. The fact that this substance is precipitable by digitonin [Schoenheimer, 1932] excludes its identity with cholestenone.

SUMMARY

The occurrence of cholestenone, characterized by its ultra-violet absorption and the preparation of cholestenone-*o*-tolylsemicarbazone, in the faeces of the dog and of the rat, after feeding on brain, has been demonstrated. The view that cholestenone is an intermediate in the conversion of cholesterol into coprosterol by the animal organism thus receives further confirmation.

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REFERENCES

Anchel, M. & Schoenheimer, R. [1938]. *J. biol. Chem.* **125**, 23.
Girard, A. & Sandulesco, G. [1936]. *Helv. chim. Acta*, **19**, 1095.
Marker, R. E., Wittbecker, E. L., Wagner, R. B. & Turner, D. L. [1942]. *J. Amer. chem. Soc.* **64**, 818.
Page, I. H. & Menschick, W. [1930*a*]. *Naturwissenschaften*, **18**, 585.
—— —— [1930*b*]. *Naturwissenschaften*, **18**, 735.
—— —— [1931]. *Biochem. Z.* **231**, 446.
Rosenheim, O. [1906]. *J. Physiol.* **34**, 104.
—— & Starling, W. W. [1933]. *Chem. Ind.* **48**, 238.
—— —— [1937]. *J. chem. Soc.* p. 377.
—— & Webster, T. A. [1935]. *Nature, Lond.*, **136**, 474.
—— —— [1941]. *Biochem. J.* **35**, 920.
Schoenheimer, R. [1932]. *Hoppe-Seyl. Z.* **211**, 65.
—— Rittenberg, D. & Graff, M. [1935]. *J. biol. Chem.* **111**, 183.