Studies of the Microbiological Oxidation of Steroids by 
Cunninghamella blakesleean a H-334

I. The Effect of Alcohols and Phenol

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The incubation of Reichstein's Compound S (11-deoxy-17α-hydroxycorticosterone) with Cunninghamella blakesleean a strain H-334 yields 17α-hydroxycorticosterone (Kendall's Compound F, hydrocortisone, Reichstein's Compound M) (Hanson, et al., 1953). The presence of cortisone (11-dehydro-17α-hydroxycorticosterone) in the transformation products was also indicated. This paper will describe some observations on the yields of these two steroids (and other side products) as affected by the addition of various alcohols and of phenol to the fermentation.

Methods

Fermentations were conducted in 100 ml of medium in 500 ml Erlenmeyer flasks. The medium (soybean meal, 5 g; dextrose, 20 g; NaCl, 5 g; K2HPO4, 5 g; brewer's yeast, Pabst, 5 g; tap water, 1000 ml; pH adjusted to 6.4) was sterilized at 121 C for 30 minutes. The flasks were inoculated with 5 ml of vegetative mycelium of C. blakesleean a H-334, which had been grown in the same medium on a reciprocating shaker at 26 C for 48 hours. The flask cultures were incubated at 28 C on a rotary shaker (Gump type, 250 rpm) for 17 hours. At the end of this period of growth, Compound S was added as a sterile ethanolic solution, and the incubation was allowed to proceed for 7 hours.

Acetone (20 ml) was then added to each flask before filtration through a Celite pad. The filter cake was washed with methylene chloride. The filtrate was extracted four times with equal volumes of methylene chloride and the pooled extracts were concentrated under reduced pressure at temperatures less than 50 C in a stream of nitrogen.

The steroids present in the extract were separated by paper chromatography (Zaffaroni et al., 1950), and detected by means of their absorption of ultraviolet light (Haines and Drake, 1950). A quantitative determination of the hydrocortisone present was made by eluting the steroid from the paper (Haines, 1952) and measuring the absorption at 242 μ. Cortisone was similarly determined by absorption measurements at 238 μ.

Experimental Results

In order to ascertain the possible effects of its solvent, the Compound S was added to the fermentations in varying volumes of sterile ethanol. Results of such an experiment are presented in table 1.
As the volume of alcohol was increased from 1.5 to 11 ml per 100 ml of fermentation broth the yield of hydrocortisone increased, whereas the cortisone yield slowly declined over the same range. The yield of both steroids was sharply reduced as the volume of ethanol was increased beyond 12 ml.

This effect is not limited to ethanol. Other water-soluble alcohols were examined by adding varying volumes to the fermentation, at the same time the steroid was added in 1 ml of sterile ethanol. The results of these experiments are presented in table 2.

With the outstanding exception of n-propyl alcohol, there was, again, an increase in the yield of hydrocortisone as the volume of alcohol added was increased. Such changes as did occur in the yields of cortisone had the net effect of increasing the ratio of hydrocortisone to cortisone formed, except in the case of t-butyl alcohol where an increased ratio occurred only after the point of maximum hydrocortisone yield was passed.

No direct comparison may be made between the percentage steroid yields in this experiment and those reported in table 1 since the experiments were performed at different times, and the extent of steroid transformation varied considerably from one set of experiments to another. Nevertheless, it is possible to conclude that in comparison with ethanol, larger volumes of propylene glycol were necessary to effect the stimulation, and the inhibition of the reaction occurred with smaller volumes of t-butyl alcohol.

In addition to hydrocortisone, cortisone and unreacted Compound S, some unidentified, ultraviolet-absorbing materials appeared in the analytical papergrams. An absorbing area at the origin of the papergrams was always present, even in the absence of steroid, but the other unidentified areas appeared only when the substrate had been added. It was observed that a steady reduction in the amount of these products (as judged by visual inspection of the intensity of absorption) occurred when phenol was added to the fermentation medium. An almost complete elimination occurred at a phenol level of 50 mg per 100 ml of medium. However, as can be seen in table 3, such a phenol concentration is detrimental to the steroid yields. In a range of 10 to 20 mg of phenol per 100 ml of medium, it was possible to obtain substantial reduction in the amount of side product formation without reducing the yield of desired product.

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Yields of hydrocortisone (17α-hydroxycorticosterone) and cortisol (11-dehydro-17α-hydroxycorticosterone), as a result of the incubation of Reichstein’s Compound S (11-desoxy-17α-hydroxycorticosterone) with Cunninghamella blakesleeana H-334, varied considerably between groups of shaker flask experiments (Mann, et al., 1955). It seemed probable that the factors affecting the microbiological transformation could be more closely controlled in a chemically defined medium. This paper will describe experiments analyzing the role of various components of an improved medium.

Methods

Czapek-Dox solution (see Thom and Raper, 1945) was employed as a prototype for constructing a defined medium. Its composition was as follows: NaNO₃, 3.0 g; KH₂PO₄, 3H₂O, 1.3 g; MgSO₄·7H₂O, 0.5 g; KCl, 0.5 g; FeSO₄·7H₂O, 0.01 g; sucrose, 30 g; distilled water, 1000 ml; adjusted to pH 7.2 before sterilization. The medium, in 100 ml volumes in 500 ml Erlenmeyer flasks, was sterilized at 15 pounds pressure for 20 minutes. The flasks were inoculated with 1 per cent of a spore suspension of Cunninghamella blakesleeana H-334 in a solution of 25 ppm Aerosol® AY, 100 per cent grade.

Preliminary experiments indicated that 48 hours of incubation on a Gump-type rotary shaker (250 rpm) at 28°C were required for adequate growth. The Compound S (10 mg) was then added in ethanol, and the incubation was allowed to proceed for 24 hours. The fermentations were run in duplicate flasks and the analytical data represent the average of the two flasks. The steroids were extracted and analyzed as previously described (Mann, et al., 1955).

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