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The 11-Oxygen Function in Steroid Metabolism*

By I. E. BUSH[1]

It seems appropriate at the present time to discuss the 11-oxygen function in steroid metabolism, a field to which VERZÁR[2] has given stimulating contributions. Much work has accumulated in the last 5 years which has not been reviewed specifically from this point of view. Indeed, it seems striking that this aspect of steroid biochemistry has received so little attention after the first flush of excitement over the therapeutic effects of cortisone was over. This is not to say that the problem has not been the subject of many and interesting pieces of work; it appears merely that such work has not yet been discussed fully from this point of view, and that certain clear lines emerging from these experimental results are in danger of being neglected and lost to view in the mass of work that has been done. In this paper, therefore, an attempt will be made to emphasize these lines, necessarily at the expense of others, with a view to framing some working hypotheses embracing present work. This attempt will involve, necessarily, some speculation, which should be recognized as such, and no attempt will be made to provide a complete review of this field which is already covered admirably by the reviews of LIEBERMAN and TEICH[3], ROBERTS and SZEGO[4], HECHTER and PINCUS[5], and SZEGO and ROBERTS[6].

Physico-chemical factors at C-11

There have been few additions to our knowledge of these factors in recent years, and, although these few contributions are extremely interesting, it is disappointing that more intensive work has not been done. However, a major recent advance is the synthesis and biological testing of the 9α-halogenated 11-hydroxy steroids, which for the first time emphasizes the importance of electronic shifts, at (or near) the 11-oxygen function, in determining its pharmacological properties.

The stereochemical properties of C-11 have long received close attention because of the chemical difficulties of introducing groups at this position. Both the oxo and β-hydroxyl groups are extremely unreactive at this position, while the 11α-hydroxyl group (equatorial configuration) is normally reactive. These findings are readily explained on purely stereochemical grounds (FIESER and FIESER[7]), and it is usual to presume that electronic factors are of little importance compared with the pronounced steric hindrance at this position. The steric factors are in fact rather critical, as shown by the fact that even such small molecules as hydrazine and hydroxylamine will not condense with the 11-oxo group, and the 11β-hydroxyl group is not esterified with acetylating agents unless powerful catalysts are used (OLIVETO *et al.*[8]). These authors also found that the 11β-acetoxy group is not hydrolyzed by refluxing methanolic sodium hydroxide, a remarkable degree of stability for esters of this type.

Further information suggesting the considerable degree of steric hindrance at C-11 comes from studies of the direction of reduction of the 11-oxo group (BERNSTEIN *et al.*[9]). Thus, reduction with metal hydrides, in which the attacking reagent is the relatively large metal hydride ion, gives almost exclusively the 11β-hydroxyl epimer (axial), while reduction with reagents attacking by means of electron transfer and proton attack affords more than 90% yields of the 11α-hydroxyl epimer (equatorial and thermodynamically more stable).

The importance of electronic factors in the properties of the 11-oxygen function has long been suspected but difficult to define or investigate. Thus, 11-oxo-17-oxosteroids show a greatly increased rate of reaction, and an altered colour, with alkaline *m*-dinitrobenzene compared with the 11-deoxy analogues, while 11β-hydroxy-17-oxosteroids show opposite changes. It is difficult to explain these effects on stereochemical grounds since this reaction involves the active methylene group at C-16. Other examples of obvious elec-

* Dedicated to Professor F. VERZÁR, for his 70th birthday on September 18, 1956.

[1] Medical Unit, St. Mary's Hospital, London, W. 2, England.

[2] F. VERZÁR and F. C. WANG, Amer. J. Physiol. *159*, 263 (1949). – F. VERZÁR, *The suprarenal cortex* (Ed. Yoffey, Butterworths, London 1952), p. 39.

[3] S. LIEBERMAN and S. TEICH, Pharmacol. Rev. *5*, 285 (1953).

[4] S. ROBERTS and C. M. SZEGO, Physiol. Rev. *33*, 593 (1953).

[5] O. M. HECHTER and G. PINCUS, Physiol. Rev. *34*, 459 (1954).

[6] C. M. SZEGO and S. ROBERTS, Ann. Rev. Biochem. *24*, 543 (1955).

[7] L. F. FIESER and M. FIESER, *Natural products related to phenanthrene* (Reinhold, New York 1949).

[8] E. P. OLIVETO, C. GEROLD, and E. HERSHBERG, Arch. Biochem. Biophys. *43*, 234 (1953).

[9] S. BERNSTEIN, L. H. LENHARD, and J. H. WILLIAMS, J. org. Chem. *18*, 1166 (1953).

tronic effets of C-11 oxygen functions include, for instance, the regular (and opposed) shifts in absorption maximum of the Δ^4-3-oxosteroids of both C_{21} and C_{19} series produced by the introduction of an 11-oxo or 11-hydroxyl group (ANTONUCCI *et al.*[10]). These effects are remarkable in being apparently transmitted over many C–C bonds in an alicyclic system.

Still more striking from this point of view are the results of FRIED and SABO[11] and GOLDFIEN *et al.*[12] on the biological activity of the 9α-halogenated hydrocortisones. These workers have shown that the order of activities, in bioassays for glucocorticoids and in man, is: F > Cl > Br > H ⩾ I (using hydrocortisone derivatives). This striking correlation with the electronegativity series, with the exception of the large iodine atom which is often abnormal in such series of compounds, suggests that in the biological activity of the 11-oxygenated adrenocortical steroids electronic shifts are at least as important as steric factors in determining the required physico-chemical properties. Other possible interpretations exist and will be discussed later.

The influence of 11-oxygen functions on metabolic pathways

With a few exceptions, the overall direction of steroid metabolism in man and other species is reductive. The reduced products, as with many other substances, are finally conjugated with sulphuric or glucuronic acid and excreted as the relatively soluble and rapidly cleared conjugates (DORFMAN and UNGAR[13]; LIEBERMAN and TEICH[3]). While the introduction of 11-oxygen functions seems to have little influence on the direction of reductions at C-20* and C-17, there is a pronounced effect on reduction at C-3, which has been studied and emphasized by DORFMAN's group (DORFMAN[14]) and by the Sloan-Kettering group (FUKUSHIMA *et al.*[15]: GALLAGHER[16]).

The reduction at C-3 involves usually the reduction of an αβ-unsaturated ketone group so that both the orientation of the resulting hydroxyl group and of the *A/B* ring junction after saturation are involved. The main findings are that 17-hydroxyl-C_{21} steroids give rise almost exclusively to 3α-hydroxy-(5β) steroids whether of C_{21} or C_{19} series, while 11-deoxy-C_{19} steroids give rise to roughly equal parts of (5α) and (5β) metabolites, as do 17-deoxy-C_{21} steroids (ENGEL *et al.*[17]; FUKUSHIMA *et al.*[18]). On the other hand, the 11-oxo-17-oxo steroids (C_{19}) give rise to about four parts of (5α) and one of (5β) metabolites. When this is combined with the fact that the 11-oxygen function is not known to be introduced normally by any tissue other than the adrenal cortex, or to be removed by any tissue of the body, it follows that estimation of the proportion of (5α) and (5β) epimers among the 11-oxygenated C_{19} steroids of urine should enable one to calculate the proportions of hydrocortisone and 11β-hydroxyandrost-4-en-3,17-dione being secreted by the adrenal cortex. It also suggests that reduction of the 17-hydroxy-C_{21} hormones at C-3 and C-4,5 occurs prior to removal of the sidechain in the formation of their 17-oxosteroid metabolites (DORFMAN[19]).

DORFMAN's original presentation was based on rather few cases and was criticized by GALLAGHER[20]. Although subsequent work appears to show that the (5α)/(5β) ratio of metabolites from any one precursor varies fairly considerably both in different subjects, and in the same subject at different times following administration (DORFMAN[21]) most workers have confirmed the general features of DORFMAN's theory which remains one of the most stimulating contributions to steroid metabolism in recent years. Results obtained in the author's laboratory over the last three years have mostly confirmed predictions from this theory, although experience with the type of equations used by DORFMAN[19] has shown that, because of the far higher yield of 11-oxygenated 17-oxosteroids from adrenosterone than from cortisone and hydrocortisone, the calculated adrenal secretion rate of the two types of hormone is subject to enormous error. In one experiment (BUSH *et al.*[22]) good agreement was obtained between the observed adrenal secretion rate of a patient undergoing adrenalectomy, and the urinary metabolites over the same period, but very slight changes in the constants assumed for the percentage conversion of hydrocortisone and 11-hydroxyandrost-4-en-3,17-dione to 17-oxosteroids, would have completely destroyed this agreement. Again the ratio of products from

[10] R. ANTONUCCI, S. BERNSTEIN, M. D. HELLER, R. H. LENHARD, R. LITTEL, and J. H. WILLIAMS, J. org. Chem. *18*, 70 (1953).

[11] J. FRIED and E. F. SABO, J. Amer. chem. Soc. *76*, 1455 (1954).

[12] A. GOLDFIEN, J. C. LAIDLAW, N. A. HAYDAR, A. A. RENOLD, and G. W. THORN, New Engl. J. Med. *252*, 415 (1955).

[13] R. I. DORFMAN and F. UNGAR, *Metabolism of steroid hormones* (Burgess, Minneapolis 1953).

* *Added in proof:* H. J. HUBENER, D. K. FUKUSHIMA, and T. F. GALLAGHER, J. biol. Chem. *220*, 499 (1956) show a striking difference in the effect of 11-oxo and 11β-hydroxyl groups on reduction at C-20.

[14] R. I. DORFMAN, Recent Progr. Hormone Res. *9*, 5 (1954); in: 5th Conference on the Adrenal Cortex (Josiah Macy Jr. Foundation, New York 1954); Ann. N. Y. Acad. Sci. *61*, 291 (1955).

[15] D. K. FUKUSHIMA, H. L. BRADLOW, K. DOBRINER and T. F. GALLAGHER, J. biol. Chem. *206*, 863 (1954). – D. K. FUKUSHIMA, K. DOBRINER, and T. F. GALLAGHER, J. biol. Chem. *206*, 845 (1954).

[16] T. F. GALLAGHER, Recent Progr. Hormone Res. *9*, 26 (1954); in: 5th Conference on the Adrenal Cortex (Josiah Macy Jr. Foundation, New York 1954).

[17] L. L. ENGEL, P. CARTER, and M. J. SPRINGER, Fed. Proc. *13*, 204 (1954).

[18] D. K. FUKUSHIMA, H. L. BRADLOW, K. DOBRINER, and T. F. GALLAGHER, J. biol. Chem. *206*, 863 (1954). – D. K. FUKUSHIMA, K. DOBRINER, and T. F. GALLAGHER, J. biol. Chem. *206*, 845 (1954). – D. K. FUKUSHIMA, A. D. KEMP, R. SCHNEIDER, H. B. STOKEM, and T. F. GALLAGHER, J. biol. Chem. *210*, 129 (1954).

[19] R. I. DORFMAN, Recent Progr. Hormone Res. *9*, 5 (1954).

[20] T. F. GALLAGHER, Recent Progr. Hormone Res. *9*, 26 (1954).

[21] R. I. DORFMAN, Ann. N. Y. Acad. Sci. *61*, 291 (1955).

[22] I. E. BUSH, J. SWALE, and J. PATTERSON Biochem. J. *62*, 16P (1956).

the administration of adrenosterone to a healthy young male agreed well with DORFMAN's figures, while a similar experiment on a man of 58, who had undergone bilateral orchidectomy and adrenalectomy for carcinoma of the prostate, resulted in a complete inversion of the expected ratio.

Further modification of DORFMAN's theory may be necessary to account for our recent finding[23] that the (5α) epimer of tetrahydrohydrocortisone[24] (5α-pregnan-3α,11β,17α,21-tetrol-20-one) is a normal and fairly plentiful metabolite of cortisone and hydrocortisone in human urine. The ratio of this steroid to the related (5β) tetrahydro derivatives is larger than suggested by BURSTEIN *et al.*[25] who were unable to find any of this (5α) metabolite after cortisone or hydrocortisone administration.

Similar effects on the steric course taken by reductions at C-3 and C-4,5 are seen in the catalytic reduction of 11-oxygenated and 11-deoxy steroids (AHRENSTEIN[26]) but the situation now seems without analogy with metabolic reductions, since two separate enzymes are probably responsible, one giving specifically (5α)-reductions, the other specifically (5β)-reductions (TAYLOR[27]; FORCHIELLI *et al.*[28]). In perfused adrenals the 11β-hydroxyl group seems to prevent other hydroxylations taking place at C-17 and C-21 (HECHTER and PINCUS[5]). However, it does not prevent hydroxylation at C-2 and C-6 by liver or the whole organism (e.g. BURSTEIN *et al.*[29]).

It is impossible at present to explain the apparently directive effect of the 11-oxygen function in the C_{19} steroid reductions, but it seems clear that neither 11-oxo nor 11β-hydroxyl groups by themselves can affect the affinity for the two reducing enzymes concerned, since they are too strongly hindered. Nor can a simple increase in solubility (affinity for the medium) be the explanation, since many steroids of far greater solubility due to polar groups elsewhere still undergo (5β) reduction preferentially. Similarly, different intracellular sites for the two enzymes (although quite a possibility in view of their widely different sedimentation rates from homogenates [FORCHIELLI *et al.*[28]]) would not explain the effect unless a specific carrier reacting with 11-oxygen functions were responsible. It is however possible that hydrogen bonding with a small molecule at C-11, or even simple hydration, would produce a considerable deformation of the β-side of the molecule which would interfere with (5β) reduction if this involves proton attack from the surface of the enzyme responsible. No such interference with (5α) reduction would occur if the same mechanism was involved, and the (5α) epimer would be favoured. The absence of such an effect in the C_{21} series could then be due to the fact that the long and flexible 17β-sidechain (SORKIN and REICHSTEIN[30]) of the natural steroids is available for association with the enzyme by the β-surface of the steroid. Since such association will be due to semipolar interactions, the forces involved will also be less affected by deformations of the rest of the molecule than the van der Waal's forces, which will play a larger role in the attraction of the C_{19} steroid *D*-ring to an enzyme site. It remains for experiment to discover whether indeed the separate reducing enzymes concerned have specificities in accord with these considerations.

Interconversion of 11-oxo and 11-hydroxyl groups

Ever since SCHNEIDER[31] isolated cortisone and tetrahydrocortisone from human urine, it has been apparent that there is a striking contrast between the form of 11-oxygenated steroids in the circulating blood and in urine. In the former, 11β-alcohols are found in far larger quantities than the related 11-ketones, while the 11-ketones predominate in urine in most cases (HECHTER and PINCUS[5]; LIEBERMAN and TEICH[3]). The very general application of this rule has been confirmed with every succeeding paper (e. g. BURTON *et al.*[32]; DE COURCY *et al.*[33]; RICHARDSON *et al.*[34]), but the significance of this rule and its now highly significant exceptions have not, to the author's knowledge, yet received close attention. A review of these findings seems overdue in view of the fact that some experiments suggest that the 11β-hydroxyl but not the 11-oxo form is responsible in the last analysis for the peculiar pharmacological activity of 11-oxygenated steroids.

The ready and general conversion of 11β-alcohols to the related ketones was obvious from comparisons of work on peripheral and adrenal venous blood (HECHTER and PINCUS[5]) and on urine (LIEBERMAN and TEICH[3]). The reverse reaction was first clearly shown by BURTON *et al.*[35] who administered cortisone acetate and recovered hydrocortisone from the urine of 5 hu-

[23] I. E. BUSH and M. WILLOUGHBY (unpublished).

[24] Reference substance kindly supplied by Prof. Dr. T. REICHSTEIN.

[25] S. BURSTEIN, K. SAVARD, and R. I. DORFMAN, Endocrinology *52*, 448 (1953); *53*, 88 (1953).

[26] M. AHRENSTEIN in: Discussion at Fed. Meetings of Biological Society, Chicago, April 1953.

[27] W. TAYLOR, Biochim. biophys. Acta *15*, 592 (1954).

[28] E. FORCHIELLI *et al.*, see: R. I. DORFMAN, Ann. N. Y. Acad. Sci. *61*, 291 (1955).

[29] S. BURSTEIN, R. I. DORFMAN, and E. M. NADEL, Arch. Biochem. Biophys. *53*, 307 (1954).

[30] M. SORKIN and T. REICHSTEIN, Helv. chim. Acta *29*, 1218 (1946).

[31] J. J. SCHNEIDER, J. biol. Chem. *183*, 365 (1950); *194*, 337 (1952).

[32] R. B. BURTON, A. ZAFFARONI, and E. H. KEUTMAN, J. biol. Chem. *193*, 769 (1951).

[33] C. DE COURCY, I. E. BUSH, C. H. GRAY, and J. B. LUNNON, J. Endocrinol. *9*, 401 (1953).

[34] E. M. RICHARDSON, J. C. TOUCHSTONE, and F. C. DOHAN, J. clin. Invest. *34*, 285 (1955).

[35] R. B. BURTON, E. H. KEUTMAN, and C. WATERHOUSE, J. clin. Endocrinol. Metab. *13*, 48 (1953).

man subjects. BUSH[36] has found that patients receiving large doses of cortisone by mouth have large concentrations of hydrocortisone in the peripheral plasma but very little cortisone. The reductive direction of this reaction was also favoured in the perfused liver or liver homogenate experiments of many workers (e.g. AMELUNG *et al.*[37]; CASPI *et al.*[38]; FISH *et al.*[39]; MILLER and AXELROD[40]). The interconvertibility of pairs of 11-oxygenated steroids has now been shown (in man) for the following substances:

Hydrocortisone ⇌ Cortisone
Corticosterone ⇌ 11-Dehydrocorticosterone
11 β-hydroxyandrost-4-en-3,17-dione ⇌ Adrenosterone
Tetrahydrohydrocortisone (5 β) ⇌ Tetrahydrocortisone (5 β)
Dihydrohydrocortisone (5 β) ⇌ Dihydrocortisone (5 β)

(BURTON *et al.*[35]; BURSTEIN *et al.*[25]; LIEBERMAN *et al.*[3]; SAVARD *et al.*[41]; SAVARD and GOLDFADEN[42]; RICHARDSON *et al.*[34]). Indeed it is now reasonable to consider these compounds as pairs, the numbers of each pair constituting a possible oxidation-reduction system.

The significance of this type of reaction is still not certain, and it might be held that it is no more interesting than the similar conversions of oxygen functions at C-17 or C-3. A case can be made however for considering this oxo/hydroxyl conversion at C-11 as of peculiar interest and of possible significance in the pharmacological properties of the C-11 oxygen function. Thus reductions at C-17 are at a relatively unhindered position and the 17α-ols predominate (MILLER and DORFMAN[43]) while oxidation of the 17-ketones occurs with both 17α- and 17β-ols (DORFMAN *et al.*[44]; WEST and SAMUELS[45]; PEARSON *et al.*[46]; UNGAR *et al.*[47]; KOCHAKIAN and NALL[48]). While little is known of the specificity and reversibility of reactions of the saturated 3-alcohols, the irreversibility of the reduction of the Δ^4-unsaturated 3-ketones in the general tissues is well established (SAVARD and GOLDFADEN[42]; UNGAR *et al.*[47]). In contrast, the reaction at C-11 is remarkably stereospecific in the reductive direction and, on the basis of the pharmacological inactivity of the 11α-epimers of hydrocortisone and corticosterone, one may assume that this also holds for the oxidative direction *in vivo*.

Interconversion of 11-oxo and 11-hydroxyl groups in man

Root steroid (11-hydroxy form)	Reactions demonstrated hydroxyl ⇋ oxo	Dominant form in man	
		Blood	Urine
Hydrocortisone	⇋	·OH	: O
Corticosterone	⇋	·OH	: O
11 β-hydroxyandrost-4-en-3,17-dione	←	·OH	·OH ?
(5 β)-pregnan-3 α, 11 β, 17 α, 21-tetrol-20-one	⇌	?	: O
(5 β)-pregnan-3 α, 11 β, 21-triol-20-one	⇋	?	: O
(5 β)-androstan-3 α, 11 β-diol-17-one	⇋	?	: O
(5 α)-pregnan-3 α, 11 β, 17 α, 21-tetrol-20-one	←	?	·OH
(5 α)-pregnan-3 α, 11 β, 21-triol-20-one	←	?	·OH
(5 α)-androstan-3 α, 11 β-diol-17-one	←	?	·OH

In the last five compounds it is not strictly true that these "reactions" have been demonstrated, since it is not known whether the reduction at C-3 and C-4,5 occur with 11-oxo, 11-hydroxy, or both forms. (See text for further interpretations.)

The special nature of this interconversion becomes more striking when one examines another aspect of its specificity. It is clear from the above papers (see also the Table) that the reaction is fully reversible for Δ^4-3-ketones of C_{19}, C_{17} 17-deoxy-, and C_{21} 17-hydroxy-series, and also for some (5β) 3-oxo and 3-hydroxy compounds. On the other hand, there is now sufficient evidence to suggest that with (5α)-3α-hydroxy steroids of all three series the reductive direction of the reaction predominates, while the oxidative direction is either impossible or extremely slow. Since this information comes from studies in which the 11-oxo forms of the related (5β) epimers were predominant in the same urine samples, there is no question of individual, or other, variation being involved. Thus, while both 5-epimers of 11-oxo-(5ε)-pregnan-3α,21-diol-20-one were found after administering corticosterone, only the 11-hydroxy form of the (5α) epimer was found (ENGEL *et al.*[17]; RICHARDSON *et al.*[34]; TOUCHSTONE *et al.*[49]). In the case of adrenosterone (or naturally secreted 11β-hydroxyandrost-4-en-3,17-dione [ROMANOFF *et al.*[50]; BUSH *et al.*[22]]) 11β-hydroxyandrosterone predominates in the urine (hydroxy/oxo forms = ca. 9·0), at the same time as 11-oxoetiocholanolone equals or pre-

[36] I. E. BUSH (unpublished).
[37] D. AMELUNG, H. J. HUBENER, L. PROHA, and G. MEYERHEIM, J. clin. Endocrinol. Metab. *13*, 1125 (1953).
[38] E. Y. CASPI, H. LEVY, and O. HECHTER, Arch. Biochem. Biophys. *45*, 169 (1953).
[39] C. A. FISH, M. HAYANO, and G. PINCUS, Arch. Biochem. Biophys. *42*, 480 (1953).
[40] L. L. MILLER and L. R. AXELROD, Metabolism *3*, 438 (1953).
[41] K. SAVARD, S. BURSTEIN, H. ROSENKRANTZ, and R. I. DORFMAN, J. biol. Chem. *202*, 717 (1953).
[42] K. SAVARD and S. H. GOLDFADEN, Fed. Proc. *13*, 288 (1954).
[43] A. M. MILLER and R. I. DORFMAN, Endocrinology *46*, 514 (1950).
[44] R. I. DORFMAN, J. E. WISE, and R. A. SHIPLEY, Endocrinology *46*, 127 (1950).
[45] C. D. WEST and L. T. SAMUELS, J. biol. Chem. *190*, 827 (1951).
[46] S. PEARSON and D. MCGAVACK, J. clin. Endocrinol. Metab. *14*, 472 (1954).
[47] F. UNGAR, A. M. MILLER, and R. I. DORFMAN, J. biol. Chem. *206*, 597 (1954).
[48] C. D. KOCHAKIAN and D. M. NALL, J. biol. Chem. *204*, 91 (1953).
[49] J. C. TOUCHSTONE, H. BULASHENKO, E. M. RICHARDSON, and F. C. DOHAN, Arch. Biochem. Biophys. *52*, 284 (1954).
[50] E. ROMANOFF, P. HUDSON, and G. PINCUS, J. clin. Endocrinol. Metab. *13*, 1546 (1953).

dominates in the (5β) series (SAVARD *et al.*[41]; BUSH *et al.*[22]). Most workers agree in finding little or no 11-oxoandrosterone in human urine compared with the 11-hydroxy form; the earlier findings of RUBIN *et al.*[51] seem to have been contradicted by later experience (DORFMAN[52]; GALLAGHER[53]). Finally, in the C_{21}-17-hydroxy series we have found appreciable quantities of (5α)-pregnan-3α,11β,17α,21-tetrol-20-one (REICHSTEIN's Substanz C) in normal human urine, and in the urine of Addisonian patients treated with cortisone, while the amount of the 11-oxo form must have been less than 1/20 this quantity to have escaped detection (BUSH and WILLOUGHBY[23]).

Whatever the basis for these findings, they clearly indicate a fairly general rule since they apply to three very different types of steroids from both C_{21} and C_{19} series. They are summarized in the Table. It will be found that the simplest hypothesis to account for them, with one exception, must involve at least two special requirements for the system involved, however many enzymes are responsible. In all cases however one has to assume that *in vivo* the 11β-hydroxy (5α) form cannot be oxidized at C-11 or else that the redox equilibrium is very much in favour of the reduced form for the (5α) form alone. In the first case, the added restriction would be required that the (5α)-reducing enzyme did not attack the 11-oxo form at all *in vivo*.

Biological function of the 11-oxygenated steroids

Considerable emphasis has been laid on the interconversion of 11-oxo and 11-hydroxyl groups because it appears not unlikely to be related to the pharmacological influence of the C-11 oxygen function. This possibility has been raised previously (BURTON *et al.*[35]; HECHTER *et al.*[54]; MILLER and AXELROD[40]). In the absence of any other satisfying hypothesis on this matter, it seems justifiable to pursue, in more detail than was possible previously, this particular line of argument, at the risk of seeming unduly speculative, if only to explore the full consequences of such an hypothesis, and to deduce from them suitable experimental tests. It is entirely possible that these interconversions are quite unrelated to pharmacological activity and belong merely to that large class of metabolic reactions by which pharmacological agents are modified until they become inactive products. The tentative hypothesis that this may not be so for the 11-oxygen function has been thought worthy of exploration for the reasons given earlier, but there exists further evidence supporting this conclusion which will be discussed in the following paragraphs*.

The type of activity described as "glucocorticoid" that is the peculiar property of the 11-oxygenated steroids is well known, and its numerous manifestations have been discussed fully by many authors (e.g. SAYERS[55]; VERZÁR and WANG[56]; SHOPPEE[57]). Attempts to define the intimate chemical mode of action of these hormones have failed, and it seems unprofitable to try and juggle with the manifold enzymes, cofactors, cells, tissues, metabolites and electrolytes, that are known to be affected by them. At the present time, it seems more valuable to approach the problem from the opposite direction; namely, to try to limit the possible modes of action by considering the known chemical and biochemical properties of this peculiar oxygen function.

The first question arising from metabolic studies is whether we have any unequivocal evidence that the oxo forms of the 11-oxygenated corticosteroids are active in the molecular processes affected by these hormones. Considerable doubt is shed on a positive answer to this question by the findings of HOLLANDER *et al.*[58] that local intra-articular injection of inflamed joints with hydrocortisone produced considerable objective improvement in function and subjective improvement as loss of pain and stiffness, whereas cortisone was ineffective. This finding has been confirmed by numerous workers and was carefully followed up by ZACCO *et al.*[59] and WILSON *et al.*[60] who examined synovial fluid from such patients for steroid metabolites. It was shown that differences in solubility and rate of disappearance of the two compounds could not account for the difference in therapeutic activity. In addition, it was shown that, while numerous metabolites were formed, the pattern of these differed for the two compounds and that very little hydrocortisone was formed from cortisone, and in some cases none.

Most other studies of the local action of these hormones *in vitro* or on prefused organs have unfortunately not involved an examination of the steroids formed from those administered. Most of these suggest that hydrocortisone is active while cortisone is very much less active or not active at all. Most of the cases in which cortisone was active involved tissues such as

* *Added in proof:* H. J. HUBENER, D. K. FUKUSHIMA, and T. F. GALLAGHER, J. biol. Chem. *220*, 499 (1956) have recently given remarkable support to this contention by showing that reduction of 11-ketones by liver homogenates occurs with Δ^4-3-oxosteroids but *not* with 3α-hydroxy-(5β)-steroids.

[51] B. L. RUBIN, R. I. DORFMAN, and G. PINCUS, Recent Progr. Hormone Res. *9*, 213 (1954).

[52] R. I. DORFMAN, In: 5th Conference on the Adrenal Cortex (Josiah Macy Jr. Foundation, New York 1954).

[53] T. F. GALLAGHER, In: 5th Conference on the Adrenal Cortex (Josiah Macy Jr. Foundation, New York 1954).

[54] O. M. HECHTER, M. M. SOLOMON, and E. CASPI, Endocrinology *53*, 202 (1953).

[55] G. SAYERS, Physiol. Rev. *30*, 241 (1950).

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liver where reduction to the 11-hydroxyl form is known to occur. In no case where cortisone was active locally is it possible to be sure that conversion to hydrocortisone did not occur (ASHTON and COOK[61]; ROBERTS and SZEGO[4]; LESLIE[62]; BALMAIN *et al.*[63]; DUBOIS[67]). In the studies of HOLLANDER *et al.*[58] and CHIU[64]; GRANT and TAYLOR[65]; SINEX[66]; COCHRAN and WILSON *et al.*[60] however, we know that the inability of the synovial tissues to reduce cortisone effectively to hydrocortisone was correlated with inactivity of the former. While in this case we are dealing with the "pharmacological" (i.e. "anti-phlogistic" or "anti-collagen disease") actions of these hormones, the results throw some doubt on the idea that both the 11-ketones and the 11β-alcohols are biologically active. As mentioned above, in the whole organism the circulating steroid is almost entirely in the hydroxyl form, whatever the administered form.

In his stimulating review, HECHTER[68] has pointed out that the hypothesis that hormones act as co-factors or similar reagents in enzymic reactions, has not yet been vindicated by results. It is necessary to emphasize at this stage that this discussion of the possible significance of oxo/hydroxyl interconversion is not meant to suggest that such a reaction must be of the *coenzyme* type. However, the possibility exists that the actual mode of action of these hormones, at the molecular level, is of this nature, the crucial reaction being the oxidation of the hydroxyl form, with reduction of the oxo form being carried out by another enzyme, not necessarily in the same tissue (e.g. absent from synovial tissues). While agreeing with HECHTER's general position, therefore, the author feels that this possibility should not be neglected.

While it is not possible to go any further (i.e. at the molecular level) on the basis of biochemical findings at present available, one can attempt to set limits to the possible chemical reactions involved in the mode of action of these hormones, using the physico-chemical facts at present known. It does not matter whether a substance acts by being coenzyme, carrier, inhibitor, or other known or unknown class of reagent, the introduction of a special type of pharmacological activity by introducing one grouping can only happen in a limited number of ways. These can be generalized as follows:

(a) The group inhibits the inactivation of the substance;

(b) it activates another region of the molecule to undergo reactions;

(c) it undergoes a specific reaction itself, usually an exchange or other reversible reaction;

(d) it alters the distribution of the root molecule at cellular or molecular levels, or at both.

In the case of the 11-oxygen function we know that, if anything, this group increases the rate of inactivation of the root substance. Again, the present evidence suggests that electronic effects on the rest of the molecule, while present, are small, although no information is available on the effects on the sidechain of the corticosteroids. We are therefore left with the last two possibilities. As far as (d) is concerned; it has already been pointed out that mere alteration of distribution, by change in solubility caused by introduction of the 11-oxygen function, is unlikely to be a sufficient explanation of its pharmacological effects. Again, there are severe steric limits placed on the formation of any stable linkage with 11-oxo or 11β-hydroxyl groups at low temperatures and pressures, so that attachment to specific groups of enzymes, proteins, or cellular surfaces by this type of groups seems highly unlikely, though not impossible. On the other hand, there is already evidence to hand that (c) can and does occur with the oxygen function in question, and that the oxidized form is inactive in a situation where reduction to the alcohol is impossible or limited.

It was suggested earlier that the pronounced effect of introducing 9α-halogen atoms is probably due to electronic effects at C-11. This is not unlikely since the inductive effect of the halogen atom α to the 11-oxygen function will be appreciable. If however oxidation/reduction at C-11 is involved in the mechanism of action of these hormones, an alternative explanation of the action of 9α-halogen atoms is possible. In view of the stereochemical specificity of this oxidation/reduction system, it may be compared with the action of the metal hydrides; in other words, the reduction probably involves proton attack from a donor approaching from the α-side of the molecule. If an enzyme is responsible, this would be the expected direction of attack, and the halogen atom might act by association with the active site on the enzyme by polar interaction or actual hydrogen bonding. This type of action would be very sensitive to steric factors, which would explain the negative effect of the very large iodine atom.

In *summary*, then, it has been suggested that present data enable us to place considerable limits on the possible modes of action of the C-11 oxygen function on physico-chemical grounds. It has also been suggested that one observed type of reaction at this position will satisfy those limits. However, it should be obvious that the large number of, as yet unknown, factors involved forbids us to go further and say that this reaction is indeed the basis of the pharmacological

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activities of this oxygen function. The suggestive correlation between certain pharmacological results and purely chemical considerations is at the moment tenuous, since many more examples are needed before such inductions can command our confidence. These correlations should, however, encourage us to perform experiments designed to confirm or invalidate the tentative theory outlined above.

Speculation is often sterile, and in the biochemical field inductive arguments from incomplete data are particularly prone to be upset by even small additions to that data. When however a suggestive arrangement of existing data can be made, and no other is yet obvious, it does no harm to examine the consequences of such an arrangement. The application of William of Occam's Razor suggests that the hypothesis that oxidation/reduction at C-11 may be the basis of the biological action of the cortico-steroids is not unworthy of experimental trial, but no one, least of all the author, will be surprised if once again William's shave is postponed by the blunting action of a Baconian Experiment.

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Zusammenfassung

Es wird versucht, die möglichen Zusammenhänge zwischen den physiko-chemischen Eigenschaften der C_{11}-Gruppe, dem Stoffwechsel und der biologischen Wirkung der 11-Oxysteroide aufzuzeigen. Die Bedeutung der reversiblen Oxydationreduktion dieser Gruppe wird besonders hervorgehoben.

On the Intraneural State of Acetylcholine*

By O. Loewi[1], New York

From experiments following soon after the discovery of the chemical transmission of nervous impulse it was concluded that the primary action of the impulse propagated in cholinergic nerves consists in increased synthesis by the nerve-endings of ACH (Acetylcholine) which subsequently is released[2]. Later it was demonstrated that the nerve-endings during nerve stimulation do indeed synthetize ACH but only secondary to its release and in an amount just compensating for the loss[3]. In fact, intact cholinergic nerves even under conditions such as isolation and rest, where continuous destruction and resynthesis is most improbable, maintain a fairly constant level of ACH in spite of the simultaneous presence of cholinesterase. The apparent protection from esterase is regarded as an indication that the ACH is present in the organs in an indiffusible state preventing contact with the esterase. The extent of this protection and the difference in fate between freely diffusible ACH and that present in an isolated, inactive organ is clearly shown in experiments[4] in which the ventricle of a frog heart was divided in two. The ACH was determined immediately in one half and after an hour in the second half: the values were found to be identical. In another experiment ACH was added at the start to the second half. After an hour the ACH content was found to be equal the original value, indicating that only the added diffusible ACH was destroyed.

There arises the question of whether the total content of ACH is indiffusible also in non-isolated, resting organs. The answer to this question is necessary for recognizing the activities by which the nervous impulse leads to the release of ACH: if the whole ACH is present within the nerve and especially its endings in an indiffusible state, the nervous impulse has to render part of it diffusible and to release it. If a part is diffusible, the nervous impulse has only to release it from the nerve-endings. There exist so far no conclusive experiments on the state of ACH–by the way also of epinephrine–in the respective nerves, because of the inadequacy of the methods used. It therefore cannot yet be decided which of the alternatives just mentioned is realized.

In order to fill this gap it seems advisable to investigate whether the state of ACH within nerves could be disclosed by application to nerve homogenates of the method of differential centrifugation. This method, by which mitochondria could be isolated for the first time, has lately been used to separate from organ homogenates granules that fix in an inactive, indiffusible state highly active, endogenous amines such as the

* In honor of Prof. Fritz Verzár on his seventieth birthday.

[1] Department of Pharmacology, New York University College of Medicine, New York.

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