Diosgenin and sterols are the only steroidal compounds available in nature in plenty. These compounds can not be directly used as drugs. However, useful steroidal compounds can be obtained from these two raw materials by chemical and microbiological transformations. Chemical processes are available for the conversion of diosgenin to pregnanolone, 16 DPA and progesterone.

Steroidal compounds lack the 11-hydroxy group which is an essential part of the corticosteroid molecule. Introduction of the hydroxy group at 11-position is possible only by microbial method.

During the course of present studies sixteen fungi, nine yeasts and eight bacteria were screened to find out their ability to convert diosgenin, androstenedione and progesterone to other compounds. It was found that diosgenin was converted to diosgenon by *Fusarium solani* and *Flavobacterium dehydrogenans*. Rothrock *et al.* (1955) obtained the same conversion by using *Penicillium chrysogenum* in 2 percent yield and Nobile (1958) took a patent for the conversion but the yield was very low (2-3 percent). In the present case *F. dehydrogenans* was able to convert diosgenin to diosgenon in 45 to 50 percent yield and *F. solani* in 15-20 percent yields (Somai and Chopra 1981). Androstenedione was attacked by *F. solani*, all the strains of *Gibberella fujikuroi* and *F. dehydrogenans*. *F. solani* and *G. fujikuroi* formed 4-androstene-3,17-dione in 10-15 percent yield. *F. dehydrogenans* formed 4-androstene-3,17-dione in 40-45 percent yield alongwith testosterone in 4-5 percent yields.
In an exhaustive list published by Charney and Herzog (1967), *F. dehydrogenans* has been reported to form androstenedione only but not testosterone whereas in the present case testosterone was also formed. Similarly *G. fujikuroi* has not been reported to bring about this conversion. However, Mamoli and Vercellone (1937 a,b) obtained testosterone by using a yeast culture which was later found to be contaminated with bacteria and the oxidation-isomerisation reaction was attributed to these contaminants. Thus *F. solani* and *F. dehydrogenans* have been found to oxidize 3-hydroxy steroids to 3-ketosteroids alongside the isomerisation of Δ⁵ to Δ⁴ irrespective of the substrate used. The two organisms thus possess an enzyme system which is specific to a part and not to the whole of the steroid molecule.

During the present studies it was found that progesterone was attacked by all the *Aspergillus* spp. screened. Out of these, *A. ochraceus* could convert progesterone to 11α-hydroxyprogesterone in higher yields (30-35 percent) as compared to other *Aspergillus* spp. which gave yields from 5-20 percent. Therefore, *A. ochraceus* was selected to carry out detailed studies on the conversion of progesterone into 11α-hydroxyprogesterone.

Since the yield of 11α-hydroxyprogesterone obtained during screening were not high, therefore, the isolated strain of *A. ochraceus* was subjected to mutation. A new high transforming mutant was isolated through ultraviolet irradiation, which converted progesterone to 11α-hydroxyprogesterone in 57 percent yield. Iisuka et al (1958) subjected *A. usami*, *A. awamori* and *A. niger* to mutagenic treatment and could get two mutants which did not produce dihydroxy compound. Similar results were obtained by Wix et al (1959) when they subjected *A. niger* to UV irradiation but with these mutants though the production
of dihydroxy compound decreased; the production of 11α-hydroxyprogesterone did not increase. Moreover, these mutants could not tolerate high substrate concentration. Sanat et al (1976) produced a mutant of A. ochraceus which converted 2 g/l of progesterone in 77 percent yield and 20 g/l of the substrate in only 43 percent yield. However, this mutant A. ochraceus TS did not produce any dihydroxy compound.

Spores of A. ochraceus have been reported to convert progesterone to 11α-hydroxyprogesterone and various conditions have been worked out by different workers using different strains of A. ochraceus (Schleg and Knight 1962, Vesina et al 1963, Sehgal et al 1968, Singh et al 1968). It has invariably been found that 6β,11α-dihydroxyprogesterone (dihydroxy compound) is also produced in small quantities along with 11α-hydroxyprogesterone. In the present investigation conditions have been optimised for the maximum production of the 11α-hydroxyprogesterone.

It was found that the optimum number of spores was 2.5x10^8 spores/ml and maximum conversion occurred at an oxygen transfer rate of 55-57 mM/l/hr with a substrate concentration of 5 g/l. Optimum conversion was obtained within 42-48 hrs up to which time there was no formation of dihydroxy compound. When the reaction mixture was further incubated the amount of 11α-hydroxyprogesterone decreased and formation of dihydroxy compound increased. However, when the substrate concentration was increased the time of incubation also increased for maximum conversion; whereas, formation of dihydroxy compound decreased. Singh et al (1968) studied three substrate concentrations vis., 5 g, 10 g and 20 g/l. 5 g/l of the substrate was converted to 11α-hydroxyprogesterone in 88 percent yield in 47 hrs, 10 g/l 90 percent in 94 hrs and 20 g/l 95 percent yield in 139 hrs. In the present
investigation the mutant of \textit{A. ochraceus} has been able to convert 20 g/l progesterone to 11\textless{} hydroxyprogesterone in 95 percent yield in 120 hrs. The conversion rate was found to be 0.167 g/l/hr during 72 hrs, 0.156 g/l/hr during 96 hrs and 0.158 g/l/hr during 120 hrs whereas the conversion rate as computed from the data published by Singh \textit{et al} (1968) was 0.125 g/l/hr in 45 hrs and 67 hrs, and 0.138 g/l/hr in 130 hrs. Thus, the rate of conversion and the final yield by the present mutant is higher than that of anyone reported earlier.

With a view to find out an economical medium for sporulation, wheat bran was tried. Though the culture sporulates profusely on wheat bran but the productivity of the spores change to some extent and there was a little decrease in monohydroxy compound and a consequent increase in dihydroxy compound (table 11). However the wheat bran can be used as a sporulation medium inspite of the physiological changes involved.

Spores require an energy source for the transformation (Vezina \textit{et al} 1963 and Singh \textit{et al} 1968). It was found that none of the carbon sources could replace glucose as energy source during the hydroxylation.

There was no significant effect of pH on hydroxylation in the range of 5.0 to 6.5.

Among the carrier solvents studied for the dissolution of progesterone, acetone was found to be the best both in the case of spores as well as mycelium. It was found that acetone was not toxic up to 10 percent concentration.

Among the four different media tested for the growth of the organism and its conversion M4 (table 2) was found to be most suitable for the purpose. Effect of pH was studied in this medium and optimum pH was found to be 6.0.

Vezina \textit{et al} (1963) have studied the effects of inhibitors and activators on progesterone hydroxylation with
the spores of *A. ochraceus*. In the present studies, the effect of inhibitors and activators on the hydroxylation with the mycelium of the organism was studied. It was found that sodium arsenite and mercuric chloride completely stopped the hydroxylation. Sodium fluoride and sodium azide reduced the yield to 10-15 percent. In the case of spores (Vesina et al. 1963), sodium arsenite does not completely inhibit the hydroxylation but brings it down from 70 to 50 percent whereas sodium azide and mercury chloride completely inhibit the hydroxylation.

In the case of transformation by mycelium, the substrate concentration had a marked effect on the products formed. The substrate concentrations tried were 5 g, 10 g, 20 g, 30 g, and 40 g/l. In case of 5 g/l, the optimum conversion was obtained in 24 hr; in 10 g/l, 48 hrs; in 20 g/l, 72 hrs; 30 g/l, 96 hrs and 40 g/l, 120 hrs. Further incubation of each set decreased the 11-ketohydroxyprogesterone and increased the dihydroxy compound. However, the incubation was not continued beyond 120 hrs. Karow and Petsiavas (1956) studied the substrate concentrations ranging between 0.9 g/l to 2.63 g/l and found that 2.0 g/l of the substrate concentration was converted in 83 percent yields whereas with 2.63 g/l of the substrate the conversion was only of the order of 9 percent. Weaver et al. (1960) tried substrate concentrations ranging from 1 to 5 g/l using *Rhizopus nigricans* and 20 to 100 g/l using *Aspergillus ochraceus*. *R. nigricans* could give yields up to 70 percent with 4 g/l of the substrate. With *A. ochraceus*, they obtained yields ranging between 70-90 percent with 20 g/l; yields of 40 percent to 65 percent with 50 g/l and 24 percent with 100 g/l of the substrate. Conversion rates as calculated from their data is between 0.205 to 0.250 g/l/hr in the case of 20 g/l and 0.278 g/l/hr in the case of 50 g/l of the substrate. The mutant of *A. ochraceus* developed in the present studies has higher conversion rate as well as higher yield from
high substrate concentration as compared to those reported earlier (Karow and Petsiavas 1956; Weaver et al 1960).

Table 26 shows the effect of substrate concentration in laboratory fermenters with a working volume of 5 liter and 12 liter. 20 g/l of the progesterone was converted in 48 hrs whereas 40 g/l took 120 hrs for optimum conversion. Karow and Petsiavas (1956) studied the conversion in a fermenter with a working volume of 3.2 litres. They could convert 4 g/l of the substrate by semicontinuous addition obtaining 80 percent yield. In the present studies the total progesterone was added in one batch and the organism was able to tolerate such a high concentration of substrate giving 90 percent yield. However, when the working volume of the fermenter was increased to 12 l the yield of 11α-hydroxyprogesterone was found to be 70 to 75 percent. This indicates that the mutant developed during the course of these investigations has a potential to convert high progesterone concentration to 11α-hydroxyprogesterone giving appreciable yield within a comparative time period. Though there are some reports of 11α-hydroxylation by A. ochraceus using both spores and mycelium but none of the strains reported in the literature has such a high potential.

During the course of studies with the spores of A. ochraceus it was observed that the hydroxylation started within 6 hrs and continued with the increase in the time of incubation. However, formation of dihydroxy compound did not start till 42 hrs of incubation at the lowest substrate concentration (Table 10 & Fig.5). As the substrate concentration was increased, the period of formation of dihydroxy compound also increased. Once the conversion was beyond 70 percent, the formation of dihydroxy compound started. If the incubation was continued beyond the period of maximum conversion, the amount of 11α-hydroxyprogesterone started decreasing with a concomitant increase in the dihydroxy compound. The ratio of 11α-hydroxyprogesterone
to dihydroxyprogesterone appears to be influenced by the concentration of progesterone. Moreover, as long as progesterone was present in the medium in sufficient quantity, the second reaction was very slow. Both the reactions seem to take place in sequential order and not simultaneously.

In the case of transformation by mycelium similar results were obtained. At low substrate concentrations dihydroxy compound was detected after 24 hrs. whereas at higher substrate concentration it was detected only after 48 hrs. Similarly at high substrate concentrations the presence of dihydroxy compound formed was low as compared to that formed at lower substrate concentrations.

In the case of fermenters the dihydroxy compound formed was much less as compared to shake flasks and with 40 g/l of the substrate, dihydroxy compound was not formed even upto 72 hrs of incubation. At 96 hrs the dihydroxy compound was only 0.5 percent which increased to 1.0 percent in 120 hrs.

Normally in other fermentations or transformations, an increase in substrate concentration results in an increase in all the reactions or sometimes there is an inhibition caused by a high substrate concentration. But in the present case, there is no inhibition caused by an increase in the substrate concentrations. Rather the rate of reaction leading to monohydroxylation increased and the rate of second reaction leading to dihydroxylation decreased. Thus this is a unique example of increase in the reaction rate with an increase in the substrate concentration.

In the case of spores the amount of dihydroxy compound formed was much less as compared to that by the mycelium. Moreover, in the spore process no aseptic conditions were necessary and neither a nutrient medium was required. Extraction was relatively simpler but spores could not tolerate high concentrations of the substrate i.e., more than 20 g/l. The rate of conversion was much less than that of the mycelium.
Thus the advantages of the spore process mentioned above were offset by the fact that mycelium could convert a higher substrate concentration. Mycelium of the organism could convert the same substrate concentration in 72 hrs and 40 g/l of the substrate in 120 hrs. Although some extraction problems were encountered in mycelium process, use of mycelium for the conversion of progesterone to 11β-hydroxyprogesterone appears to be preferable over the spore process. Perhaps this may be the reason that spore process has not yet been commercialised (Sebek 1979).

Another factor which plays an important role in 11β-hydroxylation is the oxygen transfer rate. Oxygen transfer rate was found to be different in both the cases. In case of spores the optimum OTR was found to be 55-57 mM/l/hr whereas in case of vegetative mycelium it was found to be 34 mM/l/hr. It appears that in spore process more transfer of oxygen was required for the conversion whereas in case of vegetative cells oxygen required for the conversion was much less. With the vegetative mycelium it was found that increase in the aeration rate did not affect the conversion rate beyond a certain value. At each RPM there was a definite increase in the yield from 0.5 VVM to 1.0 VVM but there was no increase from 1.0 VVM to 1.5 VVM.

Karow and Petalasvag (1956) studied the effect of aeration rate on 11β-hydroxylation in 3 liter fermenters. It was reported that there was no gross difference in the conversion rate at the aeration rates of 0.16, 0.33 and 1.0 VVM. They also studied the effect of three agitation rates i.e., 340, 561 and 760 RPM and found that at 340 RPM the conversion was only upto 60 percent, whereas at 561 and 760 RPM the conversion was upto 80 percent. These studies were conducted with initial substrate concentration of 0.4 g/l. In the case of semicontinuous addition (final steroid concentration 4 g/l), the conversion was again of the order of 60 percent at both 561 and 760 RPM. In the present studies it was found that maximum yield (90-91 percent) was obtained at 500 RPM with an aeration
rate of 1.0 VVM. At 400 and 600 RPM the yields were of the same order i.e., 82-88 percent but comparatively more dihydroxyprogesterone was found at higher RPM. At lower RPM viz., 300 the conversion was less. The higher RPM causes more dispersion of oxygen into the medium thus making more oxygen available for conversion. At low RPM, the dispersion was less, so there was less conversion. By the same reasoning it can be said that more of oxygen was available at 600 RPM, so more of dihydroxy compound was formed.

In the report by Marrow and Potsiavas (1956), practically there was no difference in the dihydroxy compound formed at 561 and 700 RPM.

Since the reaction leading to the formation of dihydroxy compound depends upon the dispersion and availability of the oxygen, not only the agitation rate but also the impeller size would have marked effect on the conversion. In other words dispersion of oxygen would depend upon the tip velocity of the impeller. The tip velocities studied in the present case were 36900, 49200, 61500, 73800/mm/minute. It was obvious (Table 25 & Fig. 10) that optimum conversion was obtained in the range of 60,000 to 70,000 mm/minute.

For any study in fermenters it is important to find out the optimum volume in the given fermenter. This helps in scaling up the process in pilot plant or industrial fermenters. The volume in simpler words is related to the height of the liquid level (H_L). The ratio between liquid height (H_L) and fermenter diameter (D_t) is a direct indication of the air dispersion and hold up of air bubbles. Effect of H_L/D_t varying between 0.48 to 1.10 was studied. It was found that at lower values of this ratio less of 11x-hydroxyprogesterone and more of dihydroxy compound was formed. Similarly at higher values more of dihydroxy compound and comparatively less of 11x-hydroxyprogesterone was formed. However, maximum yields of 11x-hydroxyprogesterone (90 percent) and minimum of dihydroxy compound (0.5 percent) was obtained at H_L/D_t ratio of 0.73 (Table 24).
From a practical standpoint the microbiological transformation of steroids represent a valuable adjunct to the chemical synthesis of the complex steroid hormones and their analogs. The pharmaceutical industry in recent years has come to be more dependent on the microbial transformation of complex molecules usually involving complex stereoisomerism. Microbial reactions have proven invaluable in the preparation of these steroidal compounds where chemical synthesis is not possible. In our country cortisones and other steroidal intermediates are imported to the tune of ₹ 2 crores. Import of prednisolone is of the order of 450 kg. The requirement for the next year 1983-1984 has been computed to be 4000 kg of Prednisolone, 625 kg of Dexamethasone and 850 kg of β-methasone. Chopra & Somal (1981) have estimated the future annual demand of 6700 kg of cortisone and 4500 kg of hydrocortisone. All these compounds are corticosteroids and possess a hydroxy group at the 11 position. Thus the most important intermediate required for all the corticosteroids is the 11α-hydroxy compound, which is not being made in the country and total requirements are met with imports. Thus it is imperative that a technology should be available for the production of 11α-hydroxy compound before we can proceed towards corticosteroids. In the present investigations an organism has been developed which introduces the 11 hydroxy group in the steroid molecule and the parameters have been standardized for the optimum production of 11α-hydroxy-progesterone.