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RECIPROCAL INTERACTIONS OF PROGESTERONE AND 17α-OH-PROGESTERONE AS EXOGENOUS SUBSTRATES OF RAT ADRENAL 21-HYDROXYLASE

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SUMMARY

Due to the small concentration and activity of 17α-hydroxylase present in the rat adrenal, the main corticoids secreated in the rat are DOC, B_k, A_k, 18-OH-DOC and aldosterone, formed directly from progesterone (I). Because of the limited amounts of 17α-OH-progesterone (II) available, the biosynthesis of S_R, F_k and E_k is restricted. Since 21-OH steroid hydroxylase (21-OH-ase) uses both I and II in corticoid biosynthesis in other species, it was considered of interest to study the comparative interactions which could exist between these two precursors and the rat adrenal 21-OH-ase, determining enzymatic constants for I and II (usual and unusual substrates, respectively). Homogenized adrenals from normal rats were incubated with various combinations of concentrations of I-7-^{3}H and/or II-^{14}C, acting as substrates and/or inhibitors of 21-OH-ase. The results showed that 21-OH-ase uses II almost as efficiently as I. The K_m values were about the same for both I and II (13.9 and 14.2 × 10^{-6} M/L), respectively, however, the V_{max} values were 54.6 and 26.0 × 10^{-7} M/L/min for I and II, respectively. The amounts of I required to saturate the 21-OH-ase was double that of II. Further kinetic studies showed that both I and II inhibit the 21-hydroxylation of the other in a reciprocal fashion. While II inhibits the 21-hydroxylation of I by competitive inhibition, I inhibits the 21-hydroxylation of II through a mixed type of inhibition. The results suggest that, rather than the existence of two different specific enzymes (one for I and another for II) as it has been postulated by others, it seems that we are dealing with a 21-hydroxylation system with two active sites. One site used only I and the other site uses I and/or II indistinctively.

INTRODUCTION

If one observes the spectrum of syndromes due to adrenal enzyme deficiencies in humans [1], probably the most frequent is the adrenogenital syndrome in which a deficiency occurs in the 21-hydroxylation of steroids (precursors or intermediates) involved in the biosynthesis of active corticosteroid hormones [1–3]. The lack of 21-hydroxylation results in an appreciable deficiency in cortisol production, associated with virilization due to androgen formation originated from accumulated 17α-hydroxyprogesterone, and over excretion of pregnantriol [4].

This enzyme defect clinically shows two distinctive varieties of the syndrome, based on whether the water-electrolyte homeostasis is either compensated (called also "non-salt losing variety") or not compensated (known as "salt losing variety") [1, 5–7].

The difference in the two varieties depends on either normal (some times hypersecretion of aldosterone) [5, 6] or defective aldosterone biosynthesis [1, 5, 6], respectively. In both instances, deficiency in cortisol production and virilization takes place [1].

Among others, two main propositions have been suggested to explain the different expressions of the 21-hydroxylation deficiency [1]:

(A) There are two different 21-hydroxylating systems, one specific for progesterone and concerned with aldosterone biosynthesis and the other specific for 17α-hydroxyprogesterone (17-OH-P) involved in cortisol biosynthesis.

(B) There is a single enzymatic system responsible for the 21-hydroxylation which, when completely defective, interferes with both, cortisol and aldosterone biosynthesis [1] with the subsequent salt and water loss, however, when partially defective, it may affect only the cortisol biosynthesis without alteration in the aldosterone production, with the subsequent regulatory control of salt and water [4].

Following the general scheme of biosynthesis, as indicated in Fig 1, one can see that starting from progesterone, various hydroxylating systems participate in the adrenocortical steroid hormone biosynthesis. The microsomal 17α-hydroxylase in the

List of Steroids: P = Progesterone = 4-Pregene-3,20-dione; 17-OH-P = 17α-hydroxyprogesterone = 17α-hydroxy-4-Pregnene-3,20-dione; DOC = Cortexone = 21-hydroxy-4-Pregnene-3,20-dione; B_k = Corticosterone = 11β,21-dihydroxy-4-pregnene-3,20-dione: Aldo = Aldosterone = 11β,21-dihydroxy-4-pregnene-18-al-3,20-dione: F_k = Cortisol = 11β,17α,21-trihydroxy-4-pregnene-3,20-dione; S_R = Substance's Reichstein S = Cortexolone = 17,21-dihydroxy-4-pregnene-3,20-dione; 18-OH-DOC = 18-hydroxy-DOC = 18,21-dihydroxy-4-pregnene-3,20-dione; E_k = Cortisone = 17,21-dihydroxy-4-pregnene-3,11,20-trione; A_k = Kendall's Compound A = 21-hydroxy-4-pregnene-3,11,20-trione.

List of Enzymes: 17α-OH-ase = 17α-hydroxylase; 21-OH-ase = 21-hydroxylase; 11β-OH-ase = 11β-hydroxylase.

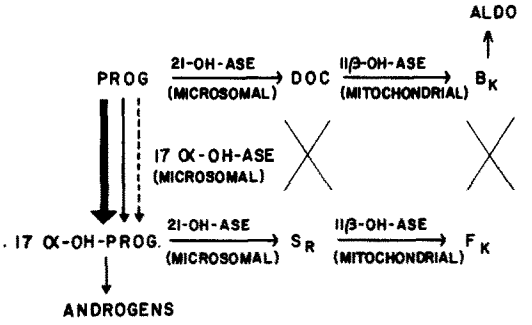

Fig. 1. Scheme of corticosteroid biosynthesis in various species exhibiting differences in the relative activity and concentration of 17α-hydroxylase, as indicated by the intensity of the corresponding arrows.
Note: for abbreviations see list of steroids and enzymes.

adrenal gland converts progesterone to 17-OH-P. These two compounds are the precursors of the two distinctive pathways shown. While progesterone is the precursor of corticosterone and aldosterone, following the upper route, the portion of progesterone which is converted to 17-OH-P becomes precursor of cortisol, following the lower route. Both, progesterone and 17-OH-P are converted through 21-hydroxylation, also in the microsomal fraction, in cortexone (DOC) and cortexolone (S_R or Substance Reichstein's S), respectively. Both steroids are, thereafter, transferred to mitochondria, where by the action of 11β-hydroxylase are converted to corticosterone and cortisol, respectively [8a].

The 17α-hydroxylase, whose relative activity and concentration varies in the adrenals of different species, determines the predominance of cortisol or corticosterone in the adrenal secretion [9–15].

The presence and high activity of 17α-hydroxylase in the human adrenals, for example, is responsible for the significant transformation of progesterone to follow the lower route of biosynthesis and, therefore, the adrenal secretion exhibits a predominance of cortisol (F_k) over corticosterone (B_k). In contrast, as it has been generally accepted, due to lack of 17α-hydroxylase in the rat adrenal, the conversion of progesterone to DOC and B_k, makes the latter, and not cortisol, the dominant corticoid secreted by the rat adrenal.

Recently, due to the high sensitivity and specificity in some of the methods now available, the presence of very small amounts of 17α-hydroxylated steroids, such as cortisol. have been detected in the rat adrenal gland [16], although these compounds are not formed in detectable amounts by the incubation procedures generally used and reported.

Progesterone is the natural substrate of 21-hydroxylase in the rat adrenal, and therefore, when it is incubated with homogenized rat adrenals, because of the absence or perhaps the extremely low effectiveness of 17α-hydroxylase [16, 17], it is not possible to detect formation of 17α-hydroxylated steroids, with the consequent predominance in the formation of DOC, 18-OH-DOC and corticosterone [18]. Besides, it has been shown that cortexone is not converted to cortexolone (8b) and that corticosterone is not converted to cortisol.

17-OH-P could be considered as a non natural substrate for the rat adrenal 21-hydroxylase, however, when it is exogenously added and incubated with rat adrenals, one expects the formation of cortexolone, cortisol and cortisone.

Since in human adrenal both 17α-hydroxylase and 21-hydroxylase are present in the microsomal fraction, and both effectively compete for the same substrate, progesterone, the results obtained from either "*in vivo*" or "*in vitro*" experiments, using this substrate in human adrenal tissue, could be difficult to interpret. In order to study and compare the 21-hydroxylation of progesterone and 17-OH-P, it was decided that the rat adrenal, whose main pattern does not include detectable 17α-hydroxylase, could provide a suitable model to work with.

Using such a model, one anticipates that progesterone could be converted to DOC and B_k without conversion to 17α-OH-steroids, while 17-OH-P used as exogenous substrate could be converted to S_R and F_k.

Since progesterone is a natural substrate, but 17-OH-P is not a common one for the rat adrenal 21-hydroxylase, our first question was to find out if 17-OH-P was converted in their corresponding 21-hydroxylated steroids by the rat adrenal homogenates and if so, in which degree this conversion occurred in comparison with that observed for progesterone, under identical experimental conditions. If both substrates were significantly transformed into their corresponding 21-hydroxylated steroids, the studies of possible interactions between both substrates (progesterone and 17-OH-P) and the probable competitive effect of each of them on the 21-hydroxylation of the other, were justified.

EXPERIMENTAL

Normal female rats of about 180 g were killed by decapitation and their adrenals removed immediately after, decapsulated and homogenized in a phosphate-buffer at pH 7.4, containing 30 mM Nicotinamide. The homogenate was prepared in such a way that 0.8 ml of it, contained one homogenized adrenal. Progesterone-[4-^{14}C] and 17-OH-P-4-^{14}C were used as substrates in parallel incubation in the preliminary comparative study at the concentrations indicated in Fig 2. However, when both steroids were incubated simultaneously as substrates, to study possible reciprocal interactions, a different isotope was used for each of the substrates: progesterone-[7-^{3}H] and 17-OH-P-4-^{14}C in the relative concentrations shown in Fig 3. In order to allow the various hydroxylases to act adequately, a NADPH regenerating system was prepared, consisting of NADP and glucose-6-phosphate in one solution and glucose-6-phosphate-dehydrogenase in a second solution. Their concen-

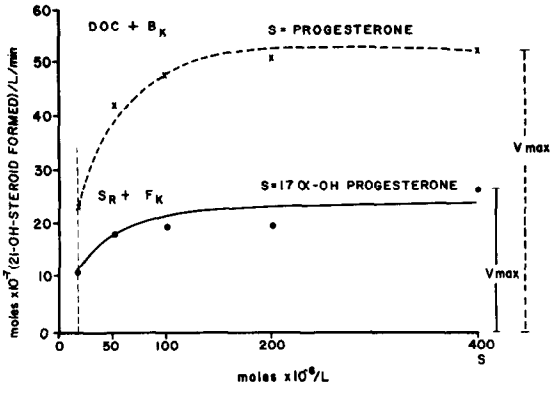

Fig. 2. Relative amounts of 21-hydroxylated steroids formed from either progesterone (×----×) or 17α-OH-progesterone (•——•) when incubated as substrates in various concentrations in the presence of one homogenized rat adrenal.

trations were such, that when 0.1 ml of each of the two solutions were added to the media, the final concentrations of the components were 0.4 mM, 3 mM and 1.0 IU/ml of media, respectively. The incubations were carried out at 37°C, under atmosphere of air, during 10 min in a Dubnoff incubator.

At the end of the incubation period, 10 ml of 0.1N HCl were added and the radioactive steroids were extracted 5 × with ether:$CHCl_3$ (4:1, v:v). The extracts contained from 95 to 100% of the initial radioactivity incubated. The extracts were analyzed by paper chromatography (18, 19, 20a) and the radioactive steroids formed were detected, eluted and aliquots counted, using a Liquid Scintillation Spectrophotometer Nuclear Chicago Corp., Mark I, applying the channel ratio technique for double isotope detection [21]. In some instances, acetylation (20b, 22) and rechromatography were required. The isolated steroids were finally identified by recrystallization in the presence of 20 mg of the authentic corresponding steroid, until constant specific activities were achieved.

Since several other enzymes are present in the incubation media, besides 21-hydroxylase, in order to determine the enzyme activity for each substrate, it was necessary to take into account all the 21-hydroxylated steroid formed from each of the two substrates. For this reason, they were isolated, identified and quantified individually. The 21-hydroxylating activity for progesterone, when it was used as substrate, was determined by adding the amounts expressed in nmol, of cortexone, corticosterone, Kendall's A and 18-OH-DOC formed. On the other hand, when 17-OH-P was the substrate, the sum of the amounts of cortexolone, cortisol and cortisone formed, expressed in nmol, was used to determine the 21-hydroxylase activity for 17-OH-P.

In the incubations in which 17-OH-P was used as substrate, a very small amount of radioactivity was detected, in some chromatograms, at 4-androstendione position, however, since at least half of the initial substrate remained intact at the end of the incubation period, in all instances, the influence of 17–20 desmolase present in the media could not affect significantly the conversion rate of 17-OH-P to cortexolone and cortisol.

RESULTS

In the first comparative study, as shown in the abscissa in Fig 2, various concentrations of either progesterone-[4-^{14}C] or 17-OH-P-4-^{14}C were incubated in parallel tubes in the presence of one homogenized

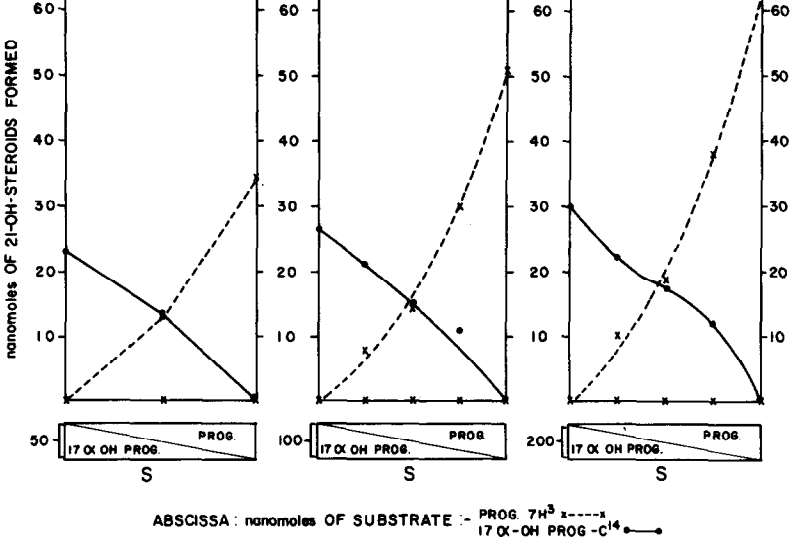

Fig. 3. Amounts of 21-hydroxysteroids formed from either progesterone-[7-^{3}H] or 17α-hydroxy-progesterone-[4-^{14}C], when incubated simultaneously at various relative concentrations of each substrate, as indicated by the diagonal line dividing the rectangles shown under the abscissa axis in each graph, but keeping constant the total initial concentration of both substrates at 50, 100 and 200 nmol/ml/adrenal in the left, center and right graphs, respectively.

adrenal obtained as an aliquote from the same pool of rat adrenals. One can see from the results that the maximum velocity or saturation level of the active sites of the 21-hydroxylating system seemed to be about double when progesterone was used as substrate (dotted line) than when 17-OH-P was used as substrate (solid line).

The results suggest that there are about twice as many active sites for progesterone than the active sites for 17-OH-P, in the rat adrenal 21-hydroxylase.

As indicated in Fig 3, when both substrates, progesterone-[7-^{3}H] and 17-OH-P-4-^{14}C were incubated simultaneously, varying their relative concentrations as it is indicated in the abscissa in the three graphs, but keeping constant the total mass of substrates, which was 50 nmol (in the left graph), 100 nmol (in the centre graph) or 200 nmol (in the right graph), it is possible to observe the curves obtained which represent the total 21-hydroxylated steroids formed from progesterone-[7-^{3}H] (dotted line) as well as from 17-OH-P-4-^{14}C (solid line).

The results show that both substrates were hydroxylated simultaneously in function to their initial relative content. However, by adding the total nmol of 21-hydroxylated steroids formed from the two substrates, there seems to be an indication of a reciprocal inhibitory effect of both steroids on the 21-hydroxylation of the other. Besides, since the points of intersection of both curves (dotted and solid lines), in the three graphs, coincide essentially with the ratio 1:1 for the two initial substrates, independently of the total concentration of the mixture, it suggests that the affinities of the two substrates for the 21-hydroxylating enzyme system are very similar.

Finally, it can be seen that for the three concentrations used, the maximum conversion of progesterone was always greater than that of 17-OH-P in the formation of 21-hydroxylated steroids. As the substrate concentration approaches and finally reaches the enzyme saturation (right graph), the maximum capacity of 21-hydroxylation of progesterone resulted to be about double that of 17-OH-P, as previously shown in Fig 2, when both substrates were incubated separately.

Since the 21-hydroxylation took place simultaneously for both substrates and, when both are present, a reciprocal inhibitory effect was suggested, further experiments were designed to determine the type of inhibition through which the progesterone and 17-OH-P could interact.

In these experiments, one of the two steroids was used as a radioactive substrate, varying its initial concentration (50, 100, 200 and 400 nmol/ml) and incubated in the absence, as well as in the presence of various concentrations (100, 200 and 400 nmol/ml) of the other steroid acting as a non-radioactive inhibitor.

The results obtained were analyzed by three graphic procedures, according to Hanes [23, 24], Woolf [23, 25] and Lineweaver–Burk [23, 26], in order to determine the characteristics of the reciprocal inhibition observed.

When various concentrations of progesterone-[4-^{14}C] (acting as substrate) were incubated in the presence of various concentrations of cold 17-OH-P (acting as inhibitor), the following results were obtained:

1. According to Hanes, the slope of the curves express the reciprocal of the maximum velocities. Since all the lines were parallel, the maximum velocity seems to remain constant in the absence as well as in the presence of various concentrations of 17-OH-P; however, the K_m value changes and the K_p values obtained from the intersection of the curves with the abscissa increase significantly as the concentration of inhibitor increases.

2. When the results were plotted according to Woolf, a significant difference was observed in the slopes of the curves, which became steeper as the concentration of inhibitor increases, indicating a significant change in the K_m value and the K_p values turn larger. At the same time, a tendency for all the curves to cross the ordinate axis at the same point was observed, suggesting a constancy in the maximum velocity values.

3. Finally, when the experimental results were plotted according to Lineweaver–Burk's method, it was possible to see that all the curves tend to cross the ordinate axis at the same point, indicating that the maximum velocity of the 21-hydroxylase remains essentially constant in the absence as well as in the presence of various concentrations of 17-OH-P. However, the K_m and K_p values, obtained at the intersection of the curves with the negative side of the abscissa, were significantly different and these values changed more as the concentration of 17-OH-P, acting as inhibitor, increases. Apparently, according with these results, the inhibitory effect of 17-OH-P on the 21-hydroxylation of progesterone is of the competitive type.

When the role of the two steroids was inverted or in other words, when 17-OH-P-4-^{14}C was used as substrate, in concentrations of 50, 100, 200 and 400 nmol/ml and incubated in the absence, as well as in the presence of various concentrations of cold progesterone (100, 200 and 400 nmol/ml), acting as inhibitor, and the experimental results were plotted again, according to the three different graphical methods described, the following results were obtained:

1. According to Hanes' method, one could see a clear inhibitory effect. The curves were not parallel and they cut the abscissa axis at different points, which means that both, the Michaelis constant as well as the maximum velocities change simultaneously, as the concentration of progesterone, acting as inhibitor increases.

2. Plotting the results according to Woolf's method, one could observe that both, the slopes of the curves,

Table 1. Apparent K_m and V_{max} values obtained for rat adrenal 21-hydroxylase, calculated according to various graphical methods, indicating a reciprocal inhibitory interaction observed between progesterone and 17α-hydroxyprogesterone on their 21-hydroxylation, when one is used as radioactive substrate and the other as cold inhibitor

Substrate-[4-^{14}C] = S Cold inhibitor = I	I nmol/ml	K_m^* and K_p values (a) × 10^{-6} M/L ①	②	③	Ave.	V_{max} values (a) × 10^{-7} M/L/min ①	②	③	Ave.	Type of inhibition
S = Prog-[4-^{14}C] I = 17α-OH-Prog	0	12.5*	13.7*	14.2*	13.5*	54.2	54.6	54.9	54.6	Competitive
	100	116.2	124.0	131.0	123.7	50.2	50.8	52.5	51.2	
	200	303.9	256.2	227.2	262.4	59.6	52.9	48.3	53.6	
	400	393.4	—	305.9	349.7	47.6	—	40.6	44.1	
S = 17α-OH-Prog-[4-^{14}C] I = Progesterone	0	13.6*	14.9*	13.5*	14.0*	27.3	24.8	24.2	25.4	Mixed
	100	30.7	30.5	30.9	30.7	20.8	20.8	20.8	20.8	
	200	54.2	58.7	59.7	57.5	18.2	18.7	18.8	18.6	
	400	68.9	80.0	—	74.5	14.2	13.9	—	14.1	

(a) Individual values and averages for K_m, K_p and V_{max}.
① Hanes Method.
② Woolf Method.
③ Lineweaver–Burk Method.

as well as the intersection with the ordinate axis were different for each of the concentrations of progesterone, used as inhibitor, indicating once more that both the K_m values as well as the maximum velocities change as progesterone concentration increases.

3. Finally, when the results were plotted according to Lineweaver–Burk, it was observed that although the point of intersection of the curves is closer to the ordinate than to the abscissa axis, there is a significant difference in the values for V_{max} as well as a significant difference in the K_m values, as the concentration of inhibitor increases.

These last results are suggesting that progesterone inhibits the 21-hydroxylation of 17-OH-P through a mixed type of inhibition, according to Dixon [23]. In other words, progesterone inhibits the 21-hydroxylation of 17-OH-P partly by competition, but at the same time, there seems to be a secondary inhibitory effect of non competitive type. The actual apparent K_m and/or K_p values as well as the corresponding V_{max} values calculated for each of the three graphic methods described, including the corresponding average values, with respect to the concentration of the steroid acting as inhibitor, in both experimental designs used, are summarized in Table 1.

The upper part of this table, in which radioactive progesterone was used as substrate and cold 17-OH-P as inhibitor, shows a relative constancy in the V_{max} values with a very clear and significant change in the K_m values, as the concentration of the 17-OH-P (inhibitor) increases. In contrast, the lower part of the table, shows that when radioactive 17-OH-P was used as substrate and cold progesterone acted as inhibitor, a progressive change takes place for both K_m and V_{max} values as the concentration of progesterone (inhibitor) increases. The results indicate that 17-OH-P inhibits the 21-hydroxylation of progesterone through a competitive type of inhibition, while the 21-hydroxylation of 17-OH-P seems to be inhibited by progesterone through a mixed type of inhibition.

DISCUSSION

When one considers the hypothesis suggesting the existence of two different 21-hydroxylases, one specific for progesterone and the other specific for 17-OH-P [1, 27] and according with the results obtained in the present study, namely the reciprocal inhibitory effect observed for both substrates on the 21-hydroxylation of the other, there seems to be an indication that such a high specificity for the two different substrates may not be entirely true. Actually, the experimental results obtained, in the present study, are more in favour of supporting the suggestion of the existence of a 21-hydroxylating system that has the peculiar characteristic of having two different active sites (A and B) with distinct properties. Therefore, the following hypothesis is proposed.

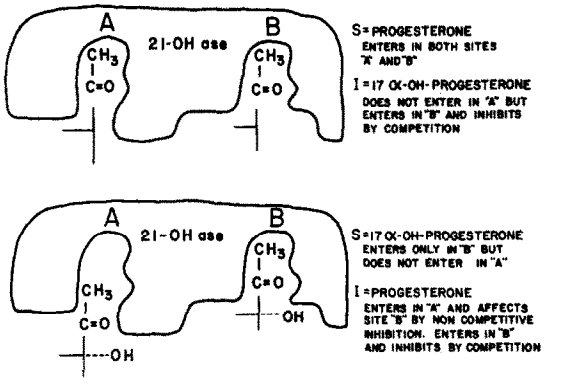

Fig. 4. Scheme showing the alternative possibilities of having one 21-hydroxylating enzyme system exhibiting two different active sites: Site A, which hydroxylates progesterone but, probably for steric impediment does not accept 17α-hydroxy-progesterone as substrate and Site B, which hydroxylates both progesterone and 17α-hydroxy-progesterone indistinctively. The various alternative possibilities for progesterone and 17α-hydroxy-progesterone, acting either as Substrate (S) or Inhibitor (I) are briefly described in the right side of the figure.

The 21-hydroxylating system, at least in the total homogenized rat adrenal, might be represented by one single enzyme with two active sites, as illustrated in Fig 4. Both, sites A and B, accept progesterone as substrate but only one of them (site B) accepts 17-OH-P as substrate. In other words, 17-OH-P, probably due to steric impediment, is unable to enter in the site A, and it uses only progesterone as substrate. In contrast, site B used both, progesterone and 17-OH-P, indistinctively.

When 17-OH-P is present as inhibitor and incubated together with progesterone used as substrate, the inhibitor does not affect site A, but enters in site B and competes with the substrate progesterone. Therefore, 17-OH-P acts as a competitive inhibitor in the 21-hydroxylation of progesterone, through site B.

On the one hand, when progesterone is present as inhibitor and it is incubated together with 17-OH-P, acting as substrate, there is a competition between progesterone and 17-OH-P for site B, since both steroids use it, indistinctively. On the other hand, simultaneously, the inhibitor progesterone enters, bounds and uses site A, requiring cofactors and probably, causing changes in the entire enzyme, which may affect the 21-hydroxylating efficiency of site B, creating a secondary effect of non competitive type, modifying the effectiveness of 21-hydroxylation of 17-OH-P by site B.

This is perhaps the reason why progesterone seems to inhibit the 21-hydroxylation of 17-OH-P through a mixed type of inhibition, partially through competitive and partially through non competitive type of inhibition.

Since the preparation used in the present study consisted of total homogenates obtained from decapsulated rat adrenals, it is important to consider the possibility that the 21-hydroxylating system present in the various adrenal zones (fasciculata, reticularis and glomerulosa) could exhibit differences regarding the characteristics of the active sites described. This remains to be elucidated.

If the results reported now for rat adrenal 21-hydroxylase occurs also in other species, namely in human adrenals, it still has to be proven. However, if one assumes that human adrenal 21-hydroxylating system could exhibit similar characteristics to those observed in the rat adrenals, it might be acceptable to speculate and discuss the present knowledge regarding the alternative explanations for the various types of adrenogenital syndromes due to lack of 21-hydroxylase in humans.

The present paper actually deals with a model which can be applied to other species, including human adrenals. Further studies and experiments to be carried out, will be aware of the peculiar property described here for the rat adrenal 21-hydroxylase.

Let us consider first, the hypothesis suggesting the existence of two different enzymes: one 21-OH-ase-A, specific for progesterone and the other 21-OH-ase-B, specific for 17-OH-P, particularly supported by the studies of Sharma and Dorfman [27] with further support given by the finding of high aldosterone production in the compensated form [6] and the differences in the ability to produce cortisol and aldosterone under ACTH administration and salt deprivation [5]. In this situation, various alternative possibilities could occur:

(a) The defect in both enzymes A and B would result in a simultaneous deficiency of aldosterone and cortisol.

(b) If only the 21-OH-ase-B is deficient while the 21-OH-ase-A remains intact, normal aldosterone would be expected to be associated with deficiency in cortisol biosynthesis.

These two situations would explain the two different varieties of the adrenogenital syndromes described, due to 21-hydroxylase deficiencies, the "salt losing" and the "non-salt losing" syndromes, respectively.

(c) There is another possibility that could correspond to the deficiency in the 21-OH-ase-A without alteration of the 21-OH-ase-B, in which case it could result in deficient aldosterone production with normal levels of cortisol. This means, a "salt losing" syndrome without virilization, a situation which has not been so far reported due to a 21-hydroxylation deficiency, but it has been considered that it can occur and actually it has been attributed to 18-hydroxylase deficiency [1].

The deficiency of either of the two enzymes, 21-OH-ase-A or 21-OH-ase-B could occur, as it has been mentioned above. The deficiency of 21-OH-ase-A has not been clearly and specifically described, while the deficiency of 21-OH-ase-B would correspond to the "non-salt losing" syndrome. However, it is very unlikely and highly improbable to consider a simultaneous deficiency in the two different eznymes in the same individual, in order to explain the "salt losing" variety, mainly if one takes into account that the frequency of this variety is so high [1]. This makes it difficult to accept the possibility of considering two different 21-OH-ases, each of them specific for each of the two substrates, progesterone and 17-OH-P.

Now let us consider the hypothesis proposing one single enzyme, postulated by Bongiovanni and Eberlein [1, 4], who found lower levels of urinary cortisol metabolites in the "salt losing" variety than in the compensated form, suggesting that there are different degrees of enzyme deficiency. If one takes into consideration the peculiar characteristics for the two distinct active sites supported in the present paper, and if the possibility is acceptable that a similar situation could occur in human adrenals, one could easily explain the two clinical varieties of the adrenogenital syndrome due to 21-hydroxylase deficiency in humans.

If the enzyme defect is limited and it alters only

site B, leaving intact site A (see Fig 4), cortisol production will not occur while aldosterone biosynthesis continues through site A, leading to the "non-salt losing" variety described. However, if the enzyme defect is more extensive, it could alter both sites A and B with the subsequent lack of both, cortisol and aldosterone production, as it occurs in the "salt losing" variety of the syndrome.

Moreover, if there is a defect in the enzyme, which could alter only site A, leaving intact site B, both aldosterone and cortisol could be biosynthesized and produced normally, and the genetic defect might be masked in such a way that it could be difficult to detect, at least, with the present methodology available. Actually, the individuals with a defective site A, the latter case, can be easily considered as normal individuals.

REFERENCES

1. Bongiovanni A. M.: In *The Metabolic Basis of Inherited Diseases*, 3rd. Edn. (Edited by J. B. Stanbury, J. B. Wyngaarden and D. S. Fredickson). McGraw-Hill, New York (1972) pp. 857–885.
2. Bongiovanni A. M. and Eder W.: *J. clin. Invest.* **37** (1958) 1342–1347.
3. Bongiovanni A. M. and Eberlein W. R.: *Metab. Clin. Expl.* **10** (1961) 917–935.
4. Bongiovanni A. M. and Eberlein W. R.: *Pediatrics* **21** (1958) 661–672.
5. Degenhart H. J., Visser H. K. A., Wilmink R. and Croughs W.: *Acta endocr. Copenh.* **48** (1965) 587–601.
6. Bartter F. C., Henkin R. I. and Bryan G. T.: *J. clin. Invest.* **47** (1968) 1742–1752.
7. Rosenbloom A. L. and Smith D. W.: *Pediatrics* **38** (1966) 215–219.
8. Dorfman R. I. and Ungar F.: *Metabolism of Steroid Hormones*. Academic Press, New York (1965) pp. 123–213; pp. 203 and 384.
9. Bush I. E.: *J. Endocr.* **9** (1953) 95–100.
10. Romanoff E. B., Hudson P. and Pincus G.: *J. clin. Endocr. Metab.* **13** (1953) 1546–1547.
11. Sweat M.: *J. clin. Endocr. Metab.* **15** (1955) 1043–1056.
12. Hudson P. B. and Lombardo M. E.: *J. clin. Endocr. Metab.* **15** (1955) 324–330.
13. Grant J. K., Forrest A. P. M. and Symington T.: *Acta endocr. Copenh.* **26** (1957) 195–203.
14. Tarrell G. L.: *Fedn. Proc.* **12** (1953) 41.
15. Zaffaroni A. and Burton R. B.: *Archs biochem. Biophys.* **42** (1953) 1–6.
16. Herrera J., León C., Morán L., Raudales L. and Bermúdez J. A. *J. steroid Biochem.* **5** (1974) 312.
17. Hofmann F. G.: *Endocrinology* **60** (1957) 382–389.
18. Domínguez O. V.: *Steroids* suppl. II (1965) 29–49.
19. Burton R. B., Zaffaroni A. and Keutmann E. H.: *J. biol. Chem.* **188** (1951) 763–771.
20. Domínguez O. V.: In *Methods for Steroid Analysis*, 1st Edition (Edited by H. Carstensen). Marcel Dekker, New York (1968) pp. 210–241; pp. 222–226.
21. Bush E. Y.: *Analyt. Chem.* **35** (1963) 1024–1029.
22. Domínguez O.V., Seely R. J. and Gorski J.: *Analyt. Chem.* **35** (1963) 1243–1247.
23. Dixon M. and Webb E. C.: *Enzymes* 2nd Edn. Academic Press, New York (1965) p. 326.
24. Hanes C. S.: *Biochem. J.* **26** (1932) 1406–1421.
25. Woolf B.: In *Allegemeine Chemie der Enzyme* (Edited by J. B. S. Haldane and K. G. Stern), Dresden and Leipzig, Steinkopff Verlag (1932) p. 119.
26. Lineweaver H. and Burk D.: *J. Am. chem. Soc.* **56** (1934) 658–666.
27. Sharma D. C. and Dorfman R. I.: *Fedn. Proc.* **22** (1963) 530.