should have an energy barrier above 70 cal./mole less than the corresponding protium–protium barrier. In cyclohexane, the 1,3–1,5-hydrogen axial interactions should be at least as large as the observed effect in cycloheptatriene and therefore relative to protium, deuterium should be more stable in the axial position (enthalpy contribution) by 140 ± 30 cal./mole.

The data support the equilibria of Ia and IIa and of Ib and IIb. No evidence was found indicating the presence of norcaradiene (III). If III were in equilibrium with Ia, the hydrogens at C-1 and C-6 would appear further upfield, the equilibrium constant would not be unity, and there would be a change in the vinyl region with temperature. (The vinyl hydrogens show no change in chemical shifts from room temperature to $-165°$.)

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(11) Sloan Foundation Fellow, 1961–1965.

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Phytolaccagenin: A Light Atom X-Ray Structure Proof Using Chemical Information

Sir:

In 1949, Ahmed, Zufall, and Jenkins[1] reported the isolation, by a long and tedious process, of the toxic principle of pokeroot, *Phytolacca americana* L. They followed their enrichment by biological testing and proposed for their final product the formula $C_{55}H_{90}O_{22}\cdot 2H_2O$, suggesting that it was a steroidal glycoside. We have repeated the isolation by a simplified, but still tedious, route and obtained the same material, for which we propose the name phytolaccatoxin.

Hydrolysis of phytolaccatoxin in either methanol–hydrochloric acid or dioxane–hydrochloric acid gives glucose and xylose, identified paper chromatographically, and a crystalline aglycone, m.p. 317–318° dec., $C_{31}H_{48}O_7$, which we have named phytolaccagenin. Chemical studies on phytolaccagenin indicated that it was not a steroid, but rather the monomethyl ester of a trihydroxytriterpene diacid of the β-amyrin series. Because of the large number of functional groups to be located and because of the difficulties involved in the preparation of enough aglycone for extensive chemical investigations, we turned to X-ray methods for the solution of our structural problems.

Attempts to prepare heavy atom derivatives of suitable crystal habit were unsuccessful, all of the products appearing as extremely fine needles. When the amorphous phytolaccagenin triacetate was converted by thionyl chloride to its acid chloride, this treated with β-bromoethylamine, and the acetate groups removed by hydrolysis with potassium carbonate in aqueous dioxane, a product was obtained which crystallized as well-formed prisms of considerable size. Combustion analyses showed, however, that the material was not the β-bromoethylamide, but rather the 2-oxazoline resulting from cyclization and loss of HBr. Despite the absence of a phase-determining heavy atom the favorable crystalline form impelled us to carry out the structure analysis on this derivative.

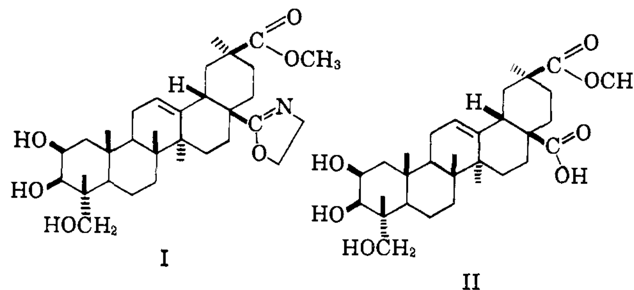
The crystals proved to be orthorhombic, space group $P2_12_12_1$, with axes a = 12.13 Å., b = 13.62 Å., and c = 18.28 Å. and four molecules in the unit cell (mol. wt. calcd., 558; found, 554). Integrated intensities for 3199 reflections were obtained photometrically from Weissenberg films, with 2648 (82.8%) being considered to be "observed." The intensities were reduced to $|F_{rel}|$ in the usual manner and a three-dimensional sharpened, origin removed Patterson function was calculated.

Twenty-nine carbon atoms (C-29 omitted) of the observed structure of ursolic acid[2] were taken as a model of the triterpene skeleton. An extensively modified version of the rotation program of Nordman[3] was then used to fit this model to the Patterson. Because of the approximations which were made to accommodate our larger model and slower (IBM 709) computer, as well as the imperfections in the model, the rotation fitting did not lead to a single, clear-cut orientation but rather to a number of possible ones. Each of these was investigated by taking advantage of the rapidity with which structure factors may be calculated for a model translated as a whole through the unit cell.[4]

The model was extended by adding C-30 (β-amyrin numbering) and by fitting the oxazoline ring about C-28 on the basis of chemical evidence that the free carboxyl group of phytolaccagenin was located at this position. The resulting model, oriented in one of the possible ways, was translated by steps of $^1/_8$ of a cell edge while structure factors were calculated after each step for the seventeen reflections with no index higher than 2. A three parameter least-squares refinement of the molecular location provided for a search of the volume bounded by the stepping points. A complete search in this fashion required less than 2 min.

Only those orientations and positions which appeared promising ($R < 35\%$) at this stage were carried further. For these, the translational search was repeated on smaller blocks about the suggested locations using data with indices to 3 (48 reflections) and 4 (103 reflections). The number of possibilities thinned rapidly and a final check on 195 reflections with a maximum index of 5 showed one set of parameters to be markedly superior.

These parameters gave R = 49% on all of the reflections (620) to $\sin\theta/\lambda$ = 0.35. A number of cycles of structure factor, Fourier, and weighted difference map[2] calculations using data with $\sin\theta/\lambda \leqslant 0.40$ permitted the correction of sizable errors in atomic positions and gradually revealed all of the missing atoms. The final structure appeared as that shown in I, proving phytolaccagenin to be II.



Refinement after the location of all of the atoms was by full matrix least squares on 479 observed reflections selected approximately at random, then by block diagonal least squares using all the data. The residual index R is currently 14.5% over all the observed reflections. Refinement is continuing.

It is important to note in connection with the structural approach outlined above that the correct solu-

(1) Z. F. Ahmed, C. J. Zufall, and G. L. Jenkins, *J. Am. Pharm. Assoc.*, **38**, 443 (1949).
(2) G. H. Stout and K. L. Stevens, *J. Org. Chem.*, **28**, 1259 (1963).
(3) C. E. Nordman and K. Nakatsu, *J. Am. Chem. Soc.*, **85**, 353 (1963).
(4) G. H. Stout, V. F. Stout, and M. J. Welsh, *Tetrahedron*, **19**, 667 (1963).

tion was characterized not by being the best at the early stages of the study, but rather by showing a very slow increase in R as the amount of data was increased. Further studies are planned as to the generality of this behavior.

The unusual features of the phytolaccagenin structure are its high degree of oxidation and the appearance of a carbomethoxy group. Other triterpene esters have been isolated,[5] but their formation has been attributed to esterification during treatment with acidic alcohol. Since phytolaccagenin can be prepared without any exposure to methanol its ester function is apparently of natural origin.

Phytolaccagenin represents one of the largest natural products to be solved without the use of a heavy atom, and its solution exemplifies the growing possibilities for the use of chemical information in the early stages of an X-ray structural analysis.

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(5) C. Djerassi and J. S. Mills, *J. Am. Chem. Soc.*, **80**, 1236 (1958); W. A. Jacobs and O. Isler, *J. Biol. Chem.*, **119**, 155 (1937).

(6) National Science Foundation Cooperative Fellow, 1960–1961.

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Evidence that Cholesterol Sulfate is a Precursor of Steroid Hormones

Sir:

Since the initial observation[1] that conjugation of steroids does not necessarily destine the hormone for excretion, two important findings have supported the idea that intact steroid sulfates may serve as biosynthetic intermediates. In a recent study, Calvin, *et al.*,[2] found that urinary dehydroisoandrosterone sulfate (DS) may be synthesized *in vivo* from pregnenolone sulfate (PS) by a pathway involving intact steroid sulfates as intermediates. They have also shown[3] that a homogenate of hyperplastic tissue is able to hydroxylate PS at C-17.

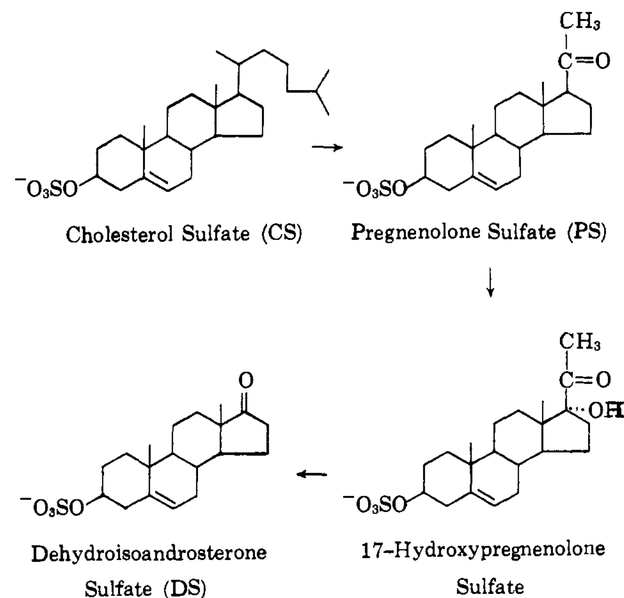
To extend these findings, cholesterol-7α-^{3}H sulfate-^{35}S (CS) (24.9×10^6 c.p.s. ^{3}H and 4.35×10^6 c.p.m. ^{35}S, ^{3}H–^{35}S ratio = 5.7) was injected into the left splenic artery of a female subject with an inoperable adrenal carcinoma. This vessel supplied 90% of the blood going to the neoplasm. The CS was injected through a small catheter introduced into the artery under radiographic visualization prior to the selective perfusion of the neoplasm with an antimetabolite.

The conjugates from the first 24-hr. urine collection were extracted into ethanol–ether (3:1).[4] After evaporation of the organic solvents, the remaining aqueous phase was extracted with n-butyl alcohol. The dry residue left after removal of the butyl alcohol contained 2.5% of the injected radioactivity. It was purified by partition chromatography on Celite using the system[5]: isooctane–t-butyl alcohol–1 M NH_4OH (2:5:5), which separates the monosulfates from other urinary conjugates. The sulfate fraction was distributed between 0.3 M pyridinium sulfate solution and chloroform.[6] Evaporation of the $CHCl_3$ afforded the pyridinium salts of the steroid sulfates (264,000 c.p.m. ^{3}H and 40,000 c.p.m. ^{35}S) which were purified further by partition chromatography on Celite using the system: isooctane–chloroform–n-butyl alcohol–methanol–0.3 M pyridinium sulfate–pyridine (4:2:0.7:2:2:0.1). At least two radioactive metabolites were eluted, the more polar of which was rechromatographed on Celite using the system: isooctane–ethyl acetate–n-butyl alcohol–methanol–1 M NH_4OH (2:4:1:2:3). One crystalline radioactive product was eluted in the third to fifth hold-back volume, and this was identified by its infrared spectrum as the ammonium salt of DS. It weighed 87 mg. and contained 92,900 c.p.m. ^{3}H and 17,100 ^{35}S. The product was recrystallized from a mixture of MeOH and acetone to constant specific activity with respect to both ^{3}H and ^{35}S in both crystals and mother liquor residues during three successive crystallizations. The data are presented in Table I, where the ^{3}H–^{35}S ratios are also shown. The latter values were essentially identical with the ratio present in the injected CS. In addition, the specific activities with respect to both isotopes remained constant following conversion of the DS to its oxime and two crystallizations of that derivative from methanol–ether. The yield of radioactivity associated with DS in the urine of the first day was approximately 0.5%.

TABLE I
CRYSTALLIZATION DATA

Sample	Specific activity with resp. to ^{3}H in c.p.m./mg.	Specific activity with resp. to ^{35}S in c.p.m./mg.	^{3}H–^{35}S ratio
1st Crystallization	1134	212	5.4
Mother liquor	970	174	5.6
2nd Crystallization	1154	210	5.5
Mother liquor	1090	200	5.5
3rd Crystallization	1141	204	5.6
Mother liquor	1155	213	5.4

SCHEME I
PROPOSED BIOSYNTHETIC PATHWAY INVOLVING STEROID SULFATES AS INTERMEDIATES



(1) K. D. Roberts, R. L. VandeWiele, and S. Lieberman, *J. Biol. Chem.*, **236**, 2213 (1961).

(2) H. I. Calvin, R. L. VandeWiele, and S. Lieberman, *Biochem.*, **2**, 648 (1963).

(3) H. I. Calvin and S. Lieberman, *ibid.*, **3**, 259 (1964).

(4) R. W. J. Edwards, A. E. Kelly, and A. P. Wade, *Mem. Soc. Endocrinol.*, **2**, 53 (1952).

(5) P. K. Siiteri, Ph.D. Dissertation, Columbia University, 1963.

(6) J. McKenna and J. K. Norymberski, *Biochem. J.*, **76**, lx (1960).