An International Symposium

on

ALDOSTERONE

Edited by

ALEX F. MULLER
M.D.

and

CECILIA M. O'CONNOR
B.Sc.

With 84 Illustrations

J. & A. CHURCHILL LTD.
104 Gloucester Place, London, W.1

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FOREWORD

R. S. MACH

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FOUR years after the famous publication of Simpson, Tait, Wettstein, Neher, von Euw and Reichstein announcing the isolation of aldosterone in crystalline form, the time has come to take stock of our knowledge of this hormone.

With this purpose in view, the Clinique Thérapeutique of the University of Geneva organized this International Symposium on Aldosterone in June 1957. We are very grateful to Dr. A. Wettstein and Dr. F. Gross of CIBA in Basle for making such a meeting possible. Our thanks go also to Dr. G. E. W. Wolstenholme of the Ciba Foundation and to J. & A. Churchill Ltd., London, for helping us to publish the papers and discussions of this Symposium.

We realize more and more that, among the hormones of the adrenal cortex, aldosterone plays a special rôle. It is the only hormone of the adrenal gland which seems to enjoy a certain freedom from pituitary control, and which is influenced directly by changes in the water and electrolyte balance. It is its vital rôle in the maintenance of homeostasis that makes its study so fascinating.

However, numerous questions still worry us. We are bothered, for instance, by the fact that all our clinical studies are based on urinary aldosterone values; that is to say, on values which represent 2–3 per cent of the aldosterone secreted.

We are also surprised at the conflicting clinical pictures produced by hyperaldosteronism. How can we explain the fact that this hormone, which in a normal person or an Addisonian causes salt and water retention, should also produce, in the Conn syndrome, polyuria with loss of potassium and intracellular retention of sodium without water? Could it be that the Conn syndrome is something other than pure hyperaldosteronism?

The paradox of oedema with secondary hyperaldosteronism also remains to be explained. Why do patients who are already swollen with water and who retain too much sodium continue to increase their aldosterone secretion?

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These are some of the problems that we met in Geneva to discuss. Those of us who were present may feel that a great deal remains to be done before the answers to all of them will be known; but the Symposium has provided us with a point of departure for our future research programmes, and it is to be hoped that, as recorded in this book, it will prove interesting and stimulating to workers in this field throughout the world.

List of those participating in or attending the Symposium on Aldosterone, 7th–8th June, 1957

J. Barlow	Presbyterian Hospital, New York
F. C. Bartter	National Heart Inst., National Insts. of Health, Bethesda
P. Bastenie	Clinique Médicale, Hôpital Universitaire Saint-Pierre, Brussels
E. E. Baulieu	Laboratoire de Chimie Biologique, Faculté de Médecine, Paris
R. V. Brooks	Dept. of Metabolic Diseases, St. Thomas's Hospital, London
P. Desaulles	Research Laboratories, CIBA Limited, Basle
E. Engel	Clinique Thérapeutique Universitaire, Geneva
E. R. Froesch	Policlinique Universitaire de Médecine, Zürich
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O. Garrod	Middlesex Hospital Medical School, London
C. J. P. Giroud	Endocrine Research Laboratory, The Montreal Children's Hospital, and Dept. of Investigative Medicine, McGill University, Montreal
J. de Graeff	Dept. of Endocrinology and Diseases of Metabolism, Academisch Ziekenhuis, Leiden
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L. Hernando	Clinique Universitaire de Médecine, Madrid
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A. Labhart	Policlinique Universitaire de Médecine, Zürich
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R. S. Mach	Clinique Thérapeutique Universitaire, Geneva
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A. F. Muller	Clinique Thérapeutique Universitaire, Geneva
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J. STAHL	Clinique Médicale B, Faculté de Médecine, Strasbourg
S. W. STANBURY	Dept. of Medicine, The Royal Infirmary, Manchester
J. S. TAIT	Middlesex Hospital Medical School, London
SYLVIA A. S. TAIT	Middlesex Hospital Medical School, London
P. VESIN	Service de Gastro-Entérologie, Hôpital Saint-Antoine, Paris
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H. P. WOLFF	1. Universitäts Klinik, Universität von München

CONTENTS

ALDOSTERONE IN URINE

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IN STUDYING the function of aldosterone in the regulation of water and salt metabolism, the availability of an accurate method for the determination of this steroid in urine is of the utmost importance. Many facets of the metabolic action of aldosterone under physiological and pathological conditions have been studied successfully by means of the bioassay procedures described by Simpson and Tait (1952), Johnson (1954), Singer and Venning (1953) and Bartter (1956).

However, all these bioassay procedures are very time-consuming, and in order to get accurate results large series of test-animals have to be used. Besides the available physicochemical methods there seemed to be room for a more quantitative chemical technique, but no chemical method for the determination of aldosterone in urine which is specific, sensitive, quantitative and rapid has been described until now.

The method developed in our laboratory, and which is discussed here, does not claim to possess all these characteristics of an ideal determination method.

After it had been found that the aldosterone molecule contains three carbonyl groups we decided to try whether aldosterone could be estimated colorimetrically by reaction with 2 : 4-dinitrophenylhydrazine. Gornall and Macdonald (1953) had found that the absorption spectra of the 2 : 4-dinitrophenylhydrazones of various ketosteroids in alkaline medium differed from one another. When we determined the absorption spectrum of the aldosterone derivative we found that this was definitely different from the absorption spectrum of a number of other ketosteroids, e.g. cortisone and cortisol. The more or less characteristic absorption curve which was obtained from the 2 : 4-dinitrophenylhydrazone of aldosterone enabled us to evaluate the homogeneity of the aldosterone fractions from urine, which were purified by means of paper chromatography. Furthermore, a quantitative estimation of the aldosterone content of these fractions was possible.

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The aspects of our method discussed here are as follows:

(1) purification of the extract;
(2) conditions for the isolation of the coloured compound;
(3) conditions under which the colour is developed and which may contribute to the specificity of the colour reaction.

PURIFICATION OF THE EXTRACT

The aldosterone-containing fraction is continuously extracted with chloroform at pH 1. The chloroform extract is purified by re-extraction with NaOH and water and evaporated to dryness. The residue is partitioned between 70 per cent methanol and a 50/50 mixture of toluene and ligroin. The methanolic solution is further purified by paper chromatography.

It was found that with the paper chromatography system which we use, aldosterone can be separated in one run from compound F, compound E, tetrahydro F and E, all the less polar steroids, and from a good deal of the interfering urinary pigments. This system contains a mixture of 98 per cent toluene and 2 per cent octanol equilibrated with an equal amount of 50 per cent methanol, and it has the great advantage that the effect of impurities on the R_F values is negligible.

Fig. 1 shows a chromatogram of 20 μg. of compounds E and F and aldosterone giving a good separation of these three steroids. The localization of aldosterone between cortisol and cortisone facilitates its identification.

However, it was found that the absorption curves of the 2 : 4-dinitrophenylhydrazine reaction product of the "aldosterone" fraction did not show the specific characteristics of the spectrum of the pure aldosterone derivative. Furthermore, when the chromatogram was sprayed with 2 : 4-dinitrophenylhydrazine it was found that at the place where aldosterone was localized a compound was present which gave a purplish-blue colour in ammonia vapour. Aldosterone could be separated from this interfering substance with the aid of a second chromatography system, for which we used the B1 system (Bush, 1952) in which the R_F values of cortisol, aldosterone, cortisone and the above-mentioned impurity are 0·03, 0·05, 0·08 and 0·13, respectively. Here again aldosterone is localized between cortisol and cortisone.

Fig. 2 shows u.v. photostats of chromatograms of cortisone, cortisol, aldosterone and a urinary extract from the Bush B1 system.

Table I summarizes some properties of the contaminating substance, which behaved in a great number of paper chromatography systems as a single compound. This substance, the identity of which is not yet known, was present in increased amounts in the urine of patients treated with ACTH and in one patient suffering from adrenogenital syndrome.

The aldosterone fraction from the second paper chromatogram is extracted with purified ethanol, and the aldosterone in the eluate determined spectrophotometrically as 2 : 4-dinitrophenylhydrazone.

Table I

PROPERTIES OF INTERFERING COMPOUND FROM FIRST CHROMATOGRAPHY

Colour	: yellow
U.V. absorption maximum	: 243 mμ
Fluorescence with NaOH	: blue
Tetrazolium-blue reaction	: negative
Porter–Silber reaction	: negative
Zimmermann reaction	: negative
2 : 4-Dinitrophenylhydrazone	: yellow with absorption maximum in NaOH at 420 mμ with ammonia—blue

CONDITIONS FOR THE ISOLATION OF THE COLOURED COMPOUND

When 2 : 4-dinitrophenylhydrazine is used as a reagent in a colorimetric estimation it is necessary to remove the excess of strongly coloured reagent from the reaction mixture. This can be done by the methods of Reich, Crane and Sanfilippo (1953), but since these methods require about 10 mg. of the phenylhydrazone, they could not be used in our case. In Gornall and Macdonald's (1953) study of the determination of 17-ketosteroids with 2 : 4-dinitrophenylhydrazine, it was suggested that the excess of reagent could be destroyed by carrying out the spectrophotometrical analysis in an alkaline medium. In our experience, however, the remaining background absorption is still very high when this method is used, and therefore we tried to remove the excess of reagent in a different way. It is well known that the 2 : 4-dinitrophenylhydrazones of various steroids (e.g. progesterone) are extremely insoluble in water. In the procedure we are using now, a precipitate of the 2 : 4-dinitrophenylhydrazone of aldosterone is formed by heating

the steroid with 0·1 ml. of the acid reagent solution in a boiling water-bath for 25 minutes. The insoluble reaction product is co-precipitated with benzoic acid, by the addition of sodium benzoate to the acid solution. The precipitate is isolated by centrifugation and is washed twice with 2 ml. of an acid washing fluid, followed by centrifugation. The washed precipitate is dissolved in 1 ml. alcoholic sodium hydroxide and the extinction of this solution is determined at 10-mμ intervals between 380 and 600 mμ.

CHARACTERISTICS OF THE ABSORPTION CURVE

The absorption spectra of the 2 : 4-dinitrophenylhydrazones of aldosterone and some other ketosteroids are shown in Fig. 3.

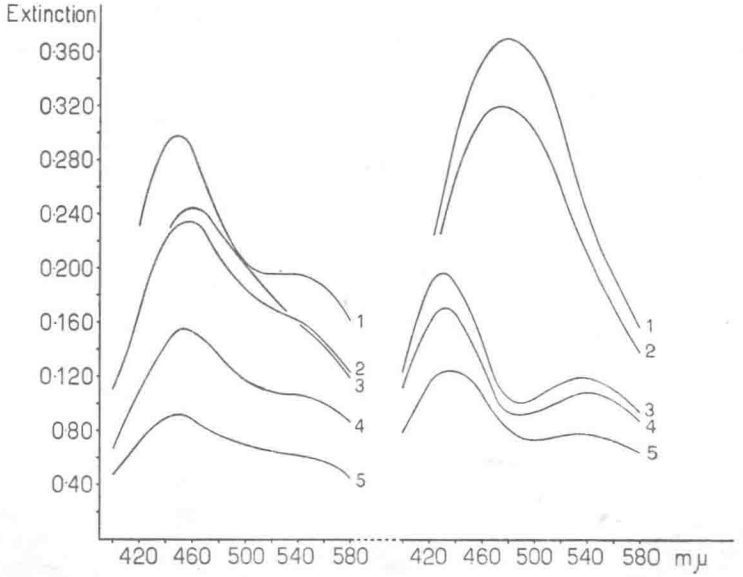

FIG. 3. Extinction curves of the reaction product of 2 : 4-dinitrophenylhydrazine with various ketosteroids.

1 = 10 μg./3 ml. progesterone
2 = 10 μg./3 ml. cortexone acetate
3 = 10 μg./3 ml. corticosterone
4 = 10 μg./3 ml. testosterone propionate
5 = 3 μg./3 ml. aldosterone

1 = 10 μg./3 ml. cortisone
2 = 10 μg./3 ml. 17-OH-cortexone
3 = 20 μg./3 ml. *cis* androsterone
4 = 10 μg./3 ml. androstane-17-ol-3-one
5 = 20 μg./3 ml. pregnenelone acetate

When standardized conditions for the formation of the coloured product are used, these curves can be split clearly in three groups according to their shape and the place where the absorption maximum occurs. Those in the first group have a symmetrical shape and

a maximum at approximately 475 mμ, and represent the 17-hydroxy-corticosteroids, e.g. cortisol, cortisone and compound S. In the second group, to which belong the 17-ketosteroids, e.g. dehydro-*iso*androsterone, *cis*androsterone, the 3-ketosteroid androstane-17-ol-3-one, and the 20-ketosteroid Δ-5-pregnen-3-ol-20-one, biphasic curves with absorption maxima at 430 and 540 mμ are found. In the third group we find an intermediary type of curve. These curves

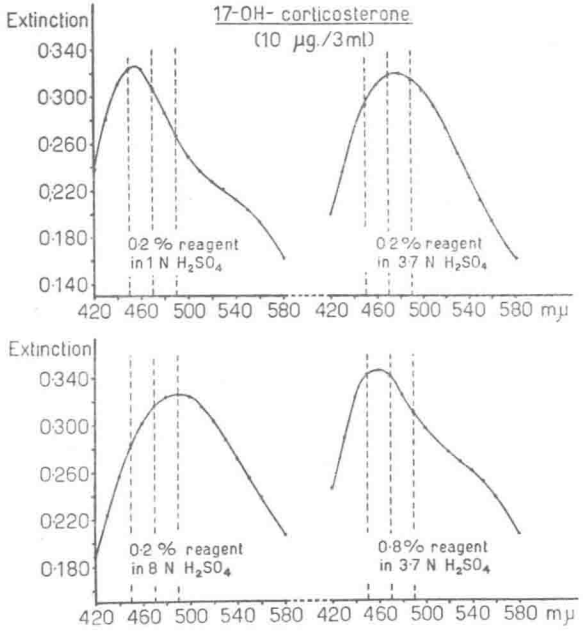

FIG. 4. Extinction curves of the reaction product of 17-OH-corticosterone with 2 : 4-dinitrophenylhydrazine prepared at different concentrations of reagent and of sulphuric acid.

are given, for instance, by aldosterone, testosterone, progesterone, corticosterone and cortexone. The first three steroids give curves with a definite shoulder. The type of curve which is obtained is partly dependent on the reaction conditions, as is shown in Fig. 4.

2 : 4-Dinitrophenylhydrazones of cortisol were prepared, using different concentrations of acid and reagent. At increasing concentrations of acid the absorption maximum is shifted to the longer wave-lengths. Under these conditions the shape of the curve also changes. When an increased concentration of the 2 : 4-dinitrophenylhydrazine reagent is used, these changes are counteracted and the

curve which is obtained is very similar to that which was found at lower concentrations of acid. A similar effect was found when cortisone and compound S were tested. Steroids which do not possess a 17-hydroxy group show these changes to a much smaller extent. The results of an expriment in which these same variables were tested, using corticosterone, are shown in Fig. 5. Although small differences in the shape of the curve can be observed, hardly

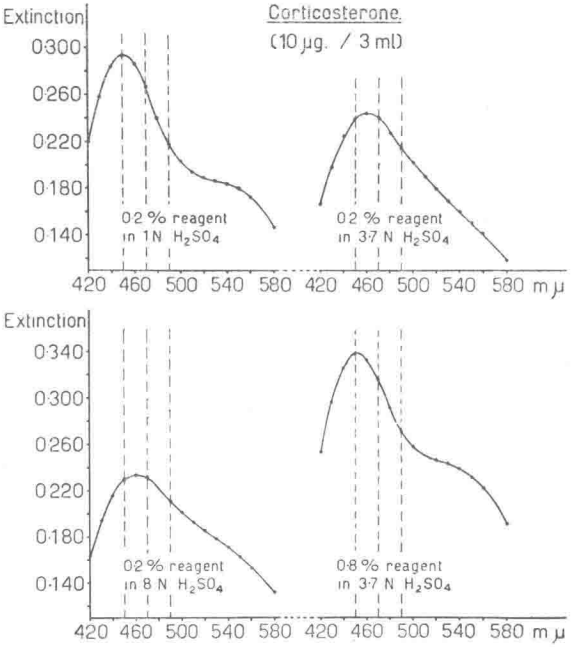

FIG. 5. Extinction curves of the reaction product of corticosterone with 2 : 4-dinitrophenylhydrazine prepared at different concentrations of reagent and of sulphuric acid.

any change in the localization of the absorption maximum was found. No changes whatsoever were found with progesterone, testosterone and androstane-17-ol-3-one. It seems that these shifts in the absorption spectrum are only found in those steroids that possess a ketol group. The effect is increased considerably when a hydroxy group is present at $C_{(17)}$. In our opinion, a possible explanation is the formation of a 2 : 4-dinitrophenylosazone at $C_{(20)}$ and $C_{(21)}$, at high concentrations of acid. This reaction is compared to the Porter–Silber reaction, where an osazone is formed from 17-hydroxy-

corticosteroids, at high concentrations of acid. The effect of increasing the concentration of 2 : 4-dinitrophenylhydrazine, which on the basis of the law of mass action seems to be in contradiction to this hypothesis, can be explained by assuming that the increased concentration of the reducing agent prevents the intramolecular oxidation-reduction reactions which, according to Weygand, precede the formation of the osazone. Estimation of the nitrogen content of the various 2 : 4-dinitrophenylhydrazones will probably clarify the exact structures of the products which are formed.

CALCULATION OF RESULTS

From the absorption spectra of the products of the reaction between 2 : 4-dinitrophenylhydrazine and the aldosterone fractions of a number of urines, and also pure aldosterone (Fig. 6), it can be concluded that:

(*a*) These urinary extracts and pure aldosterone have absorption maxima at the same wave-length.

(*b*) The shape of the curves between 380 and 600 mμ is not in accordance with the peak of the absorption maximum.

Non-specific chromogens, apparently, are still present in the urinary extracts, and these may give erroneously high values. The question might be asked: could a third paper chromatography achieve a further purification such that the remaining background absorption would be negligible? In our opinion, such a procedure seems undesirable for the following reasons:

(*a*) It is highly improbable that such a purification can be achieved by only one more chromatography.

(*b*) The introduction of a third paper-chromatography procedure implies a significant prolongation of the time of determination, and may increase the loss of aldosterone considerably.

(*c*) As indicated below, it seems possible to correct for the non-specific chromogens which are still present.

Therefore, we did not carry out further purification procedures, and we tried to calculate the aldosterone content of the urinary fractions from the absorption curves which were obtained. We are aware that this introduces some risk but, in our opinion, the method as indicated gives sufficiently reliable results to justify its use in the study of the problems discussed in the introduction to this paper.

The correction for background absorption can only be made if the shape of the absorption curve from these non-specific chromogens

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