Techniques Developed for Studying the Degradation of Steroids Manometrically

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During the past decade a great deal of attention has been devoted to studying the effects of microorganisms on steroid compounds. The majority of this work has dealt primarily with transformation type reactions and very little is known concerning the degradation of these compounds. Recently we isolated several organisms that are capable of utilizing progesterone, cholesterol, and testosterone as sole sources of carbon for growth and reproduction. To facilitate studying the dissimilation of these compounds we have employed manometric techniques. By this method we can determine the extent to which these compounds are oxidized. This information will be very helpful in elucidating the metabolic pathways.

An extensive search of the literature revealed that Santer *et al.* (1952) dissolved testosterone in ethanol before adding to the Warburg flask, while Wainfan *et al.* (1954) added an acetone preparation of colloidal cholesterol. When employing these techniques one must realize that the solvent may serve as an additional carbon source and it is difficult to accurately pipette a colloidal solution. To overcome these disadvantages we have developed simple, yet reliable, techniques that enable us to use manometric procedures to study oxygen consumption and/or carbon dioxide evolution. It is these techniques we would like to report at this time.

The organism used throughout the course of this investigation was recently isolated from a pond water sample and has not been completely identified. It was grown on a synthetic medium containing a single compound as the source of carbon and energy. The synthetic medium had the following basal composition: NaCl, 0.2%; KH_2PO_4, 0.32%; K_2HPO_4, 0.42%; NH_4Cl, 0.2%; and agar 2.0%. To this was added 0.1 ml. of a mineral salts solution consisting of $MgSO_4 \cdot 7H_2O$, 5.0%; $MnSO_4$, 0.1%; $FeCl_3$, 1.0%; and $CaCl_2$, 0.5%. The specific compound that served as a source of carbon and energy was added in a concentration of 0.1% and the pH was adjusted to 7.2. The cells were harvested from plate cultures 24 hours old, washed twice, and resuspended in M/50 phosphate buffer of pH 7.0 for manometric experimentation.

All respirometer experiments were performed in the Warburg apparatus at a temperature of 37° C with air as the gas phase. A heavy suspension of cells was added to the sidearms of the flasks. The steroid was added to the bottom of the vessel to facilitate pipetting and to allow a greater surface area for volatilization of the solvent. The exact procedure for adding the steroids will be described later. After volatilization, a known volume of buffer was added to the bottom of the flasks. The flasks were allowed to equilibrate 20-30 minutes before mixing the cells and steroid substrate.

The solvents employed for adding the steroids to the flasks were ether, ethyl acetate, chloroform, methanol, and ethanol. These solvents have been used in extracting steroids from various sources and apparently do not chemically change the molecule.

The steroids were weighed, added to 50 or 100 ml. volumetric flasks, and dissolved in the solvent. By this method, known quantities of a steroid could be added to the Warburg vessel. The volumetric flasks were refrigerated to prevent volatilization of the solvent during standing. How-

ever, since the volume decreased during cooling, the flasks were removed from the refrigerator and placed in a constant temperature room at 25° C for two hours prior to use.

The steroid concentrations were calculated so that 0.1 ml. of the solvent would contain enough substrate to allow complete and accurate measurements. This amount of solvent may be added with a graduated 0.1 ml. pipette. And the volume is small so that volatilization from the vessel is very rapid.

The solvents were volatilized by two different methods. The first method was to allow the flasks to stand either at room temperature or in a 37° C incubator. These procedures required a minimum of two hours for complete volatilization. The second method was to attach the flasks to a manifold which in turn was connected to a vacuum system. The flasks were immersed in warm water and the solvents removed under vacuum. This method is preferred since it requires approximately 15 minutes to remove ether, ethyl acetate, or chloroform. Methanol and ethanol require a longer period of treatment.

TABLE 1
THE INFLUENCE OF SOLVENTS ON THE OXIDATION OF PROGESTERONE

TIME IN MINUTES	Microliters Oxygen Uptake			
	Endogenous	Ether	Ethyl Acetate	Chloroform
20	3	24	22	28
40	7	58	55	61
60	10	97	98	98
80	14	116	119	120
100	16	154	154	156
120	19	188	193	193

The methods of adding the steroids to the vessels were then compared. The results from this experimentation are shown in Table 1. No significant differences were observed in the amount of oxygen consumed when progesterone was added in the different solvents. Additional studies also showed that the method of volatilization did not affect the oxidation of the substrate and the solvent residue did not influence the oxidative ability of the cells.

The results indicate that, of the solvents studied, ether, ethyl acetate, and chloroform were the most satisfactory. Of these, ethyl acetate or chloroform is now being used for oxidation studies. Ether has the disadvantages of volatilizing very easily during standing and is difficult to pipette. Ethanol and methanol were unsatisfactory since these solvents were difficult to remove, thereby offering an additional source of carbon for oxidation. In addition, some of the steroids are soluble in ethanol only upon heating which may prevent accurate measurements. Volatilization may be carried out satisfactorily by either method. Preliminary investigations indicate that the oxidation of steroids may be accurately measured by this procedure.

LITERATURE CITED

Santer, M., S. J. Ajl and R. Turner. 1952. Steroid metabolism by a species of *Pseudomonas*. J. Biol. Chem., 198:397-404.

Wainfan, E., C. Herkin, S. G. Rittenberg and W. Marx. 1954. Metabolism of cholesterol by intestinal bacteria *in vitro*. J. Biol. Chem., 207:843-849.