Mitochondria-Associated Ribonucleic Acid of the HeLa Cell. Effect of Ethidium Bromide on the Synthesis of Ribosomal and 4S Ribonucleic Acid

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ABSTRACT: The synthesis of the two electrophoretic classes of 4S ribonucleic acid associated with HeLa mitochondria is inhibited by ethidium bromide. The synthesis of the mitochondria-associated 28S and 5S ribosomal ribonucleic acid and the synthesis of the cytoplasmic 28S, 18S, and 5S ribosomal ribonucleic acid and cytoplasmic 4S ribonucleic acid are unaffected by ethidium bromide under identical conditions.

The methylation of the two classes of mitochondria-associated 4S ribonucleic acid is significantly different from the methylation of the cytoplasmic 4S ribonucleic acid. It is suggested that the mitochondria-associated 4S ribonucleic acid is transcribed from the mitochondrial deoxyribonucleic acid and that the 28S and 5S ribosomal ribonucleic acid in the mitochondrial fraction may be from the cytoplasm and of nuclear origin.

The mitochondrial fraction of HeLa cells contains two classes of 4S RNA separable by gel electrophoresis while the cytoplasm contains only one class (Knight and Sugiyama, 1969). These two electrophoretic classes of 4S RNA were described originally as tRNA A and tRNA B (Knight and Sugiyama, 1969). The 4S RNA B of *Escherichia coli* appears to be a class of tRNA; however, for reasons stated later in this report the 4S RNA B in the mitochondrial fraction may not be a class of tRNA. Therefore, the two electrophoretic classes of 4S RNA isolated from the HeLa mitochondrial fraction will be designated A and B only.

Nass and Buck (1969) have shown that the tRNA of rat liver mitochondria have different specificities from their cytoplasmic counterparts. They have also shown that the mitochondrial tRNA of rat liver hybridizes more efficiently to the mitochondrial DNA than the corresponding cytoplasmic tRNA and have suggested that the mitochondrial tRNA is transcribed from the mitochondrial DNA. Barnett and Brown (1967) have shown that the tRNA from the mitochondrial fraction of *Neurospora* has specificities different from the corresponding cytoplasmic tRNA.

The interaction of the intercalating dye, ethidium bromide, with DNA has been investigated by Waring (1966). Radloff *et al.* (1967) studied the binding of the dye to HeLa DNA and showed that the closed, circular mitochondrial DNA could be easily separated from the linear nuclear DNA in a cesium chloride density gradient containing saturating amounts of ethidium bromide. South and Mahler (1968) in their studies on the synthesis of RNA in isolated yeast mitochondria reported that ethidium bromide inhibits the incorporation of nucleoside triphosphates into RNA. Zylber *et al.* (1969) have shown that ethidium bromide inhibits the *in vivo* synthesis of the 12S and 21S RNA in the HeLa mitochondrial fraction.

One of the critical questions concerning mitochondrial DNA is the kind of RNA into which it is transcribed. We have approached this problem by isolating classes of RNA which exist only in the HeLa mitochondrial fraction then studying their synthesis as affected by chemicals which may selectively inhibit the transcription of nuclear and mitochondrial DNA.

In this report it is shown that the synthesis of the 4S RNA of the HeLa mitochondrial fraction is inhibited by ethidium bromide. It is also shown that the methylation of this class of 4S RNA is significantly different from the methylation of the cytoplasmic 4S RNA. The term mitochondria-associated will be used to describe those classes of RNA occurring in the HeLa mitochondrial fraction prepared as described in Experimental Procedure.

Experimental Procedure

Materials. Ethidium bromide was a gift from the Boots Pure Drug Co. [5-^{3}H]Uridine (20 Ci/mmole), [^{3}H]methylmethionine (100 mCi/mmole), and carrier-free [^{32}P]phosphoric acid were obtained from New England Nuclear Corp.

Cell Growth. HeLa cells were grown in suspension culture as described by Eagle (1959). Cultures containing ethidium bromide at 0.2–0.4 μg/ml doubled two to three times then stopped growing.

Cell Fractionation and Preparation of Mitochondria. Extracts were prepared by shearing the cells in a stainless steel ball homogenizer in a buffer of 0.01 M Tris (pH 7.4), 0.01 M NaCl, 0.0015 M $MgCl_2$, and 0.25 M sucrose. Nuclei were removed by centrifugation at 2500*g* for 3 min and a crude mitochondrial pellet was prepared by centrifugation at 9000*g* for 10 min. Cytoplasmic ribosomal and small molecular RNA were prepared from the supernatant. The pellet was resuspended in 0.01 M Tris (pH 7.4), 0.001 M EDTA, and 0.25 M sucrose, and contaminating nuclei were removed by centrifugation and the mitochondria were again deposited at 9000*g* for 10 min. When preparing small molecular weight RNA the pellet was

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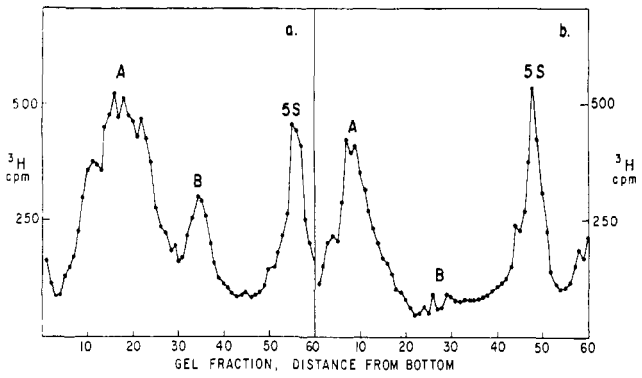

FIGURE 1: Effect of ethidium bromide on the synthesis of the small molecular weight RNA associated with HeLa mitochondria. The RNA in a culture of HeLa cells (60 ml, 25 × 10^4 cells/ml) containing ethidium bromide at 0.2 μg/ml was labeled for 18 hr with 50 μCi of [^{3}H]uridine. An identical control culture contained no ethidium bromide. The cell concentration in both cultures increased to 40 × 10^4/ml. Mitochondria-associated RNA was prepared as described in the Experimental Procedure and analyzed on 10% polyacrylamide gel, 18 cm, 8.5 hr at 6 mA/gel. The gels were fractionated and the radioactivity was measured as described in the Experimental Procedure. Migration is from right to left and only the lower region of the gel shown. (a) Small molecular weight RNA associated with HeLa mitochondria. (b) Small molecular weight RNA synthesized in presence of 0.2 μg/ml of ethidium bromide.

extracted directly with phenol as described below. When preparing rRNA associated with the mitochondria the pellet was resuspended in 0.01 M Tris (pH 7.4), 0.001 M EDTA, and 0.25 M sucrose, and layered on a 30-ml sucrose gradient for isopycnic banding. The sucrose gradient was from 15 to 55% (w/w) in 0.01 M Tris (pH 7.4) and 0.0001 M EDTA. Centrifugation for 2 hr at 24,000 rpm, 4° in the Spinco SW25.1 rotor, resulted in the banding of the cytochrome oxidase activity at ρ = 1.18. Fractions containing cytochrome oxidase activity, measured by the method of Smith (1955), were pooled and RNA prepared as described below.

Preparation of RNA. RNA was extracted by the sodium dodecyl sulfate–hot phenol (60°) method of Scherrer and Darnell (1962) from the mitochondrial pellet, the cytoplasm, and from the fractions at ρ = 1.18 containing cytochrome oxidase activity after isopycnic banding in a sucrose gradient.

Polyacrylamide Gel Electrophoresis. For analysis of small molecular weight RNA, 10% gels were prepared as described by Loening (1967). After electrophoresis at room temperature in a sodium dodecyl sulfate buffer (Loening, 1967) each gel was sliced by hand into 1-mm slices. To each slice in a scintillation vial was added 1 ml of 1 N NH_4OH and the gel–NH_3 mixture was allowed to stand overnight at room temperature. Bray's (1960) solution was added and the radioactivity was determined in a liquid scintillation counter.

rRNA was analyzed by electrophoresis in 2.7% acrylamide gels prepared in 10% glycerol as described by Weinberg *et al.* (1967) using ethylene diacrylate as the cross-linking agent. The gels were fractionated by slicing (Weinberg *et al.*, 1967) and each slice was hydrolyzed in 1 ml of concentrated NH_4OH for 1 hr at room temperature in a scintillation vial. Bray's solution was added and the radioactivity was determined in a liquid scintillation counter.

TABLE I: Stability of Mitochondria-Associated 4S RNA B.[a]

Expt	Conditions	4S RNA B (cpm)
1	18-hr label, no chase	1750
2	18-hr label, 24-hr chase	1250
3	18-hr label, 24-hr chase plus 0.2 μg of ethidium bromide/ml	1450

[a] Three cultures of 75 ml each at 20 × 10^4 cells/ml were labeled with 75 μCi of [^{3}H]uridine. After 18 hr the cells increased to 25 × 10^4/ml. The small molecular weight mitochondria-associated RNA in culture 1 was prepared as described in the Experimental Procedure and analyzed on 10% polyacrylamide gel as in Figure 1. The cells in cultures 2 and 3 were collected by centrifugation and resuspended in nonradioactive medium; no additions were made to culture 2 while ethidium bromide was added to 0.2 μg/ml to culture 3, and both cultures were allowed to grow for an additional 24 hr (cell concentration doubled). Mitochondria-associated RNA was prepared and analyzed as for culture 1. Radioactivity profiles after gel electrophoresis were obtained as in Figure 1. The total radioactivity under the 4S RNA B peak was determined and is shown in column 3.

Results

Inhibition of the Synthesis of Mitochondria-Associated 4S RNA by Ethidium Bromide. Since ethidium bromide inhibits the incorporation of nucleoside triphosphates into RNA by digitonin-treated mitochondria of yeast (South and Mahler, 1968), the effect of ethidium bromide on the synthesis of the mitochondria-associated 4S RNA was examined.

HeLa cells will grow in low concentrations of ethidium bromide (0.1 μg/ml to 0.4 μg/ml) for a period of time with no apparent initial ill effects, but growth eventually stops two to three cell doublings after the addition of the dye. To study the effect of ethidium bromide on the synthesis of mitochondria-associated small molecular weight RNA, a culture of cells was labeled for 16 hr with [^{3}H]uridine in the presence of 0.2 μg/ml of ethidium bromide and the RNA was extracted from the mitochondrial pellet and analyzed by gel electrophoresis. Figure 1a shows a polyacrylamide gel analysis of the small molecular weight RNA labeled 16 hr with [^{3}H]uridine and Figure 1b shows the RNA profile from a similar experiment in which the cell culture contained ethidium bromide. Ethidium bromide completely inhibits the synthesis of 4S RNA B and reduces 4S RNA A by *ca.* 50%. Labeling for shorter periods (2–4 hr) gave similar results. The synthesis of 5S RNA associated with the mitochondria is unaffected by ethidium bromide (Figure 1a,b). The synthesis of cytoplasmic rRNA (28, 18, and 5 S) and cytoplasmic 4S RNA are also completely unaffected by the dye at 0.2 μg/ml during the 16-hr labeling period.

Stability of Mitochondria-Associated 4S RNA B. tRNA like rRNA is relatively stable metabolically when compared with mRNA. Experiments were designed to test the stability of the 4S RNA B, and the results of such experiments are shown in Table I. Cells were labeled 16 hr with [^{3}H]uridine

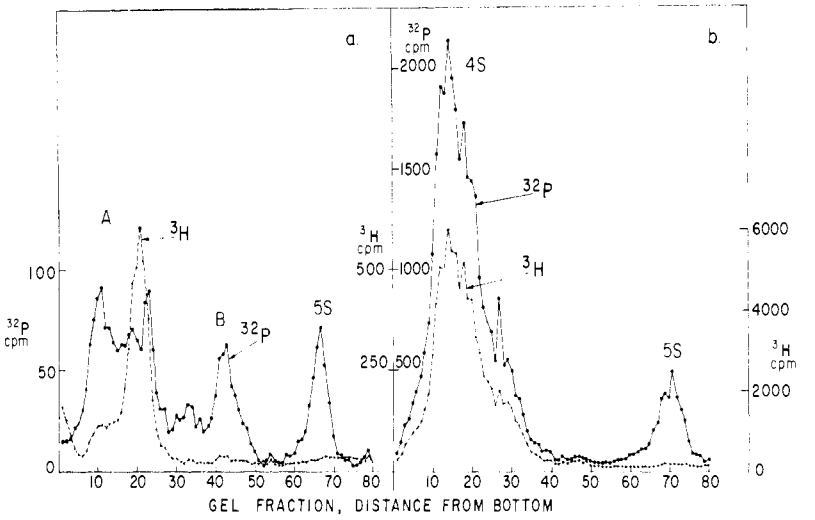

FIGURE 2: Methylation of small molecular weight RNA associated with HeLa mitochondria. A 50-ml culture of HeLa cells was grown from 20 × 10^4 to 40 × 10^4 cells per ml in presence of 10^{-4} M guanosine and adenosine. The culture was then grown to 80 × 10^4 cells/ml in presence of 1 mCi of [^{3}H]methylmethionine, 500 μCi of [^{32}P]phosphate, and 10^{-4} guanosine and adenosine. Mitochondria-associated and cytoplasmic RNA were prepared as described in the Experimental Procedure and analyzed on 10% polyacrylamide gel, 28 cm, 13.5 hr at 5.5 mA/gel. Only lower region of gel shown. (a) Mitochondria-associated small molecular weight RNA. (b) Cytoplasmic small molecular weight RNA.

and the amount of 4S RNA B was determined (summation of the radioactivity under peak B in the profile obtained from gel electrophoresis) after continued growth of the cells in nonradioactive medium with and without ethidium bromide. Experiment 1 in Table I shows the amount of 4S RNA B synthesized in 16 hr while expt 2 shows the amount of 4S RNA B present after 24-hr additional growth in nonradioactive medium. The amount of B after a 24-hr chase is 70% of the initial RNA (expt 1). This decrease is probably not significant since it has been found that the recovery of RNA from identical cultures after extraction with phenol varies from 10 to 20%. In any case the half-life of 4S RNA B compared with the half-life of mRNA (Penman *et al.*, 1963) is quite long suggesting that the 4S RNA B is relatively metabolically stable. Experiment 3 in Table I shows that 4S RNA B synthesized in the absence of ethidium bromide is stable for at least 24 hr during cell growth in the presence of ethidium bromide. Similar results were obtained for 4S RNA A.

Methylation of HeLa Mitochondria-Associated and Cytoplasmic 4S RNA. HeLa cytoplasmic 4S RNA is highly methylated (Knight and Darnell, 1967; Weinberg and Penman, 1968) and contains more methyl groups per molecule (Weinberg and Penman, 1968) than any RNA isolated from HeLa cells. A comparison of the methylation of the 4S RNA associated with HeLa mitochondria with the methylation of cytoplasmic 4S RNA could provide evidence as to the differences and similarities between the 4S RNA from the two sources.

Methylation was determined by growing cells in normal medium containing [^{32}P]phosphate and [^{3}H]methylmethionine then examining the ^{32}P and ^{3}H profiles of the 4S RNA after gel electrophoresis. The ratio of ^{3}H to ^{32}P represents extent of methylation provided that a minimal amount of ^{3}H enters the purine ring *via* one-carbon transfer from methionine. This is minimized by growing the cells in 10^{-4} M guanosine and adenosine for one cell doubling prior to labeling and during the labeling of the RNA. The 5S RNA provides a convenient internal standard for measuring ^{3}H entry into purine since it contains no methyl groups (Galibert *et al.*, 1965; Knight and Darnell, 1967). Figure 2 shows the radioactivity profiles of the small molecular weight RNA obtained from the mitochondrial fraction. Entry of ^{3}H into purine can be ignored since the 5S RNA of the mitochondrial fraction (Figure 2a) and of the cytoplasm (Figure 2b) contains no significant amount of ^{3}H.

Clearly, the methylation of 4S RNA A and B in the mitochondrial fraction (Figure 2a) is significantly different from the methylation of cytoplasmic 4S RNA (Figure 2b). 4S RNA B from the mitochondrial fraction like 5S RNA appears to be unmethylated. Mitochondria-associated 4S RNA A (Figure 2a), whose electrophoretic mobility is similar to cytoplasmic 4S RNA, has a completely different methylation pattern from cytoplasmic 4S RNA indicating that A is not a cytoplasmic contaminant.

Effect of Ethidium Bromide on the Synthesis of Mitochondria-Associated 28S rRNA. The 28S rRNA isolated from the mitochondrial fraction of BHK-21 cells (Dubin and Brown, 1967) and HeLa cells (Knight, 1969) sediments through a sucrose gradient at a slower rate than the corresponding cytoplasmic 28 S. Whether this 28S RNA is contained inside the mitochondrion or whether it is a cytoplasmic contaminant, whose size or configuration has been altered, is not known at present. As reported earlier (Vesco and Penman, 1969), the mitochondrial fraction as isolated from HeLa cells contains very little 18S RNA (Figure 3a). Vesco and Penman (1969) suggested that the 18 and 28 S found in the mitochondrial fraction may be the result of contamination in the mitochondrial fraction by membrane bound cytoplasmic ribosomes. Attardi and Attardi (1968) have also suggested that a majority of the ribosomes in the mitochondrial fraction of HeLa cells are associated with contaminating elements of the endoplasmic reticulum. Sabatini *et al.* (1966) reported earlier that the ratio of larger ribosomal subunits to smaller ribosomal subunits which are attached to membranes increases as the EDTA concentration is increased indicating a preferential detachment by EDTA of the smaller subunit. Figure 3a shows the electrophoretic profile of the high molecular weight RNA contained in the HeLa mitochondrial fraction. There is a small amount of 18S RNA compared with the 28S RNA.

Figure 3b shows the effect of ethidium bromide on the synthesis of mitochondria-associated 28S RNA under conditions where the synthesis of 4S RNA B is completely

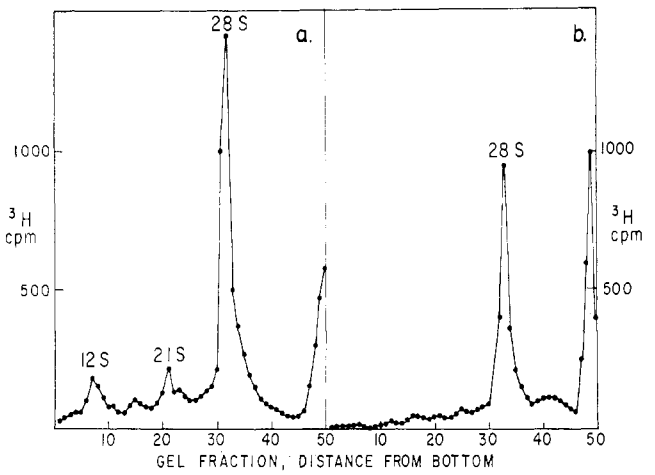

FIGURE 3: Effect of ethidium bromide on the synthesis of rRNA associated with HeLa mitochondria. The RNA in a control culture and in a culture containing 0.2 μg/ml of ethidium bromide was labeled with [^{3}H]uridine as described in Figure 1. Mitochondria-associated rRNA was prepared as described in the Experimental Procedure and analyzed on 2.7% polyacrylamide gel, 5 cm, 4 hr at 5 mA/gel. (a) Mitochondria-associated rRNA from control culture. (b) Mitochondria-associated RNA from culture containing ethidium bromide.

inhibited. The synthesis of 28S RNA is clearly not eliminated by ethidium bromide, although we have consistently observed a small reduction (20%) in the amount of 28 S synthesized when compared with that in the control culture containing no ethidium bromide (Figure 3a). As mentioned previously, ethidium bromide caused no decrease in the rate of synthesis of cytoplasmic rRNA (28, 18, and 5 S) and cytoplasmic 4S RNA during the labeling period used in these experiments.

Discussion

The experiments described in this paper show that ethidium bromide at 0.2 μg/ml completely inhibits the synthesis of mitochondria-associated 4S RNA B but does not inhibit the synthesis of cytoplasmic rRNA or cytoplasmic 4S RNA. The synthesis of mitochondria-associated 4S RNA A is inhibited approximately 50% by ethidium bromide at 0.2 μg/ml. This inhibition is not significantly increased by higher concentrations of ethidium bromide. Knight and Sugiyama (1969) suggested that 4S RNA A and B may be specific mitochondrial species; however, their sites of synthesis (nuclear or mitochondrial DNA) are unknown. Two hypotheses may be offered to explain the inhibition of synthesis by ethidium bromide of mitochondria-associated 4S RNA. (1) 4S RNA is transcribed from the mitochondrial DNA and the ethidium bromide by a specific interaction with the closed, circular DNA inhibits its synthesis. The synthesis of cytoplasmic rRNA and 4S RNA is not inhibited because at ethidium bromide concentrations used in these experiments the polymerases transcribing from linear DNA are unaffected. (2) The mitochondria-associated 4S RNA is transcribed from the nuclear DNA but the 4S RNA genes are much more sensitive to interaction with ethidium bromide than the genes for cytoplasmic RNA. An analogous situation exists with actinomycin D and the synthesis of cytoplasmic rRNA. Low levels of actinomycin D inhibit the synthesis of rRNA but not the synthesis of mRNA and tRNA (Perry, 1962; Penman *et al.*, 1968). RNA–DNA hybridization experiments in progress should allow a choice between the two aforementioned sites of synthesis. Activation of a specific degradative process for mitochondria-associated 4S RNA can be discarded since it is stable in the presence of ethidium bromide (Table I).

The methylation of 4S RNA A is significantly different from that of cytoplasmic 4S RNA while 4S RNA B is unmethylated (Figure 2) The 4S RNA B peak (Figure 2) has never shown secondary structure while that of 4S RNA A is broad and has always shown secondary structure. This observation as well as the heterogeneity of methylation within the 4S RNA A suggest that A may be a heterogeneous group of molecules while B may be relatively homogeneous as judged by gel electrophoresis. The methylation profiles of A and B reinforce the suggestion that A and B are unique to the mitochondria.

The precise biological roles of mitochondria-associated 4S RNA A and B are not known at present. It should be emphasized that 4S RNA A and B are presented as classes of RNA and not necessarily as individual, homogeneous polynucleotides. 4S RNA A may be a class of mitochondria specific tRNA. Its electrophoretic mobility and methyl content support this suggestion. 4S RNA B may not be a class of tRNA as originally suggested (Knight and Sugiyama, 1969) since it contains no methyl groups; although a class of unmethylated tRNA cannot be rigorously ruled out. The unmethylated 4S RNA B may be mitochondrial specific "5S" rRNA of much smaller molecular weight than its cytoplasmic counterpart and exist as an integral part of a ribosomal subunit (larger). The observation that most of the 4S RNA B is unattached to particles does not support this suggestion (Knight and Sugiyama, 1969). The existence of mitochondria-specific ribosomes in animal cells is unclear at present although O'Brien and Kalf (1967) have reported that the ribosomes in the mitochondrial fraction from rat liver have lower *s* values than the corresponding cytoplasmic ribosomes.

The 28S RNA that occurs in the mitochondrial fraction may in fact be a cytoplasmic 28 S with a slightly different configuration or size (Knight, 1969). No significant differences have been observed between the 5S RNA of the cytoplasm and that occurring in the mitochondrial fraction. The insensitivity of the synthesis of mitochondria-associated 5S and 28S RNA to ethidium bromide suggests that they are synthesized at sites dissimilar to those for 4S RNA A and B.

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Purification and Substrate Specificities of Bacterial Hydroxysteroid Dehydrogenases*

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ABSTRACT: Four highly specific hydroxysteroid dehydrogenases have been purified from bacteria. Substrate specificity studies reveal them to be (a) ring A/B *trans* 3β-hydroxysteroid dehydrogenase, (b) 3α-hydroxysteroid dehydrogenase unable to oxidize 3α-hydroxysteroids ring A/B *cis* with an 11-keto group, (c) ring A/B *trans* 3α-hydroxysteroid dehydrogenase, and (d) an axial 3-hydroxysteroid dehydrogenase.

The possible application of these enzymes for the quantitative determination of specific steroid groups is presented.

The highly specific DPN-dependent hydroxysteroid dehydrogenases would appear to serve as ideal analytical tools for selective microquantitation of steroid substrates in biologic specimens (Stempfel and Sidbury, 1964; Hurlock and Talalay, 1957; Carstensen, 1966). The wild-type *Pseudomonas testosteroni* (ATCC 11996) has been shown to contain two DPN-dependent hydroxysteroid dehydrogenases. β-Hydroxysteroid dehydrogenase specifically oxidizes hydroxyl groups oriented above the plane of the molecule, regardless of the carbon atom to which the hydroxyl is bound. The 3β-, 17β-, and 16β-hydroxysteroids are all oxidized to their corresponding ketosteroids (Talalay and Dobson, 1953). Steroids bearing hydroxyl groups on other carbons are not substrates. α Enzyme activity is represented by the DPN-dependent oxidiation of 3α-hydroxysteroids to corresponding ketosteroids. This specificity includes those steroids having rings A/B *trans* or *cis* in either the androstane or pregnane derivatives.

Purification and characterization of the β and α enzyme from wild-type *Ps. testosteroni* have been carried out (Delin *et al.*, 1964; Squire *et al.*, 1964; Boyer *et al.*, 1965).

Because the α and β enzymes of the wild-type *Ps. testosteroni* are so nonselective, a search for organisms with more specific hydroxysteroid dehydrogenases was attempted. Emphasis has been placed on selective specificity for 3α-hydroxysteroids. These compounds are of extreme importance in the differentiation of the known varieties of congenital adrenal hyperplasia.

Three organisms have been examined in the present work. The first organism, designated STDH-m, is a presumed mutant of *Ps. testosteroni*. It was originally obtained from a stock culture of wild-type *Ps. testosteroni*. An actual relationship between the wild type and the mutant has never been established. Teller and Bongiovanni (1963) reported this organism as having the capacity for oxidation of dehydroepiandrosterone, but not testosterone. It was suggested that

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