EXPERIMENTAL RESULTS

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A- Thin-Layer Chromatography of Some C-21 and C-19 Steroids.

Most of the earlier methods described for the identification and determination of the transformation products of progesterone and other steroidal substrates were those in which paper chromatographic techniques were adopted. All of these methods were modifications of the Zaffaroni's method (1950).

In recent years, the thin-layer chromatographic technique proved its advantages over paper partition chromatography as a rapid tool for the identification and resolution of mixtures of steroidal compounds. Thus, Straka and Malikova (1961) were able to separate some pregnanediols and pregnanetriols and reported R_f values for cortisone, cortisol, 17α-hydroxyprogesterone and progesterone. Admec *et al.* (1962) used the same technique for the separation of 11-deoxycortisol, corticosterone, cortisol and tetrahydrocortisol. Bennett and Heftmann (1962) also reported the separation of eighteen corticosteroids and pregnane derivative in submicrogram quantities. The chromatographic separation of Δ^4-androstene-3,17-dione, testosterone, $\Delta^{1,4}$-androstadiene-3,17-dione, 1-dehydrotestosterone, testololactone and 1-dehydrotestololactone obtained from biological oxidation of progesterone was recorded by Helena and Igor (1958). Takeuchi (1963) reported on the relation

between adsorpitivity and functional groups of some steroid hormones, while Lisboa (1965) found that the R_f values of a number of steroidal compounds changed by changing the proportion of ethanol in the solvent mixtures of chloroform and ethanol. Separation of some steroid hormones of the androstane and pregnane groups together with models of some steroid sapogenins on thin-layers of starch bound silica gel was carried out successfully by Smith and Foell (1962).

The use of adsorbents other than silica gel for the chromatographic separation of some steroid hormones was investigated by many workers. Nienstedt (1964) separated some C-19 and C-21 steroid hormones on magnesium trisilicate plates, while Metz (1961) reported upon the use of thin layers of Kieselghur as adsorbent for the rapid assay of enzymic steroid transformation. Thin-layers of mixed adsorbents (SiO_2 + AlO_3) were also used for the separation of steroid mixtures (Dumazert et al. 1962), while plates of silica gel containing sodium fluorescence were used for detection of some androgens (Repole et al. 1965). Identification of some Δ^4-3-oxo-C-21 steroids by induced chemical reaction on thin-layers of silica gel was given by Lisboa (1964).

The aim of the present work was to find out the appropriate thin-layer chromatographic techniques which could be applied both to mixtures of intimately related C-21 and C-19 steroid hormones and for the routine analysis of the different

steroids which might be encountered during this work as products of the transformation of progesterone with the experimental organisms. The majority of the authentic steroids used in this study were recorded to be transformation products formed by various active strains acting on different steroid substrates (Peterson 1963 and Capek et al. 1966). A collection of different solvent systems and colour reagents were used for the separation and identification of such compounds.

Methods:

Glass plates (20x20 cm.) were cleaned by washing with a detergent, tap water, distilled water and alcohol, respectively. The chromatoplates were prepared by slurrying the adsorbent silica gel " G" (Merk) in distilled warer. For five plates 30 gm. of the adsorbent were used in 60 ml. distilled water and the slurry was applied using a Desaga instrument. The plates were dried in an oven at 110°C for one hour and were then left to cool and stored in a desiccator.

Solvent systems:

For the purpose of separation of the different steroid products a number of solvent systems of reagent grade of the following compositions were used:

I - Cyclohexane : chloroform: acetic acid (80 : 10 : 10, V1V).

II- Toluene : Petroleum ether (40-60°): methanol (40:40 :20, V1V).

III - Cyclohexane: acetone : chloroform (75: 25: 25, V1V).

IV - Ethylene chloride: actone (80:20, V1V).

Solvents were mixed immediately before use.

Colour reagents :

A number of colour reagents of the following constitutions were prepared and used:

1 - Aqueous iodine : 0.2 N iodine solution in potassium iodide, (Tumova et al. 1955).

2 - Liebermann-Burchard, (Waldi 1965): 5 ml. acetic anhydride + 5 ml. conc. sulphuric acid. The mixture was added slowly while cooling to 50 ml. absolute ethanol. (After spraying, the plates were heated at 110°C for 10 min).

Authentic samples :

1 - 17α-Hydroxyprogesterone.

2 - 17α-Hydroxyallopregnane-3,20-dione.

3 - 11α-Hydroxyprogesterone.

4 - 11α-Hydroxyallopregnane-3,20-dione.

5 - 11β-Hydroxyprogesterone.

6 - 15α-Hydroxyprogesterone.

7 - 16α-Hydroxyprogesterone.

8 - Deoxycorticosterone.

9 - 17α-Hydroxypregnenolone.

10- 17α-Hydroxyallopregnanolone.

11- Cortisone.

12 - Prednisone.

13 - SubstanceS S.

14 - Corticosterone.

15 - 11α,17α-Dihydroxyprogesterone.

16 - 11α, 6β -Dihydroxyprogesterone.

17 - 11α, 16β -Dihydroxyprogesterone.

18 - Allopregnane-3β, 17α,21-triol-20-one.

19 - Cortisol.

20 - Prednisolone.

21 - Epicortisol.

22 - Androstendione.

23 - $\Delta^{1,4}$-Androstadiene-3,17-dione.

24 - Androsterone.

25 - Testosterone.

26 - 11α-Hydroxytestosterone.

27 - 6β - Hydroxyandrostenedione.

28 - Testololactone.

Samples of steroids were applied as a 0.01 % solution in chloroform or methanol or mixture of both .

Results:

The results of chromatographic separation of the different steroids are represented in Table (1). As shown in the table the first eight compounds are monohydroxy Δ^4-pregnene and allopregnane derivatives containing a ketone at

C-3 and C-20 and differ in the orientation or position of the hydroxyl function. The mixture of these compounds was completely resolved with system II. The resolution of Δ^4- and the 5 α-analogues (17 α-hydroxy allopregnane-3,20-dione, 17 α-hydroxyprogesterone ; 11 α-hydroxyprogesterone -3,20-dione and 11α-hydroxyprogesterone ; compounds 1-4) was achieved with systems I, II and IV. With these systems, the 5 α-analogues (17α-hydroxyallopregnane-3,20-dione and 11α-hydroxyallopregnane-3,20-dione, compounds 2 and 4) moved faster than Δ^4-pregnene derivatives (17 α-dydroxyprogesterone and 11 α-hydroxyprogesterone; compounds 1 and 3), respectively. The latter two compounds appeared more polar due to the presence of an $\alpha\beta$-unsaturated ketone group. The two axial-equatorial epimers, 11 α-hydroxyprogesterone and 11β-hydroxyprogesterone (compounds 3 and 5) were exclusively separated with all systems. In all cases the equatorial 11-OH group in 11 α-hydroxyprogesterone confers high polarity on the molecule compared with the axial OH-group in 11β - hydroxyprogesterone.

The second group of the C-21 steroids comprises the dihydroxy Δ^4-pregnene and allopregnane derivatives (compounds 9-17). The mixture of that group was resolved with systems II and IV. Resolution of 17α-hydroxypregnenolone (compounds 9 and 10) was achieved with systems II, III and IV. In all cases the pregnolone derivatives appeared less polar than the pregnanolone presumably due to the present of the C-5 doulbe bond. Cortisone and its Δ^1-derivative (prednisone),

compounds 11 and 12 were successfully separated with systems II and III.

The third group of C-21 steroids consists of the trihydroxy Δ^4-pregnene and allopregnane derivatives (compounds 18-21).

The separation of mixture of these compounds was accomplished with systems III and IV. Allopregnane -3β,17α,21-triol-20-one had the higher R_f value with all the systems used. Cortisol migrated less than allopregnane 3β, 17α ,21-triol-20-one due to the presence of the B-unsaturated ketone group in ring A of cortisol. The introduction of >C = C at position one in cortisol resulting in an extended conjugation with the α,β-unsaturated ketone group, caused a retardation of the mobility of prednisilone with all the systems used. Similarly the presence of equatorial OH group at C-11 in epicortisol lowered its migration rate and it appeared more polar than cortisol.

The C-19 steroids (compounds 22-28) are of the androstane type. Best resolution of these compounds was obtained with systems II and IV. Androstenedione and its Δ^1-dehydro-derivative(compounds, 22 and 23) were separated with all the systems. The introduction of a double bond at C-1 in the molecule of androstenedione led to an increase in the migration

rate of $\Delta^{1,4}$- androstadiene -3,17-dione with all systems. A phenomenon contrary to that was observed in case of cortisol and its Δ^{1}-dehydroderivative. Better separation of androsterone and testosterone (compounds 24 and 25) was recorded with system II. 11 α -Hydroxytestosterone and 6β-hydroxyandrostenedione (compounds 26 and 27) were easily resolved with all the systems except system I. As expected, the first compounds had lower R_f value with all systems due to the presence of the equatorial 11 α -OH group.

A trial was carried out to study the characteristic colours of the individual steroids by using three different colour reagents and the results are presented in Table (2).

Table (1): The R_f values of the test C-21 and C-19 steroids with different solvent systems*.

No.	Compounds	R_f x 100 with solvent systems			
		I	II	III	IV
1	17 ∝-Hydroxyprogesterone.	33	79	88	83
2	17 ∝-Hydroxyallopregnane-3,20-dione.	44	83	92	94
3	11 ∝-Hydroxyprogesterone.	5	55	75	61
4	11 ∝-Hydroxyallopregnane3,20-dione.	22	67	75	65
5	11 β -Hydroxyprogesterone.	21	69	83	74
6	15 ∝-Hydroxyprogesterone.	5	52	78	61
7	16 ∝-Hydroxyprogesterone.	5	39	75	57
8	Deoxycorticosterone.	39	78	90	89
9	17 ∝-Hydroxypregnenolone.	36	59	62	61
10	17 ∝-Hydroxyallopregnanolone.	36	54	55	51
11	Cortisone.	5	42	33	53
12	Prednisone.	5	34	43	59
13	Substance S.	7	37	44	70
14	Corticosterone.	7	83	69	78
15	11 ∝ ,17 ∝-Dihydroxyprogesterone.	5	36	48	46
16	11 ∝ ,6 β -Dihydroxyprogesterone.	5	33	39	45
17	11 ∝ ,16 β -Dihydroxyprogesterone.	5	30	19	33
18	Allopregnane-3 β,17 ∝ ,21-triol-20-one.	5	39	72	57

Table (1): (Continued).

No.	Compounds	R_f x 100 with solvent systems			
		I	II	III	IV
19	Cortisol.	5	32	40	35
20	Prednisolone.	5	32	33	28
21	Epicortisol.	5	30	17	12
22	Androstendione.	41	61	58	89
23	$\Delta^{1,4}$-Androstadiene-3,17-dione.	42	83	96	72
24	Androsterone.	36	67	68	76
25	Testosterone.	22	86	72	82
26	11 α -Hydroxytesosterone.	5	35	37	26
27	6 β -Hydroxyandrostendione.	5	53	67	56
28	Testololactone.	5	78	72	84

* Revealed by Liebermann-Burchard colour reagent.

Table (2): The different colours of the test C-21 and C-19 steroids upon spraying with the different colour reagents. The colour of each spot was observed in day light (D.L.) and U.V. lamp.

No.	Compounds	I_2/KI*	Chlorosulphonic acid		Liebermann Burchard	
			D.L.	U.V.	D.L.	U.V.
1	17∝-Hydroxyprogesterone.	weak pink	brown	brilliant blue violet	brown	brilliant blue violet
2	11∝-Hydroxyallopregnane-3,20-dione.	pale brown	brown	blue	brown	blue
3	11∝-Hydroxyprogesterone.	deep blue	brown	green yellow	brown	green yellow
4	11∝-Hydroxyallopregnane-3,20-dione.	grey	brown	blue violet	brown	violet
5	11β-Hydroxyprogesterone.	weak brown	brown	green yellow	yellow	green yellow
6	15∝-Hydroxyprogesterone.	pale brown	brown	green yellow	yellow	green yellow
7	16∝-Hydroxyprogesterone.	grey	brown	violet	brown	violet
8	Deoxycorticocosterone.	rust brown	deep blue	brilliant brick red	deep blue	brilliant brick red
9	17∝-Hydroxypregnenolone.	weak yellow	violet	blue	violet	violet
10	17∝-Hydroxyallopregnanolone.	weak yellow	violet	blue violet	violet	violet

Table (2): (Continued).

No.	Compounds	I_2/KI*	Chlorosulphonic acid		Liebermann Burchard	
			D.L.	U.V.	D.L.	U.V.
11	Cortisone.	deep blue	orange	blue	orange	blue
12	Prednisone.	rust brown	brown	brown	brown	deep brown
13	Substance S.	brown	violet	rose	violet	rose
14	Corticosterone.	rust brown	deep olive green	yellow	deep green brown	yellow
15	11α,17α-Dihydroxyprogesterone.	deep blue	brown	sky blue	brown	sky blue
16	11α,6β-Dihydroxyprogesterone.	pale brown	brown	sky blue	brown	sky blue
17	11α,16β-Dihydroxyprogesterone.	pale brown	brown	violet	brown	blue
18	Allopregnane-3β,17α,21-triol-20-one.	blue	brown	pale brown	deep brown	pale brown
19	Cortisol.	pale brown	pale brown	deep brown	brown	deep brown
20	Prednisolone.	pale brown	brown	yellow	pale brown	yellow
21	Epicortisol.	blue	pale brown	yellow	pale brown	yellow

Table (2): (Continued)

No.	Compounds	I_2/KI*	Chlorosulphonic acid		Liebermann Burchard	
			D.L.	U.V.	D.L.	U.V.
22	Androstendione.	yellow brown	blue	violet	blue	violet
23	$\Delta^{1,4}$-androstadiene-3,17-dione.	yellow	rose	grey green	deep rose	deep yellow
24	Androsterone.	pale brown	red brown	grey green	red brown	grey green
25	Testosterone.	brown	yellow brown	pink + yellow	yellow brown	pink + orange
26	11α-Hydroxytestosterone.	pale brown	deep olive green	pink + yellow	deep green	pink + orange
27	6β-Hydroxyandrostendione.	yellow brown	pale brown	blue	pale brown	blue
28	Testololactone.	violet	brown	blue violet	brown	violet

* The spots obtained with I_2/KI reagent acquired a dim colour when observed under U.V. lamp.

B- Transformation Reaction of Progesterone as a Diagnostic Feature in the Classification of Mucorales:

The most important approach in the economical synthesis of the adrenocortical hormones is the microbiological introduction of hydroxyl group into the strategic carbon atoms at C-11, C-17 and C-21 of steroids required for anti-inflammatory activity. Despite widespread ability of the fungi belonging to the order Mucorales to perform these important hydroxylations (Peterson and Murray, 1952; peterson, 1963; Capek et al., 1966; El-Refai et al., 1969; El-Kady et al. 1972 and El-Kady and Allam 1973), yet, there are no available references on making use of a suitable steroid molecule as one of the diagnostic features for the classification of Mucorales, except the limited trial reported by Eppstein et al., (1958), on the oxygenation of some steroids by three isolates, Mucor parasiticus (ATTCC 6476) and Mucor griseo-cyanus (ATTCC 1207 representing the family Mucoraceae and Helicostylum piriforme (ATTCC 8992) representing the family Thamnidiaceae of the order Mucorales.

In the present experiment, the transformation properties of 147 isolates (collected from different sources and localities in Egypt), which belong to 11 species and 5 genera, and classified under 2 families of the order Mucorales were studied .

Progesterone one of the most easily available steroids, was used as the substrate for the enzymic transformation performed by these microorganisms. To elucidate the effect of composition of the medium on the quality and quantity of the transformation reactions, two different media were used in this experiment, 4 and 6 (Table, 5 page 71). The pH of the medium was adjusted to 5.5. Each organism was cultivated on both media (50 ml/ flask), under submerged conditions on a rotatory shaker for 48 hours at 28C°, then 1 ml ethanol containing 30 mg. progesterone was added to each flask. The time course of the transformation was determined after 48 and 72 hours using thin-layer chromatographic technique and two different solvents III and IV (page 39).

The results in Table (3) show that only all isolates of Circinella muscae and of Mucor circinelloides, both belonging to family Mucoraceae, exhibited an oxidative splitting of the side chain of progesterone molecule in position C-17 with Δ^4- androsterone-3, 17-dione and testosterone as intermediary products. The following opening of ring D gave rise to testololactone as the final steroid product.

In the remaining 141 isolates of Mucorales a uniform hydroxylation process was determined in progesterone molecule. It was found that hydroxylation at C-11 was achieved in every case either at site 11 α -only or at both sites 11 α -and 11β- together. However, the quantity differed from one isolate to

another. Also none of the isolates could hydroxylate the steroid molecule at site 11β-only. 11β-Hydroxylation, beside 11 ∝-hydroxylation, regularly occurred in all isolates of Absidia corymbifera of the family Mucoraceae and in several isolates of Cunninghamella elegans and C. echinulata of the family Choanephoraceae.

With respect to the genus Rhizopus, the isolates could be classified into two groups:
The first, comprised all isolates of the species R. oryzae (22 isolates) which transformed progesterone to 11 ∝-hydroxyprogesterone as a single and final end product. The second, comprised the single isolate of R. nodosus and the 47 isolates of R. stoloniferum (R. nigricans) which transformed progesterone to 11 ∝-hydroxyprogesterone and further to the second end product 6β, 11 ∝-dihydroxyprogesterone. Beside this hydroxylation reaction performed by all isolates of R. stoloniferum some isolates gave another monohydroxylated products (21-hydroxyprogesterone and 17 ∝-hydroxyprogesterone) as well as the dihydroxylated products (11 ∝, 17 ∝- and 17 ∝, 21-dihydroxyprogesterone), and three isolates performed the polyhydroxylation of progesterone leading to the yield of 11 ∝, 17 ∝, 21-Trihydroxyprogesterone (epicortisol).

With regard to the bioconversion of progesterone by the genus Mucor, it was noted, as previously described, that

Mucor circinelloides had the ability of an oxidative splitting of the side chain of progesterone. All isolates of Mucor griseo-cyanus were able of a unique type of hydroxylation in which, beside 11 ∝ -hydroxylation, was proved the presence of 14 ∝ -hydroxylation. This monohydroxylation was not recorded in all other isolates of the test genera and species.

All isolates of Mucor racemosus (18 isolates) and Mucor hiemalis (28 isolates) were similar in some aspects; they all gave the monohydroxylated products, 11 ∝ - and 17 ∝ -hydroxy-progesterone and the dihydroxylated products 11 ∝ , 17 ∝ - dihydroxyprogesterone. The differed in some aspects; some isolates of M. hiemalis could achieve a unique monohydroxylation, 21-hydroxyprogesterone which was not recorded in any isolate of M. racemosus. On the other hand, some isolates of M. racemosus displayed a reductive reaction converting 11 ∝ -hydroxyprogesterone into 11 ∝ -hydroxyallopregnane-3, 20-dione. Also the production of trihydroxylated derivative (epicortisol) by some isolates of Mucor hiemalis, proved that these isolates owned the 11 ∝ , 17 ∝ and 21-hydroxylases.

Family Choanephoraceae was represented by 19 isolates which belong to two species of the genus Cunninghamella, C. elegans (6 isolates) and C. echinulata (13 isolates). All test isolates of C. elegans and most isolates of C. echinulata could perform hydroxylation at both 6β- and 11 ∝ - sites yielding 6β, 11 ∝ -dihydroxyprogesterone.

The results also indicate that the type of culture medium (medium, 4 and 6, Table 5) induced no influence on the quality of the metabolites, but the rate of transformation was favoured more in medium 6 containing glucose, starch and corn steep liquor than in medium (4) containing glucose, starch and peptone.

Table (3): Transformation products of the test microorganisms in the progesterone molecule.

Organism	No. of isolate	Source of isolation	Transformation products			
			Mono-hydroxy-progesterone	Di-hydroxy-progesterone	Tri-hydroxy-progesterone	Side chain degradation
Family: Mucoraceae:						
Absidia corymbifera	1	soil	11∝-; 11β-	11∝, 17∝-	-	-
A. corymbifera	8	soil	11∝-; 11β-	11∝, 17∝-	-	-
A. corymbifera	204	soil	11∝-; 11β-	11∝, 17∝-	-	-
Circinella muscae	10	grains	-	-	-	Δ^4-androsteine-3,20-dione + testosterone + testolactone.
C. muscae	39	soil	-	-	-	
C. muscae	43	wheat straw	-	-	-	
Mucor circinelloides	17	soil	-	-	-	Δ^4-androsteine-3,20-dione + testosterone + testolactone
M. circinelloides	55	soil	-	-	-	
M. circinelloides	115	soil	-	-	-	

Table (3): (Continued).

Organism	No. of isolate	Source of isolation	Transformation products			
			Mono-hydroxy-progesterone	Di-hydroxy-progesterone	Tri-hydroxy-progesterone	Side chain degradation
Mucor griseo-cyanus	160	soil	11α-; 14α-	6β, 11α-	-	-
M. griseo-cyanus	500	soil	11α-; 14α-	6β, 11α-	-	-
M. griseo-cyanus	6009	bea- t straw	11α-; 14α	6β, 11α-	-	-
Mucor hiemalis	4	soil	11α-; 17α-	11α, 17α-	-	-
M. hiemalis	5	food-stuff	11α-; 17α-	11α, 17α-	-	-
M. hiemalis	16	soil	11α-; 17α -; 21-	11α, 17α-	-	-
M. hiemalis	18	air	11α-; 17α-	11α, 17α	-	-
M. hiemalis	19	air	11α-; 17α -; 21	(11α, 17α-) ; 11α, 21-	(11α, 17α, 21-)	-
M. hiemalis	20	air	11α-; 17α-	11α, 17α-	-	-
M. hiemalis	27	soil	(11α-); 11β-; 17α-; (21-)	(11α, 17α-)	11α, 17α, 21-(11β, 17α, 21)	-
M. hiemalis	31	soil	11α-; 17α-	11α, 17α-	-	-
M. hiemalis	32	soil	11α-; 17α-	11α, 17α-	-	-
M. hiemalis	53	soil	11α-; 17α-	11α, 17α-	-	-
M. hiemalis	84	soil	11α-; 11β-; 17α-; 21-	11α, 17α-	11α, 17α, 21-(11β, 17α, 21-)	-

Table (3): (Continued).

Organism	No. of isolate	Source of isolation	Transformation products			
			Mono-hydroxy-progesterone	Di-hydroxy-progesterone	Tri-hydroxy-progesterone	Side chain degradation
M. hiemalis	102	soil	11α-; 11β-; 17α-; (21)	11α, 17α-	(11α, 17α, 21-)	-
M. hiemalis	106	soil	11α-; 17α-	11α, 17α-	-	-
M. hiemalis	107	soil	11α-; 17α-; 21-	11α, 17α-	11α, 17α, 21-	-
M. hiemalis	128	soil	11α-; 17α-	11α, 17α-	-	-
M. hiemalis	131	wheat straw	11α-; 17α-; 21-	11α, 17α-	11α, 17α, 21-	-
M. hiemalis	140	pea-nut straw	11α-; 11β-; 17α-; 21-	11α, 17α-; 11α, 21-	11α, 17α, 21-; (11β, 17α, 21-)	-
M. hiemalis	151	soil	11α-; 17α-	11α, 17α-	-	-
M. hiemalis	152	soil	11α-; 17α-	11α, 17α-	-	-
M. hiemalis	161	soil	11α-; 17α-	11α, 17α-	-	-
M. hiemalis	163	soil	11α-; 17α-	11α, 17α-	-	-
M. hiemalis	168	soil	11α-; 17α-; (21-)	11α, 17α-	11α, 17α, 21-	-
M. hiemalis	173	soil	11α-; 17α-	11α, 17α-	-	-
M. hiemalis	176	soil	11α-; 17α-	11α, 17α-	-	-

Table (3): (Continued)

Organism	No. of isolate	Source of isolation	Transformation products			
			Mono-hydroxy-progesterone	Di-hydroxy-progesterone	Tri-hydroxy-progesterone	Side chain degradation
M. hiemalis	4025	wheat grains	11α-;17α-;(21-)	11α,17α-	11α,17α,21-	-
M. hiemalis	4032	cotton seed	11α-;17α-;21-	11α,17α-	11α,17α,21-	-
M. hiemalis	4035	cotton seed	11α-;17α-	11α,17α-	-	-
M. hiemolis	6028	wheat grains	11α-;17α-	11α,17α-	-	-
Mucor racemosus	21	soil	11α-;17α-;11α-H.A.*	11α,17α-	-	-
M. racemosus	28	soil	11α-;17α-;11α-H.A.*	11α,17α-	-	-
M. racemosus	51	soil	11α-;17α-;11α-H.A.*	11α,17α-	-	-
M. racemosus	52	soil	11α-;17α-;11α-H.A.*	11α,17α-	-	-
M. racemosus	88	soil	11α-;17α-;11α-H.A.*	11α,17α-	-	-
M. racemosus	89	wheat straw	11α-;17α-;11α-H.A.*	11α,17α-	-	-
M. racemosus	97	soil	11α-;17α-;11α-H.A.*	11α,17α-	-	-
M. racemosus	126	pea-nut straw	11α-;17α-;11α-H.A.*	11α,17α-	-	-

Table (3): (Continued).

Organism	No. of isolate	Source of isolation	Transformation products			
			Mono-hydroxy-progesterone	Di-hydroxy-progesterone	Tri-hydroxy-progesterone	Side chain degradation
M. racemosus	127	soil	11α-; 17α-; 11α-H.A.	11α,17α-	-	-
M. racemosus	132	soil	11α-; 17α-; 11α-H.A.	11α,17α-	-	-
M. racemosus	133	soil	11α-; 17α-; 11α-H.A.	11α,17α-	-	-
M. racemosus	139	soil	11α-; 17α-; 11α-H.A.	11α,17α-	-	-
M. racemosus	162	soil	11α-; 17α-; 11α-H.A.	11α,17α-	-	-
M. racemosus	306	soil	11α-; 17α-; 11α-H.A.	11α,17α-	-	-
M. racemosus	313	soil	11α-; 17α-; 11α-H.A.	11α,17α-	-	-
M. racemosus	501	soil	11α-; 17α-; 11α-H.A.	11α,17α-	-	-
M. racemosus	4038	cotton seed	11α-; 17α-; 11α-H.A.	11α,17α-	-	-
M. racemosus	6023	pea-nut strew	11α-; 17α-; 11α-H.A.	11α,17α-	-	-
Rhizopus nodosus	199	soil	11α-	6B, 11α-	-	-

Table (3): (Continued).

Organism	No. of isolate	Source of isolation	Transformation products			
			Mono-hydroxy-progesterone	Di-hydroxy-progesterone	Tri-hydroxy-progesterone	Side chain degradation
Rhizopus oryzae	9	soil	11∝-	-	-	-
R. oryzae	13	soil	11∝-	-	-	-
R. oryzae	14	soil	11∝-	-	-	-
R. oryzae	15	soil	11∝-	-	-	-
R. oryzae	23	food-stuff	11∝-	-	-	-
R. oryzae	33	food-stuff	11∝-	-	-	-
R. oryzae	35	soil	11∝-	-	-	-
R. oryzae	44	soil	11∝-	-	-	-
R. oryzae	54	soil	11∝-	-	-	-
R. oryzae	58	fodder	11∝-	-	-	-
R. oryzae	62	food-stuff	11∝-	-	-	-
R. oryzae	81	pea-nut straw	11∝-	-	-	-
R. oryzae	83	soil	11∝-	-	-	-
R. oryzae	100	soil	11∝-	-	-	-
R. oryzae	108	cotton seed	11∝-	-	-	-
R. oryzae	112	pea-nut straw	11∝-	-	-	-
R. oryzae	116	air	11∝-	-	-	-
R. oryzae	121	cotton seed	11∝-	-	-	-
R. oryzae	134	soil	11∝-	-	-	-

Table (3): (Continued).

Organism	No. of isolate	Source of isolation	Transformation products			
			Mono-hydroxy-progesterone	Di-hydroxy-progesterone	Tri-hydroxy-progesterone	Side chain degradation
R. oryzae	157	pea-nut straw	11α-	-	-	-
R. oryzae	179	soil	11α-	-	-	-
R. oryzae	201	soil	11α-	-	-	-
Rhizopus stoloniferum (R. nigricans)	3	food-stuff	11α-	6β,11α-	-	-
R. stoloniferum	7	food-stuff	11α-	6β,11α-	-	-
R. stoloniferum	11	grains	11α-	6β,11α-	-	-
R. stoloniferum	22	air	11α-	6β,11α-	-	-
R. sloloniferum	34	soil	11α-	6β,11α-	-	-
R. stoloniferum	36	soil	11α-	6β,11α-	-	-
R. stoloniferum	41	soil	11α-;17α-	6β,11α-;11α,17α-	11α,17α,21-	-
R. stoloniferum	49	soil	11α-	6β,11α-	-	-
R. stoloniferum	57	soil	11α-	6β,11α-	-	-
R. stoloniferum	66	food-stuff	11α-;17α-	6B,11α-;11α,17α-	11α,17α,21-	-
R. stoloniferum	67	soil	11α-	6β,11α-	-	-
R. stoloniferum	71	soil	11α-	6β,11α-	-	-

Table (3): (Continued).

Organism	No. of isolate	Source of isolation	Transformation products			
			Mono-hydroxy-progesterone	Di-hydroxy-progesterone	Tri-hydroxy-progesterone	Side chain degradation
R. stoloniferum	73	soil	11α-	6β,11α-	-	-
R. stoloniferum	80	soil	11α-	6β,11α-	-	-
R. stoloniferum	91	soil	11α-	6β,11α-	-	-
R. stoloniferum	92	soil	11α-	6β,11α-	-	-
R. stoloniferum	93	air	11α-	6β,11α-	-	-
R. stoloniferum	94	air	11α-	6β,11α-	-	-
R. stoloniferum	99	pea-nut straw	11α-	6β,11α-	-	-
R. stoloniferum	101	air	11α-	6β,11α-	-	-
R. stoloniferum	104	soil	11α-	6β,11α-	-	-
R. stoloniferum	105	soil	11α-	6β,11α-	-	-
R. stoloniferum	110	pea-nut straw	11α-	6β,11α-	-	-
R. stoloniferum	113	cotton seed	11α-	6β,11α-	-	-
R. stoloniferum	114	pea-nut straw	11α-;17α-;21-	6β,11α-;11α,17α-;11α,21-	(11α,17α,21-)	-
R. stoloniferum	117	pea-nut straw	11α-	6B,11α-	-	-
R. stoloniferum	119	pea-nut straw	11α-	6B,11α-	-	-
R. stoloniferum	120	pea-nut straw	11α-	6B,11α-	-	-
R. stoloniferum	122	wheat straw	11α-	6B,11α-	-	-

Table (3): (Continued).

Organism	No. of isolate	Source of isolation	Transformation products			
			Mono-hydroxy-progesterone	Di-hydroxy-progesterone	Tri-hydroxy-progesterone	Side chain degradation
R. stoloniferum	123	wheat straw	11α-	6β,11α-	-	-
R. stoloniferum	124	wheat straw	11α-	6β,11α-	-	-
R. stoloniferum	125	air	11α-	6β,11α-	-	-
R. stoloniferum	129	wheat straw	11α-	6β,11α-	-	-
R. stoloniferum	130	cotton seed	11α-	6β,11α-	-	-
R. stoloniferum	135	cotton seed	11α-	6β,11α-	-	-
R. stoloniferum	137	soil	11α-	6β,11α-	-	-
R. stoloniferum	164	soil	11α-	6β,11α-	-	-
R. stoloniferum	174	soil	11α-	6β,11α-	-	-
R. stoloniferum	177	soil	11α-	6β,11α-	-	-
R. stoloniferum	180	soil	11α-	6β,11α-	-	-
R. stoloniferum	182	soil	11α-	6β,11α-	-	-
R. stoloniferum	309	soil	11α-	6β,11α-	-	-
R. stoloniferum	310	soil	11α-	6β,11α-	-	-
R. stoloniferum	311	soil	11α-	6β,11α-	-	-
R. stoloniferum	1001	soil	11α-	6β,11α-	-	-
R. stoloniferum	6011	pea-nut straw	11α-	6β,11α-	-	-
R. stoloniferum	6027	cotton seed	11α-	6β,11α-	-	-

Table (3): (Continued).

Organism	No. of isolate	Source of isolation	Transformation products: Mono-hydroxy-progesterone	Di-hydroxy-progesterone	Tri-hydroxy-progesterone	Side chain degradation
Family: Choanephoraceae:						
Cunninghamella elegans	2	soil	11α-;11β-	6β,11α-	-	-
C. elegans	6	soil	11α-;17 -	6β,11α-;11α,17α-	-	-
C. elegans	25	soil	11α-	6β,11 α	-	-
C. elegans	167	soil	11α-;11β -	6β,11α-	-	-
C. elegans	200	soil	11α-;11β -	6β,11α-	-	-
C. elegans	6019	cotton seed	11α-;11β -	6β,11α-	-	-
Cunninghamella echinulata	24	soil	11α-;11β-;17α-;21-	11α,17α-;11α,21-	11α,17α,21-	-
C. echinulata	26	soil	11α-	6β,11α-	-	-
C. echinulata	42	soil	11α-	6β,11α-	-	-
C. echinulata	60	soil	11α-	6β,11α-	-	-
C. echinulata	64	soil	11α-	6β,11α-	-	-
C. echinulata	77	soil	11α-;(17α)	11α,17α-	-	-
C. echinulata	79	soil	11α-;(11β-)	6β,11α-	-	-
C. echinulata	82	soil	11α-	6β,11α-	-	-
C. echinulata	138	soil	11α-;11β-	6β,11α-	-	-
C. echinulata	150	soil	11α-;17α-	(11α,17α-)	-	-

CTable (3): (Continued).

Organism	No. of isolate	Source of isolation	Transformation products			
			Mono-hydroxy-progesterone	Di-hydroxy-progesterone	Tri-hydroxy-progesterone	Side chain degradation
C. echinulata	301	soil	11α-	6B,11α-	-	-
C. echinulata	304	soil	11α-	6B,11α-	-	-

- = Metabolites not formed.
( ) = Metabolites formed in trace amounts.
(_) = Metabolites formed in high amounts.
*11α-H.A. = 11α-Hydroxyallopregnane-3,20-dione.

C- Quantitative Estimation of the Different Transformations of Progesterone by the Active Isolates:

It has been shown in the screening experiment (Table, 3) that M. hiemalis (isolate No. 19) gave a high yield of 11 α, 17 α -dihydroxyprogesterone and epicortisol, while isolates No. 27 and 102 of the same mould gave a high yield of both 21-hydroxyprogesterone and epicortisol. Cunninghamella echinulata (isolate No. 77) gave a high production of 17 α - hydroxyprogesterone, while R. stoloniferum (isolate No. 114) gave a high yield of epicortisol.

The aim of this experiment was to determine quantitatively the transformation products and capacities of these organisms.

Quantitative analysis of the different transformation products encountered during this work was carried out as previously described. Preparative thin-layer chromatographic analysis using silica gel GH254 and standard equipment was used.

Cultivation of the organisms:

50 ml. Of medium No. 1 (Table 5, page 71)were dispensed in each of 250 ml. Erlenmeyer flasks. The flasks were sterilised, inoculated with the test fungus and incubated at 28C°. After 48 hours, 25 mg. of progesterone dissolved in 1 ml. ethanol were added to each flask and the fermentation was continued for another 48 hours.

Extraction:

At the end of the fermentation period, the contents of each flask (medium + mycelium), were homogenized with 150 ml. chloroform, and the combined chloroform extracts of each organism were washed separately with half its volume water, dried over anhydrous sodium sulphate, filtered and then distilled to give semi-solid residue. The crude transformation mixture of each organism was raised to constant volume and fractionated using preparative thin-layer technique and the following solvent system as a mobile phase: cyclohexane: acetone: chloroform (75: 40: 40 , v./v./v.)

The results in Table (4) reval that C. echinulata (isolate No. 77) was the most active test isolate. The residual progesterone was the least in its presence, combined with the highest production of 11 α-and 17 α-hydroxyprogesterone and 11 α ,17 α-dihydroxyprogesterone.

This organism was, thus, selected for further experiments:

Table (4): Concentration of residual progesterone and the different transformation products yielded by the test isolates.

Transformation products mg/100 mg added progesterone.	Organisms				
	Mucor hiemalis, isolate No. (19).	*Mucor hiemalis*, isolate No. (27).	*Mucor hismalis*, isolate No. (102).	*Cunninghamella echinulata*, isolate No. (77).	*Rhizopus stoloniferum*, isolate No. (114).
Residual progesterone.	34.2	37.3	40.5	22.7	32.8
11α-Hydroxyprogesterone.	14.3	8.2	8.5	20.2	12.2
11β-Hydroxyprogesterone.	-	4.2	-	-	5.2
17α-Hydroxyprogesterone.	12.4	7.4	10.8	37.5	7.4
21-Hydroxyprogesterone.	7.6	13.8	11.7	-	6.2
11α,6β-Dihydroxyprogesterone.	-	-	-	-	6.0
11α,17α-Dihydroxyprogesterone.	9.2	6.3	10.5	12.3	9.2
11α,21-Dihydroxyprogesterone.	6.8	traces	traces	-	6.8
11α,17α,21-Trihydroxyprogesterone. (epicortisol).	12.4	8.2	14.2	-	9.3
11β,17α,21-Trihydroxyprogesterone.	-	9.7	-	-	-

D- Large Scale Production of 17 α -Hydroxyprogesterone Using Cunninghamella echinulata, Isolate No. 77.

1- Selection of the fermentation medium

The experimental organism was cultivated in a 2-litre Earlenmeyer flasks, each contained 500 ml of the medium. Six types of media of different compositions were employed (Table, 5). The pH of the medium was adjusted to 5.5 . The data presented in Table (6), clearly show that there was a noticeable difference in the concentration of each transformation product with the different fermentation media. Medium 6 which contained glucose, starch and corn-steep liquor proved to be the most suitable for the production of 17α -hydroxyprogesterone, only 24 % of the added progesterone remained unchanged and 30 % of progesterone were transformed to 17 α -hydroxyprogesterone. Medium 1 and 4 were less conducive to 17 α -hydroxylation than medium 6 (30 % and 26 % of progesterone were transformed to 17α -hydroxyprogesterone respectively). Medium 2,3 and 5 were inferior to the previous media.

Table (5). Composition of the different media (gm/litre).

Constituents	Media					
	1*	2	3	4	5	6
Glucose	40	20	20	20	-	20
Dextrin	-	20	-	-	-	-
Hydrol	-	-	20	-	-	-
Starch	-	-	-	20	-	20
Molasses	-	-	-	-	100**	-
Peptone	1.0	5.0	5.0	5.0	-	-
Corn-steep liquor(solid)	-	-	-	-	7.0	7.0
Yeast extract	1.0	-	-	-	-	-
Asparagine	0.7	-	-	-	-	-
$MgSO_4 . 7H_2O$.	1.0	1.0	1.0	1.0	1.0	1.0
KH_2PO_4	0.7	0.7	0.7	0.7	0.7	0.7

* The medium formulated by capek et al. (1964).

** 100 gm equivalent to 44.5 gm of glucose.

Table(6). Assay of the different transformation products obtained after 48 hours of adding progesterone to different fermentation mediua cultivated by C. echinulata (isolate No. 77).

Transformation products (mg./ culture)*	Media					
	1	2	3	4	5	6
Residual progesterone	140.2	250.4	275.3	180.2	300	120
17 ∝-Hydroxyprogesterone	150.6	90.6	80.7	130.8	752	180.2
11 ∝-Hydroxyprogesterone	132.3	111.3	101.8	105.2	507	109.3
11 ∝,17∝-Dihhdroxyprogesterone	70.3	55.2	31.2	.	30.6	61.4

* The values in this work are the arithmetical means of triplicate analysis.