was refluxed for 16 hr. and then cooled. Dilution with water precipitated needles (5.19 g.) which were identified as **1-(4-biphenylyl)cyclopentanecarboxamide (IVd)**, m.p. 149–151° after one crystallization from acetone. An analytical sample melted at 150–151°; λ_{max} 257 mμ (ε 23,400); $\nu_{max}^{CS_2}$ 1692 (amide) cm.$^{-1}$.

Anal. Calcd. for $C_{18}H_{19}NO$: C, 81.47; H, 7.22; N, 5.28. Found: C, 81.80; H, 7.20; N, 5.28, 5.27.

The above filtrate was extracted with ether and acidification of the aqueous solution gave only traces of acidic material. The combined ether extracts were washed with water, dried, and evaporated to dryness leaving a yellow amorphous solid (2.62 g.). This solid was suspended in a 5% aqueous solution (50 ml.) of sodium hydroxide and refluxed for 1 hr. After cooling, the undissolved solid (crude amide) was separated by filtration and the filtrate was acidified with dilute hydrochloric acid. Extraction with ether and working up in the usual way gave **1-(4-biphenylyl)-cyclopentanecarboxylic acid (IVc)** as a white solid (0.21 g.) which melted at 187–189° after one crystallization from aqueous methanol. An analytical sample melted at 200–201°; λ_{max} 256 mμ (ε 24,200); $\nu_{max}^{CS_2}$ 1695 (carboxylic acid) cm.$^{-1}$

Anal. Calcd. for $C_{18}H_{17}O_2$: C, 81.15; H, 6.87. Found: C, 80.79; H, 6.98.

Acknowledgment.—The authors gratefully acknowledge the technical assistance of Mr. R. Luz and Mr. R. Guthrie and thank Dr. G. Papineau-Couture and his associates for analytical and spectral data. They also wish to express their appreciation to Dr. D. Dvornik and to Dr. L. G. Humber for helpful suggestions and discussions during the course of this work.

A Correlation of Drug Concentration with Sterol Biosynthesis Inhibition in the Liver[1]

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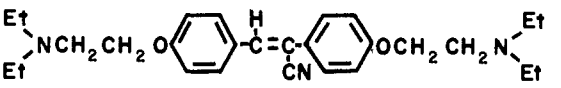
The time course of sterol biosynthesis inhibition of two 2,3-diphenylacrylonitriles which block the conversion of desmosterol to cholesterol is compared with triparanol in orally treated rats. The data thus obtained are correlated with drug concentration in the liver and with the effects of these compounds on sterol biosynthesis *in vitro*.

The liver is regarded as a primary site for the synthesis as well as the metabolism of cholesterol.[2] That a feed-back mechanism is operative in the liver has been shown by cholesterol-feeding experiments which produce higher than normal concentrations of cholesterol in the liver. These livers have a reduced capacity to synthesize new cholesterol.[3,4] Chemical agents also affect liver cholesterol. Estrogens which effectively lower serum cholesterol have been shown to alter both liver sterol content and the ability of these livers to synthesize cholesterol.[5,6] Triparanol, a sterol biosynthesis inhibitor, does not materially change liver sterol concentration, but does alter liver sterol composition. Desmosterol, which is present in minute amounts in normal rat liver, replaces much of the cholesterol in the livers of triparanol-treated animals.[7,8]

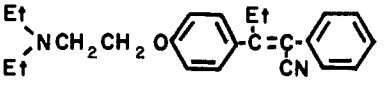
In the course of studies of sterol biosynthesis inhibitors[9] that block the conversion of desmosterol to cholesterol, we became interested in correlating drug concentration at the site of action with degree of inhibition. By administering a labeled precursor to animals pretreated with the drug we hoped to determine the extent of biosynthesis inhibition by measuring the relative amounts of labeled desmosterol and cholesterol in the liver and to measure at the same time the drug content of the liver. This report describes an experiment in which the drug content of the liver was correlated with its effect as indicated by the comparison of radioactive liver sterols following mevalonate-2-C^{14} injection. For this purpose two compounds, 2,3-bis[*p*-(2-diethylaminoethoxy)phenyl]acrylonitrile (I) and *trans*-3-[*p*-(2-diethylaminoethoxy)phenyl]-2-phenyl-2-pentenenitrile (II),[9] were compared with triparanol (Chart I).

CHART I

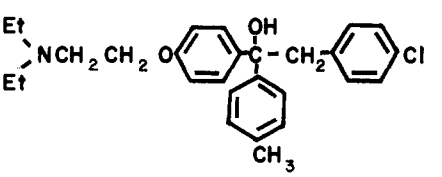
I.



II.



TRIPARANOL



(1) Based on a presentation at the 142nd National Meeting of the American Chemical Society, Atlantic City, N. J., Sept. 10, 1962.

(2) D. Kritchevsky, "Cholesterol," John Wiley and Sons, Inc., New York, N. Y., 1958, pp. 75, 118.

(3) I. D. Frantz, Jr., H. S. Schneider, and B. T. Hinkleman, *J. Biol. Chem.*, **206**, 465 (1954).

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(5) G. S. Boyd and W. B. McGuire, *Biochem. J.*, **62**, 19p (1956).

(6) G. S. Boyd, *Federation Proc.*, **21**, 86 (1962).

(7) W. M. Stokes and W. A. Fish, *J. Biol. Chem.*, **235**, 2604 (1960).

(8) J. Avigan, D. Steinberg, H. E. Vroman, M. J. Thompson, and E. Mosettig, *ibid.*, **235**, 3123 (1960).

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Results and Discussion.—A relative order of activity within a group of diphenylacrylonitriles has been established from their ability to reduce the apparent plasma cholesterol of rats.[9] Determinations of degree of inhibition of sterol synthesis in rat liver homogenates by several members of this group gave similar results. The *in vitro* effect of drug concentration on the relative amounts of cholesterol and desmosterol synthesized from mevalonate in these experiments is shown in Fig. 1. A comparison of the drugs at the concentration that produced 50% inhibition of the conversion of desmosterol to cholesterol indicates that I is one-third as active as triparanol and II is approximately 4 times as active as triparanol. These *in vitro* relative activities are in general agreement with the data obtained *in vivo;* however, the observed difference (II is 0.9 times triparanol) undoubtedly results from absorption and transport of drug in feeding experiments.

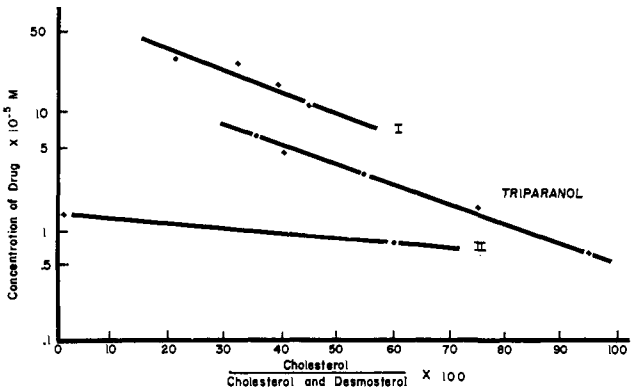

Fig. 1.—*In vitro* dose response comparison of triparanol with I and II. Inhibition of the conversion of desmosterol to cholesterol is expressed as the per cent of radioactive cholesterol found in the total radioactive sterols (cholesterol and desmosterol).

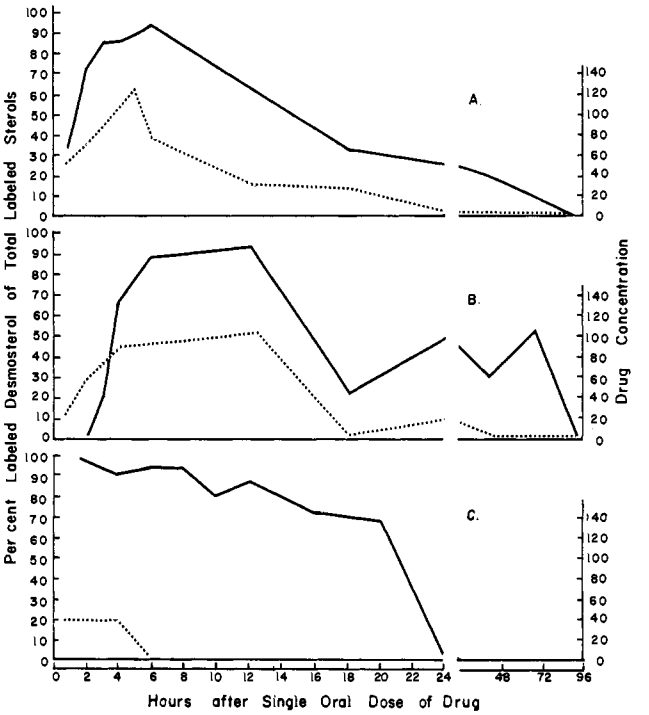

Fig. 2.—Correlation of liver drug content with biosynthetic inhibition: A, triparanol; B, compound I; C, compound II. The solid line shows the per cent of radioactive desmosterol found in the total radioactive sterols, and is read from the scale at the left; the dotted line is read from the right and is recorded as micrograms of drug per gram of liver.

The time course of biosynthetic block was obtained by analyzing the liver sterols derived from labeled mevalonic acid at various times after drug administration. The proportion of labeled desmosterol in the labeled liver sterols then serves as a direct measure of biosynthesis inhibition. Inhibition of sterol biosynthesis (as indicated by the labeled desmosterol content of the total labeled liver sterols) and the concentration of the drug in the liver plotted as a function of time following a single 40 mg./kg. dose of drug, are shown in Fig. 2. At 1 hr., the first period examined, triparanol was found in the liver and at that time desmosterol constituted 35% of the radioactive sterols. The drug content of the liver increased quite rapidly and reached a peak at 5 hr. The peak of sterol biosynthesis inhibition was reached at approximately the same time, at which point the conversion of desmosterol to cholesterol was almost completely blocked. Thereafter the level of drug in the liver decreased rapidly as did the effect on biosynthesis (Fig. 2A).

The results of a similar experiment with the bisether (I) is shown in Fig. 2B. The concentration of I in the liver builds up more slowly than does triparanol, reaching a maximum concentration at 4–5 hr. Maximum drug concentration in the liver also results in practically complete block of the conversion of desmosterol to cholesterol. This state was maintained from the fourth to the twelfth hour after which drug content and biosynthesis inhibition declined. At 24 hr. there was an apparent increase of drug concentration and blocking effectiveness. Although the cause of this phenomenon is not known, the formation of an active metabolite cannot be excluded.

Treatment of rats with 40 mg./kg. of II resulted in almost complete inhibition of the reduction of desmosterol to cholesterol at the second hr. as shown in Fig. 2C. The effect then diminished slowly until the 20th hr., after which it declined rapidly. Correlation of the biosynthesis effect with the presence of the drug in the liver in this instance, however, was poor. The drug was present in the liver for the first 4 hr. at levels of about 20 γ/g. Thereafter none could be detected by an analytical method sensitive to 3 γ/g. The reason for this pronounced biological effect in the absence of detectable concentrations of the administered drug have not been established, but a reasonable suggestion is that II is rapidly transformed *in vivo* to a chemical species not detectable by the assay employed for the parent compound.

In the course of these experiments we have observed sterol biosynthesis inhibition resulting from known liver concentrations of a drug both *in vivo* and *in vitro*. Although a direct comparison of the amount of drug required to produce a specific degree of inhibition in each experiment is not strictly valid, it is still interesting to obtain this comparison. It should be remembered, however, that the form in which a drug is presented to an enzyme system *in vivo* is probably quite different from the mechanics of *in vitro* experiments. The lack of similarity of drug presentation is

further complicated by absorption, transport, and possibly metabolism effects encountered *in vivo*.

Expressing drug concentration in the *in vitro* experiment in terms of the weight of tissue used to prepare the homogenate, a concentration of 60 γ of triparanol/g. of liver *in vitro* results in 50% inhibition of the reduction of desmosterol to cholesterol (Fig. 1). This can be compared with the 90% inhibition produced *in vivo* at a concentration of 100 γ g. (Fig. 2). On this basis of comparison, I was substantially more active *in vivo* than *in vitro*. A concentration of 100 γ of I g. of liver *in vivo* achieved 90% inhibition of biosynthesis, whereas *in vitro* 180 γ g. inhibited the biosynthetic reaction only about 50%.

Experimental

General Method for Isolation and Identification of Desmosterol in Rat Liver Sterols.—The livers from 6 rats were blotted to remove excess moisture, combined (total weight approximately 45 g.), and refluxed vigorously for 1 hr. in a solution of 10% potassium hydroxide in 50% aqueous ethanol. For each gram of wet liver, 3 ml. of solution was used. After cooling, the reaction mixture was diluted with an equal volume of water and extracted with three 150-ml. portions of distilled hexane. The hexane in turn was washed with 1% sodium hydroxide, water, dried over sodium sulfate, and concentrated to dryness *in vacuo*. The resulting nonsaponifiable fraction (approximately 150–200 mg.) was chromatographed on a deactivated alumina column[10] designed to separate sterols from other nonsaponifiable residues. An aliquot of the benzene eluate, which contains approximately 80 mg. of rat liver sterols, was assayed by paper chromatography (see below) with desmosterol[11] and cholesterol controls. The amount of desmosterol present was estimated visually by comparison with known amounts of control sterols. At intervals, rat liver sterol composition was verified by gas–liquid chromatography,[12] and in general good agreement was obtained with the method of visual estimation.

By the procedure thus described, control (untreated) rat liver contains 1.98 ± 0.12 mg. of sterol/g. of wet liver. The sterol is pure cholesterol. A typical result of a group of 6 rats receiving 40 mg./kg. of triparanol for 6 days is 1.97 mg. sterol/g. of wet liver; the composition of the sterols was 50% cholesterol and 50% desmosterol.

In Vivo **Experiments.**—Pairs of Charles River Sprague–Dawley male rats each weighing 150 g. were given a single oral 40 mg./kg. dose of drug and sacrificed at intervals thereafter. Two hr. prior to sacrifice each animal received an intraperitoneal injection of 2 μc. of sodium DL-mevalonate-2-C^{14}. Rats that were to be sacrificed at 1 and 2 hr. received drug and mevalonate simultaneously. At sacrifice the livers were removed, combined, weighed, and homogenized in 10 ml. of water. One aliquot was assayed for sterols, and another aliquot was assayed for the drug. The sterol analysis was carried out by adding 2.5 ml. of 10% potassium hydroxide in 50% aqueous ethanol to each ml. of homogenate, which was then refluxed under nitrogen for 1.5 hr. The petroleum ether extractable, nonsaponifiable fraction was chromatographed on deactivated alumina.[10] An aliquot of the fraction eluted with benzene, containing cholesterol and desmosterol, was chromatographed in a system consisting of Whatman No. 1 paper impregnated with a 15% solution of 2-phenoxyethanol in acetone, and developed in hexane saturated with 2-phenoxyethanol for 3 hr. The chromatogram was dried for 1 hr. at 100°, sprayed with 10% phosphomolybdic acid in ethanol, and heated at 180° for 3 min.[13]

The papergrams were scanned for radioactivity with suitable controls and sectioned into thin strips. Individual strips were extracted with 15 ml. of standard toluene scintillator and counted in a Packard Tri-Carb liquid scintillation counter.

Calibration curves for drug assays were constructed by adding known amounts of drug to liver homogenates, followed by extraction and spectrophotometric assay. A typical procedure is described. All extractions and washings were carried out by mechanical shaking for 30 min. followed by centrifugation. Increasing amounts of drug stock solution were added to 5 identical aliquots of rat liver homogenized in water. The homogenate was made basic by the addition of 10 *N* sodium hydroxide solution and extracted with 60 ml. of hexane containing 1.5% isoamyl alcohol. Fifty ml. of the hexane extract was washed with 10 ml. of pH 8 buffer, then a 25-ml. portion of the hexane was extracted with 5 ml. of 0.1 *N* hydrochloric acid. The acid solution was read at the appropriate wave length in a Beckman DU spectrophotometer, and the observed optical density plotted against increasing concentration. Satisfactory results were obtained for all assays. Triparanol was assayed similarly by conversion to the anhydro derivative.[14]

The relevant spectral data are: I, $\lambda_{max}^{0.1\ N\ HCl}$ 335 mμ (ϵ 23,500); II, $\lambda_{max}^{0.1\ N\ HCl}$ 285 mμ (ϵ 14,500); triparanol, $\lambda_{max}^{0.1\ N\ HCl}$ 314 mμ (ϵ 16,200).

In Vitro **Experiments.**—Incubations were carried out as described by Bucher and McGarrahan.[15] Each flask contained 1.5 μc. of sodium DL-mevalonate-2-C^{14} of specific activity 3.3 mc./mmole. The total volume of each incubation was 3.5 ml. After a 3-hr. period of incubation, the mixture was saponified by the addition of 6 ml. of 15% alcoholic KOH and allowed to stand for 17 hr. at 60°. The sterols were extracted with hexane, isolated by chromatography on alumina, separated by paper chromatography, and counted as described in the *in vivo* experiments. Two flasks were averaged for each point. The data are plotted with the per cent of radioactive cholesterol (of the total labeled sterols, cholesterol, and desmosterol) as a function of the log dose (Fig. 1).

(10) P. B. Schneider, R. B. Clayton, and K. Bloch, *J. Biol. Chem.*, **224**, 175 (1957).

(11) Desmosterol was synthesized in these laboratories according to the method of U. H. M. Fagerlund and D. R. Idler, *J. Am. Chem. Soc.*, **79**, 6473 (1957).

(12) Gas–liquid chromatography of sterols was determined by the method of S. R. Lipsky and R. A. Landowne *Anal. Chem.*, **33**, 818 (1961).

(13) We wish to thank Mr. M. J. Lynch for developing this chromatographic system. The full details of this as well as additional systems for separating cholesterol and desmosterol will be submitted by Mr. Lynch to *J. Chromatography*.

(14) G. Gould, *Progr. Cardiovascular Diseases*, **2**, 492 (1960).

(15) N. L. R. Bucher and K. McGarrahan, *J. Biol. Chem.* **222**, 1 (1956).