21-ACETOXY-12α : 17α -DIHYDROXYPREGN-4-ENE-3 : 20-DIONE AND ITS ANTICORTISONE PROPERTIES

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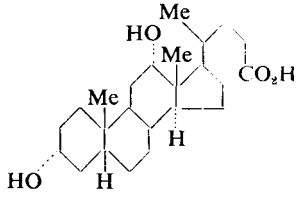
SOME fifty years ago Still[1] and Wishart[2] noted that jaundice exercised a profound and beneficial effect upon the syndrome of rheumatoid arthritis. Initial attempts by Hench[3] to apply this finding to the clinical treatment of rheumatism by inducing artificial hyperbilirubinæmia by intravenous injection of bile salts and bilirubin, however, were not entirely successful, only slight transitory benefits, not comparable with those produced by naturally-acquired jaundice, being observed. More encouraging results were subsequently reported by MacCallum and Bradley[4] and by Gordon, Stewart and MacCallum[5] who induced jaundice with icterogenic serum in volunteer rheumatoid arthritic patients. Their results led them to conclude that the remissions induced in rheumatoid arthritis by an attack of jaundice were striking and showed that the disease was capable of undergoing considerable regression.

Parallel with these investigations Hench, in America, was continuing studies initiated in 1929 on the beneficial effects of pregnancy and jaundice upon rheumatoid arthritis. By 1949 he was able to state[6] that "the inherent reversibility of rheumatoid arthritis is activated more effectively by the intercurrence of jaundice or pregnancy than by any other condition or agent thus far known." He further conjectured that the beneficial effects thus encountered were due to a hypothetical "antirheumatic Substance X" which was not a disintegration product from a damaged liver, but a compound specific in nature and function and normal to the human organism. As temporary remissions of arthritis were known to follow procedures capable of stimulating the adrenal cortices, Hench turned his attention to the adrenal hormones, finally associating "antirheumatic Substance X" with cortisone[6]. The dramatic results, thereby achieved have inevitably given a powerful stimulus to the furtherance of studies on the relation between structure and action in the steroid group.

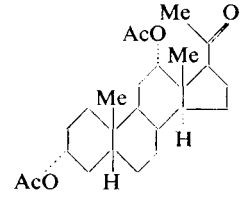
Early in 1951 a programme of work was initiated in these laboratories on the preparation of certain analogues of cortisone for study as anti-phlogistic agents. We had been greatly impressed by the clinical evidence linking remissions in rheumatoid arthritis with intercurrent jaundice. We had also accepted in large measure the explanation of this effect on the basis of Selye's "General Adaptation Syndrome" whereby jaundice is regarded as a stressor agent which imitates the physiological defence mechanism of the body by calling for increased production of antiphlogistic steroids[7]. At the same time we thought the results were capable of a different interpretation and one worthy of consideration.

Disturbance of the excretory mechanism of the liver leads to jaundice

through reabsorption into the blood stream of the bile pigments, bile salts, cholesterol and other constituents normally voided through the bile ducts into the intestine. Reabsorption of the bile salts would clearly lead to a raised level of desoxycholic acid (I) (and cholic acid) in the



(I)



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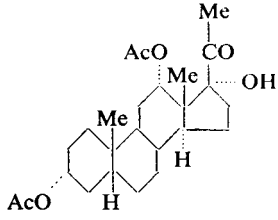

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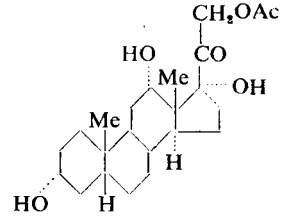

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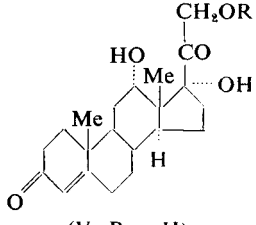
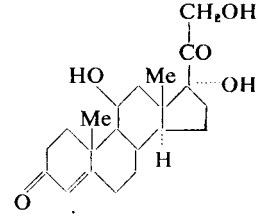


(V; R = H)
(Va; R = Ac)



(VI)

blood stream[8] and hence to a raised level of this steroid in the blood perfusing the adrenals. These glands, in turn, were known[9] to convert compounds such as cholesterol into hydrocortisone (VI) and could therefore also possess the capacity to transform desoxycholic acid (I), or a precursor thereof, into 12α:17α:21-trihydroxypregn-4-ene-3:20-dione (V) which bears a close structural resemblance to the antiphlogistic hormone hydrocortisone (VI). If this were indeed the case, it follows that (V) might correspond to the "antirheumatic Substance X" postulated by Hench as responsible for the remissions produced by jaundice in arthritic patients.

It was against this background that we began the tedious and difficult transformations required to convert (I) into (Va). Desoxycholic acid was first converted into 3α:12α-diacetoxypregnan-20-one (II). The 17α-hydroxyl group was then introduced to give 3α:12α-diacetoxy-17α-hydroxypregnan-20-one (III). The constitution assigned to this compound was confirmed by (i) dehydration by the Darzen method when 3α:12α-diacetoxypregn-16-en-20-one was obtained, identical with an

authentic sample prepared by the method of Djerassi[10] and (ii) chromic acid oxidation to a product, $C_{23}H_{34}O_5$, which, from its melting point and optical rotation appeared to be 3α:12α-diacetoxy-testan-17-one[11]. Hydrolysis of (III), followed by introduction of a 21-acetoxyl group, furnished 21-acetoxy-3α:12α:17α-trihydroxypregnan-20-one (IV). Partial oxidation of this compound gave a product which was undoubtedly the desired 21-acetoxy-12α:17α-dihydroxypregnane-3:20-dione. Its alternative formulation as the corresponding 12-keto-3α-hydroxy-derivative was rendered unlikely by the observation that the material failed to react with ethyl chloroformate, a reagent specific for C_3-hydroxyl groups[12]. (IV), in contrast, passed smoothly into the 3-cathyl-derivative, converted into 12α:21-diacetoxy-3-cathyl-17α-hydroxypregnan-20-one by acetylation. Bromination of the foregoing 3-keto compound, followed by dehydrobromination, furnished 21-acetoxy-12α:17α-dihydroxypregn-4-ene-3:20-dione (Va). The overall yield of (Va) from (I), however, was very low. This fact was largely accounted for by the use of more than 20 stages in its preparation. Thus an 80 per cent. yield at each stage, even if it could invariably be reached, would only give an overall yield of *ca.* 1 per cent. In addition, the marked interference exercised by the 12α-hydroxyl group upon the introduction of the 17α-hydroxyl group with consequent drop in yield, combined to make the preparation of (Va) a formidable undertaking. It may be of interest to record, in this connection, that nearly 100 kg. of desoxycholic acid were used in devising the process and preparing sufficient material to allow a preliminary biological evaluation of the product.

21-Acetoxy-12α:17α-dihydroxypregn-4-ene-3:20-dione (Va), obtained in this way, separated from acetone-hexane in white needles, m.pt. 195° to 197° C. Found: C, 68·3; H, 8·0. $C_{23}H_{32}O_6$ requires C, 68·3; H, 7·9 per cent., $[\alpha]_D^{24°\,C.}$ + 146° (c, 0·422 in chloroform). The presence of the 3-keto-Δ^4 system in this compound followed from its ultra-violet absorption spectrum which showed a maximum at 240 mμ, ϵ_{2380} 16,600 (in *iso*-propanol). The 12α-hydroxyl group was characterised by acetylation. The α-ketol side chain was revealed by use of the 3:3′-dianisole bis-4:4′:(35-diphenyl) tetrazolium chloride reagent[13] with which a purple coloration was obtained on paper chromatograms. Infra-red absorption spectra measurements, kindly determined by Dr. A. E. Kellie (Courtauld Institute of Biochemistry) provided independent confirmation for the presence of a free hydroxyl group, a 3-keto-Δ^4 system and of an acetylated α-ketol side chain in the compound.

Examination of (Va) for cortisone-like activity proved disappointing. Thus the compound was unable to produce involution of the thymus in infantile rats at a dose level effective for cortisone. In addition, it failed to raise the liver glycogen level in the adrenalectomised rat[14]. Examination for androgenic[15], progestational and œstrogenic activity showed the material to be inactive at the dose levels employed. Its effect on mineral metabolism, kindly determined by Dr. J. F. Tait and Mrs. S. A. Simpson (Middlesex Hospital Medical School) employing a bioassay based on the depression of the urinary $^{24}Na/^{42}K$ ratio in adrenalectomised rats[16],

revealed that it possessed not more than 2 per cent. of the activity of desoxycorticosterone employed as a standard.

In spite of these negative results, which clearly did not support the hypothesis that (V) must correspond to Hench's "antirheumatic Substance X," we nevertheless retained a conviction that the steroid was sufficiently closely related in structure to the adrenal cortical hormones to show some form of biological activity. A purely empirical search for such action, however, was contra-indicated by the relative inaccessibility of the material. We therefore turned our attention to some theoretical concepts relating changes in structure of active compounds to the effect of such changes upon biological activity, hoping thereby to obtain some indications for further work.

Last year we[17] presented a discussion to the British Pharmaceutical Conference on the mechanism of biological action and put forward therein the novel concept of specific (σ) and non-specific (ν) pharmacodynamical groups. In accordance with these views the molecule of a biologically active compound was pictured as being partly embedded in the receptor system, the σ-groups lying within the system and the ν-groups resting upon its surface. Any changes in the σ-groups were held to alter the delicate spatial relationship between the biologically active compound and the receptor centres thus making juxtaposition impossible with consequent loss of activity. Limited structural changes in the surface ν-groups were nevertheless regarded as permissible for quantitative retention of activity. It was pointed out, however, that such changes could also lead to structures lacking the activity of the biologically active compound but able to take its place in the receptor system thereby blocking the approach of the active molecule. Inhibition would consequently occur.

Applying these concepts to the cortisone structure, it appeared that the 3-keto, 20-keto, 17-hydroxyl, 21-hydroxyl and the Δ^4-unsaturated linkage could be regarded as σ-pharmacodynamical groups as any changes therein lead to loss of antiphlogistic activity[18]. The 11-substituent, however, seemed to fall within the ν-category as both the 11-keto and 11 β-hydroxy compounds show similar biological action[18]. Modification of this pharmacodynamical centre, as in (V) could therefore have the effect of altering quantitatively the antiphlogistic action of the compound or else could lead to a steroid with inhibitory properties. As the former alternative was ruled out by the experimental evidence outlined on p. 863, above we turned our attention to the second possibility, namely that (Va) may have the properties of an anticortisone. In so far as the preliminary results indicate, this has indeed proved to be the case.

We have already referred to the action of cortisone in restoring the liver glycogen of the adrenalectomised rat[14]. We now find that simultaneous administration of (Va) with cortisone acetate leads to a markedly smaller increase in the level of the liver glycogen compared to controls receiving cortisone acetate alone. As (Va), *per se*, did not appear to influence glyconeogenesis (p. 863 above) these results indicate that (Va) inhibited the action of the cortisone acetate, i.e., it functioned as an *anticortisone* in this particular assay.

It cannot, of course, be inferred from these observations that (Va) must necessarily inhibit all the biological functions of cortisone in the animal organism. The full extent of its antihormonal action has still to be determined. The results obtained do, nevertheless, indicate the importance of further study of the properties of (Va) in relation to the pathological states adversely affected by the antiphlogistic steroids. For example, cortisone is known to be able to suppress the inflammatory reaction irrespective of the nature of the causative agent, thereby enabling pathogenic organisms to disseminate rapidly[19]. Again, under certain conditions it appears to prevent the rapid production of antibodies[20,21]. It also exercises an undesirable effect upon the growth of certain neoplasms in that it induces the production of metastases[22,23,24]. There are, consequently, many potential uses for inhibitors of cortisone action.

In conclusion, it seems opportune to speculate on the possible existence of natural steroids with such inhibitory functions. The liver is known to synthesise cholesterol from acetate and thence convert it into cholic acid, desoxycholic acid, etc. by oxidative and reductive procedures[25]. In addition, it is now clear that the liver also contains enzyme systems able to convert simple steroids into cortisone[26,27,28]. Thus nearly all the mechanisms required to transform (I), or a cholesterol-like precursor thereof, into (V) are proven to operate in the liver. Legitimate grounds consequently exist for the assertion that (V) may possibly be formed in the body and thus play a part in the dichotomy of the inflammatory response.

Experimental

Examination of 21-*Acetoxy*-12α:17α-*dihydroxypregn*-4-*ene*-3:20-*dione* (Va) *for Cortisone-like Activity.*—(i) Dr. S. W. F. Underhill and Miss B. M. Manly, B.Sc., of these laboratories, have kindly examined (Va) for its hypotrophic effect on the thymus of nestling rats[29]. They found that the compound was inactive at a total dose level of 3 mg./22 g. rat.

(ii) The glyconeogenetic activity of (Va) was determined in the following way[30]: Young male albino rats of B.D.H. stock whose ages ranged from 50 to 150 days were weighed and adrenalectomised under ether anæsthesia. A high protein diet was provided *ad libitum* to the fourth postoperative morning and 1 per cent. sodium chloride drinking water to the fifth post-operative morning. In some of the preliminary studies food was given twice daily at regular intervals for a limited period, but in spite of training prior to adrenalectomy this feeding routine was found to be unsatisfactory and was therefore discontinued. After removal of the drinking water the compound under test was injected subcutaneously in aqueous suspension, the dose being given in 4 portions at 2-hourly intervals. The volume of the suspension used was adjusted such that each animal received 0·5 ml./150 g. body weight, at each injection. Hepatectomy under hexobarbitone anæsthesia (0·2 ml. to 0·4 ml. of a 10 per cent. aqueous solution intraperitoneally) was performed 2 hours after the last injection. Immediately on removal each liver was washed in iced saline solution, dried with filter paper, weighed and digested in 15 ml.

of 30 per cent. potassium hydroxide in a boiling water bath for approx. 60 minutes. The glycogen was precipitated by the addition of 16·5 ml. of 95 per cent. ethanol, heating to boiling on the water bath and cooling. The precipitated glycogen was separated by centrifuging (2000 r.p.m.) for 10 minutes. It was dissolved in 5 ml. of distilled water, transferred to a 100-ml. round-bottomed flask and hydrolysed by heating on a boiling water bath for 3 hours with 10 ml. of N sulphuric acid. The solution, on cooling, was neutralised with N sodium hydroxide using 3 drops of phenol-red as indicator. The volume of each hydrolysate was made up to 50 ml. and each portion strained through cotton wool. Aliquots of 5 ml. each were mixed with an equal volume of Schaffer Hartmann reagent[31] in small conical flasks and placed in a vigorously boiling water bath for 15 minutes. This was followed by rapidly cooling in iced water to 35° to 40° C., adding 2 ml. of 5N sulphuric acid and placing for 2 minutes in a bath maintained at 35° to 37° C. The mixture was then titrated with 0·005N sodium thiosulphate solution using starch mucilage as indicator and the results calculated as described by Somogyi[31]. Table I shows the values obtained, the liver glycogen content being expressed as a percentage of fresh liver weight.

TABLE I

Rat	Number of animals	Compound	Total dose mg./150 g. of body weight	Mean percentage glycogen (fresh liver weight)
Intact (Control)	28	—	—	0·37
Adrenalectomised (Control)	5	—	—	0·024
Adrenalectomised	3	(Va)	4	0·036
	3	(Va)	8	0·037
	3	(Va)	16	0·034

Examination of Table I shows that the results obtained with the control groups accord well with earlier values given by Pabst, *et al.*[30], who report liver glycogen values of 0·4 per cent. and 0·016 per cent. for intact and adrenalectomised rats, respectively. (Va) is seen to exert no significant effect. Cortisone acetate, in contrast, is found to restore the liver glycogen content, typical results obtained being given in Table II.

TABLE II

Number of rats	Dose of cortisone acetate mg.	Dose of Va in mg./150 g. of body weight	Mean percentage glycogen (fresh liver weight)
7	0·25	—	0·087
3	0·25	4·0	0·04
2	0·25	12·0	0·0
7	0·5	—	0·103
3	0·5	8·0	0·08
3	0·5	12·0	0·053
7	1·0	—	0·301
2	1·0	12·0	0·093
4	1·0	16·0	0·17

Examination of 21-*Acetoxy*-12α:17α-*dihydroxypregn*-4-*ene*-3:20-*dione* (Va) *for Androgenic, Progestational and Œstrogenic Activity.*—Dr. S. W. F. Underhill and Mr. W. S. Parr have kindly examined (Va) for androgenic[32], progestational[33] and œstrogenic[34] activity using the methods indicated but found no evidence for activity at doses of 50 mg., 200 mg., and 3 mg., respectively.

Examination of 21-*Acetoxy*-12α:17α-*dihydroxypregn*-4-*ene*-3:20-*dione* (Va) *for Inhibition of the Glyconeogenetic Activity of Cortisone in the Adrenalectomised Rat.*—Rats were adrenalectomised and the liver glycogen content determined as described above. Our aim to use not less than 3 animals at each dose level was not fully realised as the numbers were reduced to 2 in two instances owing to post-operative deaths. The results obtained are recorded in Table II.

Examination of Table II shows the effect of cortisone acetate in restoring liver glycogen. A linear relationship was found between log dose and liver glycogen per cent. of fresh liver weight (cf. Pabst *et al.*[30]). Comparison of groups of rats in which one group received (Va) additionally to the cortisone acetate shows that (Va) inhibits the glyconeogenetic effect produced by the cortisone acetate.

Summary and Conclusions

1. 21-Acetoxy-12α:17α-dihydroxypregn-4-ene-3:20-dione (Va), a structural analogue of hydrocortisone (acetate), has been prepared from desoxycholic acid.

2. A preliminary evaluation of its biological properties has been made.

3. It has been found that this compound (Va) inhibits the glyconeogenetic activity of cortisone acetate in the adrenalectomised rat.

4. The possible implications of this result are discussed.

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DISCUSSION

The paper was presented by DR. F. HARTLEY.

DR. G. E. FOSTER (Dartford) said that there was a theory that adrenocorticotrophic hormone stimulated the adrenals to produce cortisone, and he wondered whether the substance described in the paper would inhibit adrenocorticotrophic hormone as well as cortisone activity.

DR. F. HARTLEY, in reply, said that the action of adrenocorticotrophic hormone was not merely to liberate cortisone, and it would be important to consider to what extent their substance might reverse the action of other adrenal cortex secretions. The latter were capable of influencing liver glycogen, carbohydrate metabolism and androgenic and shock-inhibiting characteristics, so that while at first sight it might seem attractive, in his view it would lead to difficulties in interpreting the results if such a blunderbuss method of stimulating adrenal activity as adrenocorticotrophic hormone were used in an attempt to antagonise the carbohydrate metabolism factor. It was emphasised in the paper that the results could not be interpreted at present as meaning that the compound would antagonise all the actions of cortisone. That that cautionary remark was justified was shown by the fact that the help of Dr. Long and his colleagues at the Medical Research Council had been sought to ascertain the behaviour of the substance in the guinea-pig tuberculin sensitivity test for cortisone. It was injected intraperitoneally, whereas cortisone was injected subcutaneously. The substance was without effect on the cortisone action on the tuberculin insensitivity. It was however still permissible to speculate, and although cortisone was usually thought of in terms of its anti-inflammatory activity, and more popularly its antirheumatoid value, it was as well to recognise its other properties such as stimulation of dormant tuberculosis. Steroids with anticortisone properties might therefore open up a new picture in chemotherapy. It was conceivable that among the steroids there would be found a material not only capable of combating the spread of infection but perhaps even of acting prophylactically.