this stability is decreased, the net result being a gradual depolymerization to more stable but still biologically active lower molecular weight forms. Thus a molecular weight of this material at any time after disruption of its natural bond to the protein would be an average of the cyclic esters present.

Bolley (1964) observed the formation of a somewhat similar complex mixture of lactones, lactides, simple esters, and linear and cyclic polyesters. This mixture, which he termed "estolides," was formed from ricinoleic acid upon heating or from methyl ricinoleate upon addition of sodium methylate catalyst and mild heating under vacuum. This reaction is not spontaneous, however, and must be catalyzed by the alkali catalyst and heat.

In preparation of the lipase and the cofactor all steps are conducted at 0–4°; the evaporation of butanol is the only point which involves heat. A chromatographic analysis of the butanol extract before and after removal of the solvent by heat yields the same pattern shown by C, Figure 1. Therefore, we may conclude that the components observed are not heat-induced artifacts. The natural occurrence of large cyclic structures such as those proposed here is by no means new. Woodward (1957) and Hochstein *et al.* (1960) have found such systems in a variety of natural sources.

*Biological Activity of Related Compounds.*—It was of interest to determine whether materials other than ricinoleic acid derivatives would also exhibit cofactor activity and what properties or functional groups they might have in common. The results in Figure 4 show that monoricinolein, diolein, oleic acid, and the methyl half ester of dodecenyl succinate exhibit varied degrees of cofactor activity. It was shown earlier that $\alpha$-tocopherol succinate can replace the cofactor (Ory and Altschul, 1962a). However, this compound is completely inactivated by saponification. Thus, it appears that both some type of carboxyl function and a long hydrocarbon chain are required for cofactor activity. The fact that triglycerides are not themselves active as cofactors might suggest that surface activity is another requirement.

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REFERENCES

Ackman, R. G., Linstead, P., Wakefield, B. J., and Weedon, B. C. L. (1960), *Tetrahedron 8*, 221.
Altschul, A. M., Ory, R. L., and St. Angelo, A. J. (1963), *Biochem. Prepn. 10*, 93.
Bangham, A. D., and Dawson, R. M. C. (1960), *Biochem. J. 75*, 133.
Bolley, D. S., Baker Castor Oil Co. (1964), personal communication.
Desnuelle, P. (1961), *Advan. Enzymol. 23*, 129.
Dieckert, J. W., Carney, W. B., Ory, R. L., and Morris, N. J. (1958), *Anal. Chem. 30*, 1442.
Hanahan, D. J. (1952), *J. Biol. Chem. 195*, 199.
Hanahan, D. J., Rodbell, M., and Turner, L. D. (1954), *J. Biol. Chem. 206*, 431.
Hanahan, D. J., and Vercamer, R. (1954), *J. Am. Chem. Soc. 76*, 1804.
Hochstein, F. A., Els, H., Celmer, W. D., Shapiro, B. L., and Woodward, R. B. (1960), *J. Am. Chem. Soc. 82*, 3225.
Kates, M., and Gorham, P. R. (1957), *Can. J. Biochem. Physiol. 35*, 119.
Ory, R. L., and Altschul, A. M. (1962a), *Biochem. Biophys. Res. Commun. 7*, 370.
Ory, R. L., and Altschul, A. M. (1962b), *Biochem. Biophys. Res. Commun. 7*, 375.
Ory, R. L., St. Angelo, A. J., and Altschul, A. M. (1962), *J. Lipid Res. 3*, 99.
Weiss, H., Spiegel, H. E., and Titus, E. (1959), *Nature 183*, 1393.
Woodward, R. B. (1957), *Angew. Chem. 69*, 50.

# Estriol Biosynthesis by Sow Ovary

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Incubation of 16$\alpha$-[4-$^{14}$C]hydroxyprogesterone with minced sow ovary has led to the formation of [4-$^{14}$C]estriol. A pathway is presented for the genesis of estriol based on the compounds isolated and identified. The results are in agreement with the biosynthetic route as proposed for progesterone. The presence of an active C-16 dehydrase enzyme system is found which appears to operate in preference to the 17$\alpha$-hydroxylase enzyme system.

The formation of estriol from estradiol-17$\beta$ by human fetal liver slices has been demonstrated by Engel *et al.* (1958) and also with isolated rat livers (Hagopian and Levy, 1958). Surprisingly enough, no estriol biosynthesis has been observed from estradiol-17$\beta$ in adult liver (Engel *et al.*, 1958; Ryan and Engel, 1953). Ryan (1959) has been able to show conversion of C-16-oxygenated steroids to estriol in human placenta, while in a preliminary note the conversion of 16$\alpha$-hydroxyprogesterone to estriol in sow ovary was reported (Kadis, 1964). It is the purpose of this report to elucidate further the nature of the latter reaction through the identification of intermediate products.

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EXPERIMENTAL

Incubation techniques used and isolation of compounds have been described elsewhere (Warren and Salhanick, 1961a) with one major change. For removal of fats, a partition between 90% methanol and Skellysolve B was used.

Eight per cent sodium hydroxide was used to separate the incubated material into neutral and phenolic fractions which were chromatographed separately (Kadis, 1964). The neutral fraction was chromatographed in a hexane-benzene system for 20 hours; this left all steroids on the paper except 16-dehydroprogesterone (Table I). To the overrun known 16-dehydroprogesterone was added and chromatographed according to the outline in Table I. Of the compounds remaining on the paper, the 4-androsten-16$\alpha$-ol-3,17-

TABLE I
STRUCTURAL PROOF OF STEROID PRODUCTS

| Steroid | Reaction | $R_F$ Identical with that of | Zaffaroni System | Specific Activity (cpm/mg) |
|---|---|---|---|---|
| 16α-Hydroxyprogesterone | | 16α-Hydroxyprogesterone | Benzene (0.30) | $8.5 \times 10^5$ |
| | | | Benzene-chloroform (0.74) | |
| 4-Pregnene-16α,17α-diol-3,20-dione | | 4-Pregnene-16α,17α-diol-3,20-dione | Benzene (0.24) | 1,870 |
| | | | Benzene-chloroform (0.58) | 1,803 |
| | Periodic acid | Keto-aldehyde | Benzene-chloroform (0.2) | 1,781 |
| 4-Androsten-16α-ol-3,17-dione | | 4-Androsten-16α-ol-3,17-dione | Benzene (0.41) | 786 |
| | | | Benzene-chloroform (0.63) | 780 |
| | Reduction | 4-Androstene-16α,17-diol-3-one | Benzene-chloroform (0.1) | 765 |
| | Periodic acid | Dialdehyde | Benzene-chloroform (0.21) | 736 |
| 16-Dehydroprogesterone | | 16-Dehydroprogesterone | Hexane (0.39) | 10,525 |
| | | | Hexane-benzene (0.85) | 10,450 |
| | Epoxidation | 16,17-Epoxyprogesterone | Hexane (0.22) | 10,260 |
| 4-Pregnene-16α,20α-diol-3-one | | 4-Pregnene-16α,20α-diol-3-one | Benzene-chloroform (0.09) | 325 |
| | | | Chloroform (0.17) | 322 |
| | $CrO_3$-oxidation | 16-Ketoprogesterone | Hexane-benzene (0.19) | 287 |
| Estriol[a] | | Estriol | Benzene | 462 |
| | Methylation | Estriol-3-methyl ether | Alumina | 437 |
| | | | Chloroform (0.78) | 432 |

[a] Part of the data taken from the paper by Kadis (1964).

dione was located slightly ahead of the 16α-hydroxyprogesterone, while the remaining compounds were either on the origin or between the origin and the 16α-hydroxyprogesterone. Elution of this latter area, followed by addition of authentic steroids, and chromatography according to Table I led to adequate separations of material.

An ultraviolet scanner composed of a Corning no. 7-54 ultraviolet transmitting filter and a germicidal lamp was used to locate the steroids on the paper.

Steroids were obtained from commercial sources except for the 16α-hydroxyprogesterone (Dr. John Babcock, Upjohn Co.) and 4-androsten-16α-ol-3,17-dione (Dr. Kenneth J. Ryan).

*Preparation of Derivatives Other than Listed by Warren and Salhanick (1961a).*—REDUCTION.—The steroid was dissolved in absolute ethanol and an excess of sodium borohydride added to the solution at 0°. After 90 minutes, glacial acetic acid was added, the alcohol removed, water added, and the product extracted with ethyl acetate.

OXIDATIONS.—The chromic anhydride–pyridine method of Poos *et al.* (1953) was used.

EPOXIDATION.—The method described in the patent section of *Chemical Abstracts* ((1956) *50*, 6517) of the Farbwerke Hoechst A.-G. was utilized.

## RESULTS

Table I lists the products isolated from the reaction in detectable amounts. None of the compounds found, with the possible exception of 4,16-pregnadiene-3,20-dione (16-dehydroprogesterone), was unexpected. 4-Pregnene-16α,17α-diol-3,20-dione has been isolated from hyperplastic adrenals (Villee, 1964), while the other compounds are what would be expected if one accepts the general pathway of estrogen biosynthesis from progesterone (Dorfman, 1963). No attempt was made to isolate C-19-oxy compounds. It was found, however, that a very polar material extracted with the neutral fraction would not move in the chloroform system. Attempted acetylation of the material did not yield any acetylated compound.

The large amount of dehydration was unexpected, and, in fact, it appears from the data that this reaction takes preference over 17α-hydroxylation. Since 4-androsten-16α-ol-3,17-dione was formed in reasonable quantities, it was assumed that the side-chain-cleaving enzyme (a desmolase) was operating. To prove this point, authentic 4-pregnene-16α,17α-diol-3,20-dione was incubated under the same conditions as reported here, and the products were isolated. 4-Androsten-16α-ol-3,17-dione was found to be present, and its authenticity was proved by the methods listed in Table I.

It also appeared that some 16-ketoprogesterone might have been formed. Upon complete examination of the fraction thought to contain the compound, no significant quantity could be detected.

## DISCUSSION

Because estriol is the principal urinary estrogen, its biosynthesis from estrone and estradiol-17β has been of considerable interest. Estriol is found in a variety of endocrine tissues (Merrill, 1958), but the exact nature of its biosynthesis still remains unsolved. Ryan and co-workers at Western Reserve University have been instrumental in elucidating many aspects of estriol biosynthesis (e.g., Ryan, 1959). Recently Magendantz and Ryan (1964) have isolated 5-androstene-3β,16α-diol-17-one as a new estriol precursor from

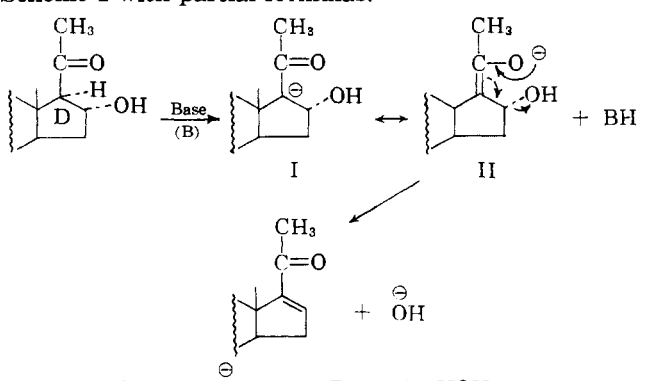
fetal blood. That 16$\alpha$-hydroxylated compounds can be converted into estriol has been documented in both human placenta (Ryan, 1959) and in sow ovary (Kadis, 1964). Failure to demonstrate in adult endocrine tissue and liver a 16-hydroxylase enzyme system for estrone or estradiol-17$\beta$ leads one to wonder by what mechanism estriol is formed. That is, is a 16-hydroxylated nonphenolic compound the real precursor of estriol rather than estradiol-17$\beta$ or estrone?

*In vivo* studies with $^{14}$C-labeled estrone or estradiol-17$\beta$ leads to the formation of estriol, as found through urinary studies (Beer and Gallagher, 1955). These findings, however, do not indicate the site of 16$\alpha$-hydroxylation.

The presence of a 16$\alpha$-hydroxylase enzyme system has been demonstrated in human ovary (Warren and Salhanick, 1961b), in sow ovary (Kadis, 1964), and in swine adrenals (Rao and Heard, 1957). Little *et al.* (1963) have isolated 16$\alpha$-hydroxyprogesterone in human placenta, thereby indicating the presence of a 16-hydroxylase. The 16-hydroxylase in placenta must be specific for progesterone or pregnenolone, since Bolté *et al.* (1964) do not find a 16-hydroxlase capable of acting upon either estrone or estradiol-17$\beta$. Because a desmolase enzyme system has not been demonstrated in placenta, it seems unlikely that 16$\alpha$-hydroxyprogesterone can serve as a precursor of estriol in that tissue. On the other hand, ovarian and adrenal tissues are capable of converting C-21 steroids to C-19 compounds (Dorfman, 1963). Experiments performed in our laboratory have shown that [4-$^{14}$C]androstenedione does not give rise to a 16$\alpha$-hydroxylated product upon being incubated with sow ovary. It therefore appears that during pregnancy estriol biosynthesis might proceed on the fetal side rather than the maternal side of the placental barrier.

In the present set of experiments the formation of the small amount of 4-pregnene-16$\alpha$,20$\alpha$-diol-3-one was surprising. In contrast, Warren and Salhanick (1961a) found that with human ovary the major product isolated from progesterone incubations was 4-pregnen-20$\alpha$-ol-3-one.

The unexpected dehydration reaction to form 16-dehydroprogesterone as the major product demonstrates the presence of an active dehydrase enzyme. This type of enzyme has been found in rat testes (Stylianou *et al.*, 1961a) and human liver (Stylianou *et al.*, 1961b), as shown by the formation of 16-dehydro compounds when testosterone was incubated. In our own laboratory, we have evidence that 17$\alpha$-hydroxyprogesterone is also converted to 16-dehydroprogesterone by sow ovary (Armstrong and Kadis, unpublished observations). That such a conversion should occur with 16$\alpha$-hydroxyprogesterone is not too surprising if one looks at a possible mechanism of dehydration illustrated in Scheme I with partial formulas:

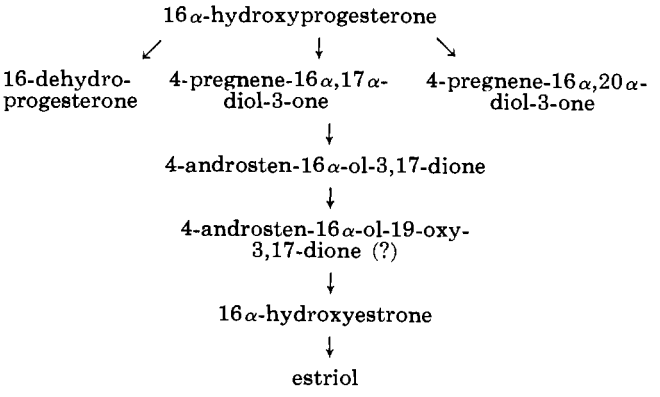



*Scheme 1*

Abstraction of the acidic hydrogen at C-17 by base to form the anion I would be favored owing to resonance stabilization of the enolate anion II. The final driving force of the reaction would be the removal of the hydroxyl group as an anion which in turn reacts with the conjugate base (BH) to form water and the free base.[1]

Experiments performed in our laboratory with sow ovary using unlabeled 16$\alpha$-hydroxyprogesterone as the substrate indicated that no 4-pregnene-16$\alpha$,17$\alpha$-diol-3,20-dione was formed (Ball and Kadis, unpublished results). When 16$\alpha$-[4-$^{14}$C]hydroxyprogesterone serves as the substrate, conversion on a small scale is noted. This would indicate that the 16$\alpha$-hydroxyl group interferes with the 17$\alpha$-hydroxylase enzyme system. If this latter assumption is correct, the large amount of 16-dehydroprogesterone produced might be explained. Attempted isolation of 4-[4-$^{14}$C]pregnene-16$\alpha$,17$\alpha$-diol-3-one from 17$\alpha$-[4-$^{14}$C]hydroxyprogesterone incubations has failed. When authentic material is chromatographed radioactivity remains, but upon periodic acid oxidation of the chromatographed 16,17-diol the specific activity is essentially zero. This result has been obtained in two separate experiments.

The pathway by which the estriol arises can then be depicted as illustrated in Scheme 2, in which the 17-




*Scheme 2*

ketone has not been reduced until the last step. No phenolic compound other than estriol could be detected in significant amounts.

## References

Beer, C. T., and Gallagher, T. F. (1955), *J. Biol. Chem. 214*, 335, 351.

Bolté, E., Mancuso, S., Eriksson, G., Wiqvist, N., and Diczfalusy, E. (1964), *Acta Endocrinol. 45*, 535.

Dorfman, R. (1963), *in* Pituitary-Ovarian Endocrinology, Dorfman, R., and Castro, M. N., eds., San Francisco, Holden-Day, p. 1.

Engel, L., Bagget, B., and Halla, M. (1958), *Biochim. Biophys. Acta 30*, 435.

Hagopian, M., and Levy, L. (1958), *Biochim. Biophys. Acta 30*, 641.

Kadis, B. (1964), *Biochim. Biophys. Acta 82*, 649.

Little, B., Shaw, A., and Purdy, R. (1963), *Acta Endocrinol. 43*, 510.

Magendantz, H., and Ryan, K. (1964), *Federation Proc. 23*, 275.

Merrill, R. (1958), *Physiol. Rev. 38*, 463.

Poos, G., Arth, G., Beyler, R., and Sarett, L. (1953), *J. Am. Chem. Soc. 75*, 422.

Rao, B., and Heard, R. (1957), *Arch. Biochem. Biophys. 66*, 504.

Ryan, K. (1959), *J. Biol. Chem. 234*, 2006.

[1] Protonation of the C-16-hydroxyl group followed by the loss of water because of the enolate anion is another possibility.

Ryan, K., and Engel, L. (1953), *Endocrinology 52*, 287.
Stylianou, M., Forchielli, E., and Dorfman, R. (1961a), *J. Biol. Chem. 236*, 1318.
Stylianou, M., Forchielli, E., Tummillo, M., and Dorfman, R. (1961b), *J. Biol. Chem. 236*, 692.
Villee, D. (1964), *J. Clin. Endocrinol. Metab. 24*, 442.
Warren, J., and Salhanick, H. (1961a), *J. Clin. Endocrinol. Metab. 21*, 1218.
Warren, J., and Salhanick, H. (1961b), *J. Clin. Endocrinol. Metab. 21*, 1376.

# Gas Adsorption and Surface Structure of Bone Mineral*

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Adsorption techniques have been applied to anorganic bone. Weight loss as a function of degassing temperature has been correlated with surface areas as determined by B. E. T. nitrogen adsorption. Pore size distribution in the bone mineral has been estimated on the basis of adsorption experiments and by use of the mercury porosimeter. From these results, as well as from the results of adsorption calorimetry, we suggest a model in which partially discrete and more or less randomly oriented crystals of submicroscopic dimensions are welded together by ionic forces and by adsorbed water bridging the grain boundaries in certain areas of contact. This model seems consistent with previously proposed models based on other experimental evidence such as X-ray and electron microscopic data.

A program of adsorption studies on anorganic bone has been conducted for several years in the Amherst Laboratory and in part at Carleton University. Various aspects of the work have been described in several publications (Dry and Beebe, 1960; Holmes and Beebe, 1961; Gale and Beebe, 1964). In this paper we shall discuss the effects of degassing temperature on the specific surface area. We shall give data on the pore size distribution of the bone mineral and present a possible model for its submicro crystal morphology.

Neuman and Neuman (1958) discuss in some detail the complexity of the problems with which investigators are confronted in attempting to characterize bone mineral with respect to both chemical composition and crystal morphology. The above authors point out that the tiny crystallites in bone, which are of submicroscopic dimensions, can be studied effectively by line broadening of X-rays and especially by means of the electron microscope. They cite the work of Finean and Engstrom (1953) and of Robinson and Watson (1952) as applications of these methods. They also discuss the work of Ascenzi (1955), who concludes from his electron microscopic studies that bone mineral is a continuous phase rather than an aggregation of more or less discrete crystals.

Neuman and Neuman summarize the present position in the following paragraph: "From the present status of information on the crystals of bone mineral, it seems reasonable to suppose that some of the divergence among investigators is caused by experimental artifacts. Some may also come from the examination of specimens in different stages of maturation. Thus a section beginning to mineralize would be expected to show collagen fibers associated with small, isolated, fragmentary crystals. As mineralization continued to the exclusion of interstitial water, the growth of crystals within the fibers and epitactic overgrowth of preexisting crystals would give the appearance of an almost continuous mineral phase."

The techniques of gas-solid adsorption have long been applied to the investigation of solid surfaces, especially those of catalysts, pigments, and porous adsorbents such as charcoal and silica gel. By these techniques it has been possible to deduce such properties as specific surface area, pore size distribution, and state of the adsorbing surface, whether energetically homogeneous or heterogeneous and whether polarizing or nonpolarizing. Rather extensive adsorption studies have been made on certain synthetic hydroxyapatites and bone chars (Barrett *et al.*, 1951a,b, 1952). With such experiments in mind it seemed to us that information supplementing the electron microscope and X-ray work might be obtained from adsorption studies on anorganic bone.

In order to bring the bone mineral into a reproducible state for the adsorption work, it is desirable to heat the sample in vacuo at 450°, usually for 24 hours. By this procedure virtually all adsorbed water can be removed without drastically altering the hydroxyapatite structure within the crystals, although there is some evidence for crystal growth during this heating process (Robinson and Watson, 1952). We feel that any crystal growth, although it would alter the extensive properties of the crystals, would not alter the intensive properties of the crystal surfaces. Thus a surface site might well possess the same energy for adsorption whether this site resided in the face of a crystal having a width of 60 Å or of 300 Å.

From the point of view of an interest in the bone structure *in vivo*, it is unfortunate that the bone mineral must be subjected to such drastic treatment in preparation for the adsorption studies. We feel, however, that the morphology of the dehydrated bone mineral would still bear a reasonably close resemblance to that in the parent bone. The point should be stressed

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