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Transformation of Progesterone by *Rhizopus nigricans* REF 129 as Influenced by Modification of the Fermentation Medium

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Hydroxylation of progesterone with *Rhizopus nigricans* REF 129 at C-11, C-6, C-17, C-21 as well as formation of 11α-hydroxy-5α-pregnane-3,20-dione was investigated when the fermentation medium was modified in a different manner. The various transformation products of progesterone were found to be affected qualitatively and quantitatively by the composition of the medium employed. Mg^{2+}, PO_4^{3-} proved to be indispensable for the transformation processes. Mn^{2+} and Fe^{2+} stimulated the reactions. Similarly, acetate, malate and riboflavin enhanced the transformation processes. Formation of the various derivatives of progesterone, except for 11α-hydroxy-5α-pregnane-3,20-dione and 6β,11α-dihydroxyprogesterone, was also favoured by the addition of adenine, adenosine, uracil and guanine to the fermentation medium.

The very important discovery of the microbiological 11α-hydroxylation of progesterone and other steroids by Peterson and Murray[1] served as a new and vigorous stimulus for studies on microbiological transformation of steroids. By using common industrial microbiological methods, chemical transformation of steroids have now been effected in many different ways.[2] However, the studies made for the elucidation of various physiological and biochemical aspects of the microbiological hydroxylation of steroids are still of fragmentary nature. O'Connell *et al*[3] had studied the effect of the composition of the fermentation media on the conversion of substance S (17α,21-dihydroxy-4-pregene-3,20-dione) to cortisone (17α,21-dihydroxy-4-pregnene-3,11,20-trione) and cortisol (11β,17α,21-trihydroxy-4-pregnene-3,20-dione) by *Cunninghamella blakesleeana*. Zetsche[4] also showed that the enzyme system of *Curvularia lunata*, responsible for the introduction of 6β;7α;11β; 14 α hydroxyl groups to progesterone, was affected by the conditions of the culture.

In a previous communication the authors[5] reported the role of some redox agents and trace elements on the transformation of progesterone with *Rhizopus nigricans* REF 129. It was found that the addition of potassium ferricyanide or cadmium ions to the fermentation medium inhibited the formation of 11α-hydroxy-5α-pregnane-3,20-dione and 6β,11α-dihydroxyprogesterone with the subsequent increase of the yields of 11α-hydroxyprogesterone and 11α,17α,21-trihydroxy derivative. However, the formation of the other transformation products, namely, 17α-hydroxyprogesterone; 21-hydroxyprogesterone and 11α, 17α-dihydroxyprogesterone were unaffected. Identity of the different transformation products of progesterone with this local strain of *R. nigricans* were previously confirmed by the authors.[6]

In this paper, we should like to report our studies on the role of some factors that influence the hydroxylation of progesterone with *R. nigricans* REF 129, in a trial to throw some light on the identification of the required cofactors for hydroxylases systems of microbiological origin.

Experimental

Cultivation. The local strain of *R. nigricans* REF 129 was selected from the centre of culture of the Microbiological Chemistry Research Lab., National Research Centre, Cairo. Cultivation was performed in 250-m*l* Erlenmeyer flasks each containing 75 m*l* of the following medium:[5] glucose, 40.0; KH_2PO_4, 0.74; $MgSO_4 \cdot 7H_2O$, 1.0; asparagine, 1.825 g/*l*. The pH was adjusted to 5.5. The flasks were sterilized at 1 atm for 20 min and inoculated with 2-m*l* spore suspension of 48 hr old culture of the pure organism. The culture flasks were agitated on reciprocal shaker (110 strokes/min, amplitude 7 cm) at 30°C±2 for 48 hr; 100 mg of progesterone dissolved in 2 m*l* ethanol was then added to each flask and fermentation was continued for another 72 hr. Throughout the course of this study, grading of the amount of growth by visual estimation

1) D. H. Peterson and H. C. Murray, *J. Amer. Chem. Soc.*, **74**, 1871 (1952).

2) D. H. Peterson, "Biochemistry of Industrial Microorganisms," Rainbow and Rose (Eds.), Acad. Press, London and N. Y. (1963), Ch. 10, p. 537.

3) P. W. O'Connell, K. M. Mann, E. D. Nielson and F. R. Hanson, *Appl. Microbiol.*, **3**, 16 (1955).

4) K. Zetsche, *Archiv für Mikrobiologie*, **38**, 237 (1961).

5) L. A. R. Sallam, A. H. El-Refai and I. A. El-Kady, *J. Gen. Appl. Microbiol.*, in press.

6) L. Sallam, A. El-Refai and I. A. El-Kady, This Bulletin, **43**, 1239 (1970).

has been a routine observation.

Extraction and Analysis of the Mixture of Transformation Products. At the end of the fermentation period, the contents of each flask (medium+fungus) were homogenized in a blender (16000 rpm) with twice its volume of chloroform(150 m*l*). Extraction was repeated 3 times in order to assure that all the transformation products were extracted. The combined chloroform extracts were washed with half its volume 5% sodium bicarbonate followed by an equal volume distilled water, dried over anhydrous sodium sulphate, filtered, then distilled to give a semi-solid residue which was then dissolved in a measured volume of chloroform: methanol (1 : 1, v/v) mixture. The mixture was referred to as "tested material".

Qualitative analysis of the different transformation products encountered during this work was carried out as previously described.[7] The preparative thin-layer chromatographic analysis using silica gel G and standard equipments were used for quantitative determination of the different components of the transformation mixture. A measured volume of the tested material was applied as a streak across the bottom of the glass plate (20×20 cm) covered with a layer of silica gel G (1 mm thickness). The plates were developed with cyclohexane : acetone : chloroform (75 : 25 : 20, v/v). After development, the bands of the individual steroids were marked, scraped off from the plate and quantitatively eluted with spectroscopic ethanol. The concentration of each compound was determined by UV spectrophotometric measurement. The band corresponding to 11α-hydroxy-5α-pregnane-3,20-dione was eluted and the eluate was evaporated in a tared beaker whereby its exact weight was determined.

Results

Inorganic Constituents. In considering the inorganic ions affecting the transformation of progesterone when *R. nigricans* was grown on the basal medium, certain ones seemed to be indispensable. In order to test this idea and investigate the need for other inorganic ions, the basal medium was modified by the omission of the ion under investigation and the addition of various levels of this ion in different experimental series. The data were compared with yields obtained from incubation in the basal medium from which the compound under investigation was omitted.

Effect of $MgSO_4 \cdot 7H_2O$. Study of the effect of the addition of various levels of $MgSO_4 \cdot 7H_2O$ to the fermentation medium(Table 1) showed that the formation of the different transformation products of progesterone was markedly stimulated by the addition of the magnesium salt. Addition of 1.5 g/*l* $MgSO_4 \cdot 7H_2O$ seemed to be optimal for the formation of 11α-, 17α- and 21-monohydroxyprogesterones, as well as 6β,11α- and 11α,17α-dihydroxy derivatives. Although the concentration of 6β,11α-dihydroxyprogesterone did not appear to be affected by the addition of higher levels of $MgSO_4 \cdot 7H_2O$, the concentrations of the other products were gradually decreased under these conditions. However, the concentration of 11α,17α,21-trihydroxyprogesterone was markedly increased with the increase of the magnesium salt level.

Effect of KH_2PO_4. Omission of KH_2PO_4 from the medium markedly reduced the amounts of progesterone transformed (Table 2). Thus, about 23% and 82% of the added progesterone was transformed when the experimental organism was grown on the medium without added phosphate and on the medium supplemented with 1.5 g/*l* KH_2PO_4, respectively. Similarly, the formation of the different derivatives of progesterone was enhanced with phosphate addition. Maximal yields of 11α-, 17α- and 21-monohydroxyprogesterones, as well as 11α,17α,21-trihydroxy derivative were obtained with 1.5 g/*l* KH_2PO_4 treatment. However, the optimal level for the formation of 6β,11α- and 11α,17α-dihydroxyprogesterones seemed to lie at 0.5 g/*l*. On the other hand, the addition of lower concentrations of phosphate exerted no noticeable effect on the formation of 11α-hydroxy-5α-pregnane-3,20-dione. However, inhibition of the formation of this product was observed with higher

Table 1. Hydroxylation of progesterone by *R. nigricans* as influenced by varying the $MgSO_4 \cdot 7H_2O$ level of the fermentation medium

Product (mg/culture)	Control*	$MgSO_4 \cdot 7H_2O$ levels (g/*l*)				
		0.25	0.50	1.00	1.50	2.00
Residual progesterone	82	60	45	27	20	23
21-Hydroxyprogesterone	0.8	1.5	2.1	3.0	3.4	2.4
17α-Hydroxyprogesterone	0.6	1.4	2.7	3.8	4.4	2.0
11α-Hydroxy-5α-pregnane-3,20-dione	0.9	2.5	3.5	4.6	6.6	5.4
11α-Hydroxyprogesterone	4.9	17.2	22	29.5	32	31.4
11α,17α-Dihydroxyprogesterone	1.8	5.1	8.3	11	12.6	10.3
6β,11α-Dihydroxyprogesterone	1.0	3.2	3.6	4.2	4.7	4.7
11α,17α,21-Trihydroxyprogesterone	1.0	4	7.3	11.5	13.2	14.6

* Control medium having the following composition (g/*l*): glucose, 40.0; KH_2PO_4, 0.74; asparagine, 1.825.

7) A. El-Refai, L. Sallam and I. El-Kady, *J. Gen. Appl. Microbiol.*, **15**, 309 (1969).

Table 2. Hydroxylation oe progesterone by *R. nigricans* as influenced by varying the KH_2PO_4 level of the fermentation medium

Product (mg/culture)	Control*	KH_2PO_4 levels (g/*l*)				
		0.25	0.50	1.00	1.50	2.00
Residual progesterone	86.2	47	30	22	18	20
21-Hydroxyprogesterone	0.3	2	3	3	4.8	4
17α-Hydroxyprogesterone	0.3	2	2	3	4	3
11α-Hydroxy-5α-pregnane-3,20-dione	0.6	2.6	3.0	3.0	3.0	2.4
11α-Hydroxyprogesterone	5	22	25	30	38	34
11α,17α-Dihydroxyprogesterone	1	7	14	12	10	8
6β,11α-Dihydroxyprogesterone	1	4	6	5	4	3
11α,17α,21-Trihydroxyprogesterone	0.6	5	8	11	15.9	14.8

* Control medium having the following composition (g/*l*): glucose, 40.0, $MgSO_4 \cdot 7H_2O$, 1.0, asparagine, 1.825.

Table 3. Hydroxylation of progesterone by *R. nigricans* as influenced by varying the $MnSO_4$ level of the fermentation medium

Product (mg/culture)	Control*	$MnSO_4$ levels (g/*l*)				
		0.01	0.05	0.1	0.15	0.2
Residual progesterone	23.6	18.2	15.0	12	10.4	8.8
21-Hydroxyprogesterone	3.3	4.2	4.8	5.5	6.4	7.0
17α-Hydroxyprogesterone	3.3	4.5	5.4	6.6	6.9	6.9
11α-Hydroxy-5α-pregnane-3,20-dione	9.3	4.4	5	5.8	6.4	6.7
11α-Hydroxyprogesterone	30	30.0	30.0	26	24	22.2
11α,17α-Dihydroxyprogesterone	12.5	13.2	13.6	14.4	14.8	15.7
6β,11α-Dihydroxyprogesterone	5.5	5.5	5.9	6.6	6.6	7
17α,21-Dihydroxyprogesterone	—	4.2	4.2	5.3	6.8	7.4
11α,17α,21-Trihydroxyprogesterone	11.1	12	12.6	13.3	14	14.5

* Control medium having the following composition (g/*l*): glucose, 40.0, $MgSO_4 \cdot 7H_2O$, 1.0; KH_2PO_4, 0.74; asparagine, 1.825.

levels of phosphate.

Effect of $MnSO_4$. The data presented in Table 3 show that the addition of $MnSO_4$ to the basal medium stimulated the transformation processes. However, 11α-hydroxylation does not appear to be affected by such addition. The yield of 11α-hydroxyprogesterone was appreciably decreased upon the addition of 0.1—0.2 g/*l* of $MnSO_4$. 17α, 21-Dihydroxyprogesterone, a compound not detected when the experimental organism was grown on the basal medium (control treatment), was detected on the addition of manganous salt. The concentration of this product increased with the increase of the level of the manganous salt.

Effect of $FeSO_4 \cdot 7H_2O$. Addition of the ferrous salt exhibited different effects on the various transformation reactions (Table 4). Hydroxylation at C-17 or C-21 was appreciably enhanced on the addition of the different levels of ferrous salt. On the other hand, the concentration of 11α-hydroxy-5α-pregnane-3,20-dione decreased at the higher levels of $FeSO_4$. The transformation of progesterone to 6β,11α-; 11α,17α- as well as to 11α,17α,21-trihydroxy derivatives did not seem to be appreciably influenced by the addition of ferrous salt to the medium. Hydroxylation into both 17α- and 21-positions was observed when the experimental organism was grown on the basal medium to which ferrous salt was added. Under the experimental conditions employed, the concentration of this product increased with the increase of the level of the ferrous salt.

Organic Acids. The effect of the addition of some organic acids, mostly the intermediates of TCA cycle, to the fermentation medium, on the transformation of progesterone with the experimental organism is presented in Table 5. It was found that the addition of these acids exerted different effects on the transformation reactions. Thus, although the addition of citrate, *cis*-aconitate, fumarate, pyruvate and α-ketoglutarate showed no noticeable effect on the transformation processes, yet malate, succinate, acetate, and oxalate affected the reactions in a different manner. The concentrations of the residual progesterone obtained in the case of malate and acetate were about 46% and

TABLE 4. HYDROXYLATION OF PROGESTERONE BY *R. nigricans* AS INFLUENCED BY VARYING THE $FeSO_4 \cdot 7H_2O$ LEVEL OF THE FERMENTATION MEDIUM

Product (mg/culture)	Control*	$FeSO_4 \cdot 7H_2O$ levels (g/l)				
		0.01	0.05	0.1	0.15	0.2
Residual progesterone	23.6	16.8	12	15	17.2	20
21-Hydroxyprogesterone	3.3	4	5.2	5.2	5.2	6.0
17α-Hydroxyprogesterone	3.3	5.2	5.5	6.2	7.3	7.7
11α-Hydroxy-5α-pregnane-3,20-dione	3.9	3.6	3.4	2.6	2.2	2.2
11α-Hydroxyprogesterone	30	30.0	30.0	25.6	20.7	16.8
11α,17α-Dihydroxyprogesterone	12.5	13.4	12.4	15.6	16.5	17.4
6β,11α-Dihydroxyprogesterone	5.5	5.5	6.4	7.8	7.8	7.8
17α,21-Dihydroxyprogesterone	—	3.3	3.6	4.2	5.6	5.8
11α,17α,21-Trihydroxyprogesterone	11.1	12.2	13.4	14.4	12.2	12.2

* Control medium having the following composition (g/l): Glucose, 40.0; $MgSO_4 \cdot 7H_2O$, 1.0; KH_2PO_4, 0.74; asparagine, 1.825.

TABLE 5. EFFECT OF ADDITION OF SOME ORGANIC ACIDS* ON THE HYDROXYLATION OF PROGESTERONE BY *R. nigricans*

Product (mg/culture)	Control	Citrate	Malate	*cis*-Aconitate	Acetate	Oxalate	Fumarate	Pyruvate	α-Ketoglutarate	Succinate
Residual progesterone	23.6	22.7	11.0	22.0	6.4	36.5	24.4	22.5	24.5	10.5
21-Hydroxyprogesterone	3.3	3.3	3.8	3.3	5.3	2.0	3.0	3.3	3.0	3.2
17α-Hydroxyprogesterone	3.3	3.3	3.6	3.3	6.0	1.8	3.5	3.3	3.0	3.3
11α-Hydroxy-5α-pregnane-3,20-dione	3.9	4.1	4.5	4.1	3.9	3.0	4.3	4.3	3.0	4.2
11α-Hydroxyprogesterone	30.0	32.4	30.8	33.8	36.2	25.5	30.0	32.7	33.0	30.0
11α,17α-Dihydroxyprogesterone	12.5	12.0	17.3	12.0	15.0	10.0	10.8	12.7	12.5	15.2
6β,11α-Dihydroxyprogesterone	5.5	5.5	7.4	5.5	16.2	4.7	5.5	5.8	5.5	5.5
11α,17α,21-Trihydroxyprogesterone	11.2	11.4	16.2	11.5	17.2	10.0	10.6	11.6	11.5	19.2

* These acids in concentration 1 g/l of each were added to the fermentation medium. The initial pH value of the medium was adjusted to pH 5.5 in each treatment. Control medium has the same composition as that in Table 4.

TABLE 6. EFFECT OF ADDITION OF SOME BIOLOGICALLY ACTIVE COMPOUNDS* ON THE HYDROXYLATION OF PROGESTERONE BY *R. nigricans*

Product (mg/culture)	Control**	Adenine	Xanthin	Uracil	Guanine	Adenosine
Residual progesterone	23.6	10.8	2.0	6.1	10.7	12.5
21-Hydroxyprogesterone	3.3	6.7	5.2	6.4	8	7.2
17α-Hydroxyprogesterone	3.3	6.9	5	6.4	8	7.7
11α-Hydroxy-5α-pregnane-3,20-dione	3.9	1.2	4	1.0	2	2
11α-Hydroxyprogesterone	3.0	36	38.4	35.3	31.7	32
11α,17α-Dihydroxyprogesterone	12.5	16.2	15.5	20	18.6	16.7
6β,11α-Dihydroxyprogesterone	5.5	2	5.5	2.1	1.2	2.0
11α,17α,21-Trihydroxyprogesterone	11.1	15.3	18.2	17.4	15.6	14.8

* Added in concentrations of 100 mg/l each.
** The same composition as in Table 4.

27% of that recorded with basal medium (control treatment). The addition of malate favoured the formation of both 6β,11α- and 11α,17α-dihydroxyprogesterones as well as 11α,17α,21-trihydroxy derivative, rather than the transformation of progesterone to the monohydroxy derivatives. Similar results were recorded in the case of succinate treatment, however, in contrast to malate, the formation of 6β,11α-dihydroxyprogesterone was not stimulated in the case of succinate. On the other hand, the yields of the various transformation products except for 6β,11α-dihydroxyprogesterone and 11α-hydroxy-5α-pregnane-3,20-dione were markedly increased when acetate was added to the basal medium. Oxalic acid appeared to exert an inhibitory effect on the transformation processes.

Biological Bases and Nucleoside. The effect of adding some purine, pyrimidine bases as well as a nucleoside (adenosine) on the different transformation processes of progesterone was also investigated. As shown in Table 6, the addition of xanthin and uracil to the fermentation medium increased the concentrations of the transformed progesterone (compare with control treatment). Addition of adenine, adenosine, guanine and uracil stimulated the formation of 17α- and 21-hydroxyprogesterones. The yields of these two products greatly increased as compared with those recorded with the control treatment. Moreover, a noticeable increase in the concentrations of 11α-hydroxyprogesterone; 11α,17α-dihydroxyprogesterone and 11α,17α,21-trihydroxy derivative was observed. In contrast, the formation of 11α-hydroxy-5α-pregnane-3,20-dione and 6β,11α-dihydroxyprogesterone were greatly reduced in the cases of uracil, guanine and adenosine treatments. The addition of xanthine increased also the concentrations of various transformation products except 11α-hydroxy-5α-pregnane-3,20-dione and 6β,11α-dihydroxyprogesterone.

Vitamins. Addition of some individuals of the vitamin B group, namely, nicotinic acid, thiamine and riboflavin to the fermentation medium proved to enhance the transformation reactions (Table 7). The concentration of the residual progesterone greatly decreased with nicotinic acid and thiamine. Moreover, progesterone was almost completely consumed in the case of riboflavin treatment. An appreciable increase in the yields of the various transformation products was generally observed under these experimental conditions, maximal yields being obtained with riboflavin.

Discussion

The nature of the fermentation medium employed is a decisive factor influencing the microbiological transformation of steroids. We have shown that the transformation of progesterone with a local strain of *Rhizopus nigricans* REF 129 was greatly affected by the composition of the fermentation medium. Various transformation products were found to be affected quantitatively as well as qualitatively by the nature of the fermentation medium. In considering the inorganic ions required for an adequate transformation of progesterone with the experimental organism, certain ions seemed to be indispensable. Only Mg^{2+} and PO_4^{3-} were considered as possibly superfluous constituents of the medium. Omission of either one of these two ions proved to inhibit the transformation processes. The activities assayed when the organism was grown on medium without added Mg^{2+} or PO_4^{3-} might be due to the presence of traces of these two ions as impurities already present in the analytical reagent grade chemical ingradient used in the preparation of the medium. Under these conditions the growth of the fungus was greatly inhibited. Similarly, the addition of Fe^{2+} or Mn^{2+} stimulated the hydroxylation process especially at C-17 and C-21. On the other hand, formation of 11α-hydroxy-5α-

TABLE 7. EFFECT OF SOME VITAMINS OF THE B GROUP ON HYDROXYLATION OF PROGESTERONE BY *R. nigricans*

Product (mg/culture)	Control*	Vitamins**		
		Nicotinic acid	Thiamine	Riboflavin
Residual progesterone	23.6	5.4	7.0	traces
21-Hydroxyprogesterone	3.3	5.0	3.3	3.3
17α-Hydroxyprogesterone	3.3	5.0	3.3	3.6
11α-Hydroxy-5α-pregnane-3,20-dione	3.9	4.6	4.6	6.0
11α-Hydroxyprogesterone	300	37.4	38	40.8
11α,17α-Dihydroxyprogesterone	12.5	14	15.2	16.7
6β,11α-Dihydroxyprogesterone	5.5	7.0	6.1	6.1
11α,17α,21-Trihydroxyprogesterone	11.1	14.3	15	16.2

* The same composition as Table 4.

** Vitamins were added to the Sterile medium under aseptic conditions in 750 μg/75 ml each.

TABLE 8. TRANSFORMATION ASSAY OF 21-HYDROXYPROGESTERONE* BY *R. nigricans* WITH THE ADDITION OF Fe^{2+} AND Mn^{2+}** IONS TO THE FERMENTATION MEDIUM

Treatment	Incubation period/hr	Transformation products (mg/culture)			
		21-Hydroxy-progesterone	11α,21-Dihydroxy-progesterone	17α,21-Di-hydroxypro-gesterone	11α,17α,21-Trihydroxy-progesterone
Control***	24	4.5	5.2	—	—
	48	—	9.4	—	—
Control+Fe^{2+}	24	4.2	3.0	2.2	—
	48	—	4.1	3.2	2.1
Control+Mn^{2+}	24	5.0	2.2	2.2	—
	48	—	3.5	2.6	3.2

* 21-Hydroxyprogesterone (10 mg/75 m*l* medium) was charged to 48 hour old culture and fermentation was continued for 24 and 48 hr.

** Fe^{2+} and Mn^{2+} ions were added as 0.1 g/*l*.

*** Control medium: The same composition as in Table 4.

pregnane-3,20-dione decreased on the addition of Fe^{2+} to the medium. A peculiar finding is the presence of 17α,21-dihydroxyprogesterone, in addition to the other derivatives of progesterone, when the experimental organism was grown on the basa lmedium supplemented with Fe^{2+} or Mn^{2+} ions. This product which was not detected on cultivation of the organism on other medium modifications was proposed by the authors[8] to be formed momentarily, by the introduction of 17α-hydroxyl group into 21-hydroxyprogesterone, during the course of transformation to 11α,17α,21-trihydroxyprogesterone. Addition of Fe^{2+} or Mn^{2+} ions to the medium was accompanied with the formation of comparatively lower yields of 11α-hydroxyprogesterone, although the yields of both 17α- and 21-hydroxy derivatives were appreciably increased. It seems, therefore, that the presence of Fe^{2+} or Mn^{2+} stimulated hydroxylation at C-17 and C-21. As a result of the comparatively lower activities of 11α-hydroxylase system, catalyzing the transformation of 17α,21-dihydroxyprogesterone to 11α,17α,21-trihydroxy derivative, the dihydroxylated product was accumulated.

Formation of 17α,21-dihydroxyprogesterone from 21-hydroxyprogesterone was confirmed, however, when 21-hydroxyprogesterone was charged as substrate to fermentation medium supplemented with Fe^{2+} or Mn^{2+} (Table 8). Under these conditions, 21-hydroxyprogesterone was transformed after 24 hr into 11α,21-dihydroxyprogesterone and 17α,21-dihydroxy derivative. After 48 hours of fermentation, 11α,17α,21-trihydroxyprogesterone was detected.

The importance of Mg^{2+}, PO_4^{3-} and Fe^{2+} ions for the microbial hydroxylation of steroids had been reported by some workers.[3,4] Sweat and Bryson,[9] also showed that 11β-hydroxylase of mammalian system is markedly enhanced by the addition of Mn^{2+} and to a lesser extent by Mg^{2+}. Evidence from studies carried out by many workers[10-13] clearly confirms the hydroxylation reaction to be an aerobic reaction requiring conditions for oxidative phosphorylation, and that reduced triphosphopyridine nucleotide (TPNH) is an important cofactor. Diphosphopyridine nucleotide (DPN^+), adenosine triphosphate (ATP), triphosphopyridine nucleotide (TPN^+), nicotinamide, magnesium ions, and intermediates of the Kreb's citric acid cycle were needed as cofactors in the formation and maintenance of TPNH in its reduced state.

In a trial to identify the required cofactors for the hydroxylation processes, the role of intermediates of TCA cycle, some purine and pyrimidine bases and nucleoside as well as some vitamins, on the transformation of progesterone by the experimental organism was investigated. Among the different organic acids tested, acetate, malate and succinate appeared to stimulate the hydroxylation reactions. Similarly, riboflavin, thiamine and nicotinic acids increased the capacity of the organism to transform progesterone. Maximal yields of the various progesterone derivatives were obtained with riboflavin whereby progesterone was practically quantitatively transformed after 3 days of fermentation. Ryan and Engel[14] noted that nicotinic acid amide stimulated the hydroxylation of steroids in mammals presumably because nicotinic acid amide participate in the synthesis of DPNH or TPNH. Zetsche[4] also showed

8) A. El-Refai, L. Sallam and I. El-Kady, *J. Gen. Appl. Microbiol.*, **16**, 137 (1970).

9) M. L. Sweat and M. J. Bryson, *Arch. Biochem. Biophys.*, **96**, 186 (1962).

10) P. Talalay, *Physiol. Revs.*, **37**, 362 (1957).

11) J. K. Grant, *Biochem. J.*, **64**, 559 (1956).

12) H. K. Grant and A. C. Brownie, *Biochem. et Biophys. Acta.*, **18**, 433 (1956).

13) M. L. Sweat and M. D. Lipscomb, *J. Amer. Chem. Soc.*, **77**, 5185 (1955).

14) K. J. Ryan and L. L. Engel, *ibid.*, **78**, 2654 (1956).

that malate and riboflavin the favour hydroxylation of progesterone at 7α,11β and 14α by *Curvularia lunata*.

Addition of adenine, adenosine, xanthin, uracil or guanine to the fermentation medium seemed to enhance the transformation reactions. However, in the cases of adenine, adenosine, uracil and guanine, formation of 11α-hydroxy-5α-pregnane-3, 20-dione as well as 6β,11α-dihydroxyprogesterone was noticeably restricted. Such results are encouraging because the latter two compounds were considered to be undesired products.

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