THE CONTROL OF ALDOSTERONE SECRETION

J. P. COGHLAN, B.Sc.

Research Biochemist, Ionic Research Laboratory

D. A. DENTON, M.B., B.S.

Senior Medical Research Fellow, National Health and Medical Research Council, Ionic Research Laboratory

J. R. GODING, M.B., B.S.

Senior Research Fellow, Wool Industry Fund of the Commonwealth, Ionic Research Laboratory

R. D. WRIGHT, M.S., D.Sc., F.R.A.C.P.

Professor of Physiology

(From the Department of Physiology, University of Melbourne, Victoria, Australia)

SOME GENERAL CONSIDERATIONS

Clinical

There is evidence that increased secretion of aldosterone is a contributory cause of oedema occurring in kidney, liver and heart disease. The term 'contributory' is used in the sense of implying that a number of factors act concurrently in the developed clinical state, and it would be unusual for oedema formation to be simply and commensurately attributable to any one of them alone. Thus administration to normal men of doses of aldosterone considerably larger than the inferred daily secretion in man with congestive cardiac failure did not produce oedema.¹ Oedema is not a characteristic sign in the aldosterone secreting tumours which were first described by Conn.^{2,3} Similarly with adrenalectomized dogs, Gross and Lichtlen⁴ found that very large doses of aldosterone did not produce oedema. On the other hand, Davis, Pechet, Ball and Goodkind⁵ found that aldosterone secretion was increased in dogs with experimental right heart lesions which caused congestive failure, and in dogs with thoracic inferior vena cava constriction producing ascites. If such dogs were adrenalectomized, administration of adequate electrolyte-active hormone was essential for oedema formation to occur.⁶ The developed clinical syndromes are similar to many other physiological responses and pathological states in so far as the 'cause' is identifiable as the concurrent action of a number of factors which are severally necessary and jointly sufficient. In the particular instance of cardiac oedema, substantial studies have been made to evaluate other factors

postulated in the 'forward' and 'backward' failure theories, and we do not propose to enter this discussion here.

It is this multiplicity of concomitant and frequent phenomena in clinical conditions which makes elucidation so difficult. As an example we may consider the enormous excretion of aldosterone by nephrotic patients. Using a promising new modification of the original isotope dilution method^{7,8} of estimating aldosterone secretion, Ulick, Laragh and Lieberman⁹ found up to 6,600 γ /day. The etiology of nephrosis is unknown and therefore the direct effect of the etiological conditions on the adrenal cannot be tested. It is possible that the nephrotic kidney produces a substance which activates the adrenal but this hypothesis cannot be tested until such material is identifiable by assay. Through unknown mechanisms the excess secretion may result from disturbance of electrolyte levels or distribution or from abnormal distribution of body fluid. Abnormal metabolic degradation of aldosterone by the liver or other tissues may cause excess secretion through some feed-back mechanism to the adrenal. The elucidation of any clinical condition is impossible without understanding every phenomenon in the disease mechanism to the stage where each can be separately identified both qualitatively and quantitatively.

Experimental Physiology

The principal physiological questions involved in the control of aldosterone secretion are, (i) the

anatomical site of the receptor (or receptors) regulating aldosterone secretion, (ii) the exact nature of the stimulus to it, (iii) the site of origin, mode of transmission and nature of the stimulus to the adrenal, and (iv) the manner of the adrenal response. There appears to be some conflict of opinion as to the present status of knowledge in relation to these questions. The opening sentence of the Year Book of Endocrinology (1958-59) editorial dealing with the adrenal cortex states, 'One of the most fascinating developments of the past year is the discovery of the way the body controls the release of aldosterone.' Though certain of the studies cited in support of this statement are evidently of great importance, it is, in our view, an over-optimistic assessment of the present position. In fact on many crucial issues there are flatly contradictory findings in recent literature. This is as likely to be a source of difficulty and confusion for someone seeking a general review of the field as it is for those directly concerned in experimental investigation of the questions cited above. For this reason it may be helpful at the outset to state four main factors in experimental approach, the consideration of which may identify the causes of some conflicting findings.

Factor 1—The Operation of Measurement

The primary aim of animal experiment is to have control of all the relevant conditions and be able to find the effect of controlled variation of one or more of these conditions. *It is essential that the process of measurement should not introduce an uncontrolled or undesired variable.* In all cases, it may be assumed that the base-line levels will be found in the confident conscious animal. For work on the conscious animal, training to indifference to the observer and what he does is essential. When the functional tissue for investigation is anatomically deep-seated and difficult of access, surgical procedures of considerable complexity over a long period may be required in order to contrive the necessary direct access in the conscious undisturbed state.

Pavlov was the first person to enunciate clearly the principle and take advantage of aseptic surgery to implement this approach. He recognized that the crude damage to the physiology of the organism which occurs in the acute surgical experiment under anaesthesia may evoke a chain of reactions which frequently overshadows or radically modifies the normal function under scrutiny. As Verney has pointed out, initial experiments with postpituitary extract made on anaesthetized animals led to the belief that the physiological role of this substance was to promote diuresis. In the course of his own work, Verney¹⁰

found that a normal water diuresis did not occur in anaesthetized or frightened dogs, and we have shown that normal responses to rapid changes of Na balance do not occur in anaesthetized or frightened sheep.¹¹ An outstanding example of successful implementation of this principle of approach was the development by Lockett, O'Connor and Verney¹² of the renal artery loop preparation permitting direct access to the arterial supply and venous drainage of the kidney in a conscious dog.

However, in advocating this principle, particularly where it is recognized that higher nervous activity plays an important role in the physiological regulation concerned, it obviously cannot be overlooked that facts of great importance have been discovered by the method of acute experiment. Apart from examples such as Harvey's observations by vivisection, and the discovery of secretin, the early observation by Verney and Starling¹³ of the profuse urine flow in the heart-lung-kidney preparation was the point of departure for Verney's analysis of the control of secretion of antidiuretic hormone. The simple three organ preparation provided the basic fact of absence of an inhibitory influence, but it is extremely unlikely that the discovery that the physiological stimulus to antidiuretic hormone control was contemporaneous with a small variation in the osmotic pressure of carotid artery blood could have been made except by the survival type of experiment. In other publications^{14, 15} we have set out in detail the desiderata for valid determination of the cause and manner of secretion of a hormone by an endocrine gland. They are:

1. All observations should be made on conscious undisturbed animals in normal relationship with their environment. Thus the results derived are those holding for a normal animal.
2. The physiological stresses causing a response dependent upon the gland should be precisely defined and standardized, and be capable of graded variation.
3. The arterial blood supply of the endocrine gland under investigation should be exclusively accessible so that the effect of supposed active agents can be tested by direct local injection.
4. The nerve supply of the gland, when relevant, should be accessible for stimulation or blocking.
5. The venous effluent exclusively from the gland should be accessible.
6. There should be one or more biological systems for quantitative assay of the activity of the venous effluent.
7. The chemical assay of the venous effluent must be developed until it detects quantitatively each substance known to be active on the bio-

FIG. 1.—Transplant 12. A photograph showing the skin loop containing the carotid artery, jugular vein, and the left adrenal gland. A pneumatic cuff inflated to 300 mm. of Hg. has been applied to the loop cranial to the transplant. A needle attached to polythene tubing has been inserted into the carotid artery caudal to the transplant, and an infusion is being made into the arterial blood supply of the adrenal gland. The transplant operation was carried out two years earlier.

logical indicator, and the overall effect of these substances must correspond quantitatively to the action of the entire venous effluent.

Some experimental procedures aiming to meet the desiderata. It would be ideal for investigation of aldosterone regulation if it were possible to have a continuous record of the adrenal blood flow and the arteriovenous difference in aldosterone concentration in a conscious animal under appropriate physiological stress. The amount of blood removed for analysis should not, of itself, stimulate secretion unless desired. In circumstances where it is impossible to contrive this end or as a matter of convenience, an alternative would be to have a continuous index of the concentration of aldosterone in peripheral blood. This concentration is dependent upon the rate of secretion, the rate of destruction and excretion, and the volume of fluid in which the hormone is distributed. Formally, the possibility of storage has to be considered also, and this includes the question of protein binding. In the instance of concurrent chemical and biological assay of peripheral blood, a difference could be attributable to the fact that protein-bound steroid was biologically inactive. Whereas with cortisol this is an important consideration, evidence to date is against storage or significant protein binding of aldosterone. If one assumes that the rate of degradation and excretion of aldosterone is proportional to the peripheral concen-

tration, the short half-life of aldosterone could result in the peripheral concentration following a smoothed-out fashion variations in rate of secretion. In that chemical assay of aldosterone in peripheral blood requires large volumes and is still in the developmental stage, the better alternative is a biological indicator.

The parotid gland of the ruminant is such an indicator,¹⁵ responding with approximately 60 to 80 minutes delay to starting or stopping an ipsilateral intra-arterial infusion of aldosterone. Corrections for parotid secretion rate variations¹⁶ and increased sensitivity of the gland in that depletion¹⁷ are necessary to make the salivary $\text{Na}^+ \text{K}^+$ a satisfactory index of peripheral blood aldosterone. Notwithstanding the limitations, the salivary $\text{Na}^+ \text{K}^+$ is a valuable continuous index of the secretion of aldosterone in the conscious animal. The great advantage of the parotid fistula preparation in the ruminant is that desiderata 1, 2 and 6 are met. With respect to 2, the fistula depletes the animal of Na^+ rapidly, and the degree of deficiency can be controlled by administering NaHCO_3 by rumen tube or allowing the animal an appropriate amount of NaHCO_3 or NaCl to drink.^{18, 19}

To meet the desiderata 3, 4 and 5 outlined and, in particular, to determine whether the adrenal is stimulated directly by the local changes of ionic composition of arterial blood, an adrenal transplant

paration has been developed in this laboratory. The left adrenal gland of the Merino sheep is transplanted to a combined carotid artery-jugular skin loop in the neck.²⁰ Thus the composition of adrenal arterial blood may be locally altered and the adrenal steroid output can be directly measured in the adrenal venous blood of the conscious undisturbed animal (Fig. 1).

The implementation of the desiderata has revealed a number of facts about adrenal secretion which would have been difficult to determine in a convincing manner in any other way, e.g., changes in ionic composition of adrenal arterial blood directly affect aldosterone secretion. However, ionic changes do not account for the known variation of adrenal secretion seen with change of Na^+ balance, and it is clear that some additional factors act upon the adrenal via the blood stream. When the transplants were used for cross-circulation experiments, it was shown that the blood of a Na^+ -depleted adrenalectomized animal stimulated the adrenal transplant of a sheep in normal Na^+ balance to produce electroactive adrenal steroid.¹⁴

The new method of aldosterone assay in blood developed by Kliman and Peterson^{21, 22, 23} permits accurate assay of small serial samples of adrenal blood so that parotid salivary Na^+/K^+ ratio may be related with aldosterone production over long periods of time without loss of blood and its contained aldosterone becoming an undesired variable in the experimental situation. The concurrent use of both indices, i.e. episodic chemical assay of adrenal venous blood, and continuous biological assay of peripheral blood, to some extent circumvents the shortcomings resulting from the use of either method alone. With the survival type of experiment there is the overall advantage that the trained animal can be used as its own control over a period of years, and the results of chromatographic assay can be compared under identical conditions with the effect of various rates of intravenous injection of aldosterone itself and other steroids. Thus the analysis outlined in desideratum 7 can be made rigorously.

Procedures for meeting desideratum 5 have been described by Hume and Nelson²⁴ and by Weaver and Eik-Nes.²⁵

Investigation by acute experiment. Most of the difficulties of interpretation arising from defects of design in these regards are exemplified in the reports of Farrell and co-workers.²⁶⁻³² Using anesthetized or decerebrated animals, the total venous outflow from one adrenal is collected for a number of hours to provide one sample for a single chemical analysis. Sometimes specimens are pooled. No baseline for the individual animal is

FIG. 2.—Plurality of cause.

determined. The effect of anaesthesia, nervous ablation and injury produce an undetermined change of aldosterone production and the exsanguination (with or without epinephrine) gives a mounting, uncontrollable, not uniform stimulus to aldosterone production.²⁷ Against such a background, the effects of tissue extracts and nervous lesions and ablations produce their effects. Farrell's experiments provide a clear statement of the rate of aldosterone production in the specific conditions of the experiments but no certain basis for the analysis of physiological regulation. Some of these methodological objections and difficulties of interpretation apply to the results of other workers, including the experiments in this laboratory on the effect of acute nervous ablations on adrenal secretion under various conditions of Na^+ balance.¹⁵

Factor 2—The Plurality of Causes

William of Occam stated, 'It is vain to do with more what can be done with fewer.' Frequently the assumption is made that one result has one cause and Occam rather than experiment is called on to justify the assumption. There are many causes of increased aldosterone secretion. It does not follow that the known causes all operate through the same mechanism. Sodium depletion, exsanguination and caval constriction each cause increased aldosterone secretion. Water restriction, exsanguination and severe exercise cause oliguria. There is no more reason to assume that the total mechanism for producing the result will be the same for each of the conditions in the first group than there is for doing so for those in the second group.

To give a diagrammatic illustration of this argument in the case of aldosterone secretion: With acute loss of blood, the adrenal may be stimulated via a chain of physiological mechanisms represented by the sequence, A, B, C, D, E (Fig. 2). With Na^+ deficiency, the causal system may

involve action on the adrenal by more than one pathway and, as a corollary, interruption of one pathway may not preclude action via another. In the two instances, the operation of the causal chain might require the existence of certain standing conditions (represented as M and N). Assuming this analysis to be representative of the state of affairs in the conscious undisturbed animal, it is formally possible that the combination of, e.g., Na^+ deficiency or exsanguination with anaesthesia and surgical injury could institute a causal chain which did not operate under other circumstances and which would continue to act despite disruption of the normal physiological chain. This is shown as P, R, on the diagram.

John Mill made the following statement: '... It is not true ... that one effect must be connected with only one cause, or assemblage of conditions; that each phenomenon can be produced only in one way. There are often several independent modes in which the same phenomenon could have originated. One fact may be the consequent in several invariable sequences; it may follow, with equal uniformity, any one of several antecedents, or collection of antecedents. Many causes may produce mechanical motion: many causes may produce some kinds of sensations: many causes may produce death. A given effect may really be produced by a certain cause, and yet be perfectly capable of being produced without it.'³³

Another possible source of misapprehension is the transference of notions derived from the results of one operational set of conditions to fill in gaps in a superficially similar but actually different set of conditions. It must be uncertain that the effect of mid-collicular decerebration on the aldosterone secretion in the Na^+ depleted animal will be the same as its effect on aldosterone secretion resulting from exsanguination even though both investigations use the anaesthetic-traumatized animal. Section or ablation in the nervous system may produce acute effects by paralysis, irritation or release; long term results are modified by compensation and plasticity. Comparisons must therefore be on the basis of precisely defined anatomical, chronological and physiological similarity and variation.

The possibility of variation in physiological organization for one functional result in higher mammalian types must be considered also, but it is unlikely that such a basic mechanism as the regulation of ionic content would show any radical differences.

Factor 3—Chemical Methods

A third reason for conflicting findings is variation of reliability of chemical methods for detection of aldosterone in biological fluids. This is

FIG. 3.—Chemical formulae.

particularly so in the case of measurement of aldosterone in urine.^{34, 35, 29}

Factor 4—The Criteria for Hormonal Activity of Extracts of Biological Material

A putative hormone may be isolated from biological excreta, or the supposed site of origin. The criteria for the full categorization of its role as a hormone are (i) demonstration of its presence at the site of origin, (ii) the disappearance of the physiological effect attributed to it upon removal of the site of origin, (iii) demonstration of activity by infusion of the material exclusively into the arterial blood supply of the target organ. This eliminates the possibility of indirect action via an intermediate chain of causation. (iv) Demonstration of transmission by the blood stream at a concentration commensurate with the elicited physiological stress.

Tissue extracts active on an organ are not necessarily in a hormonal relation to that organ, e.g., a bronchial extract may activate gastric secretion because of the histamine content.

Deane, Shaw and Greep³⁶ showed that variation of Na^+ and K^+ balance caused cytological changes of the zona glomerulosa indicative of altered activity. There is evidence from the studies of a number of workers that there is a hormonal stimulus to aldosterone secretion. Farrell²⁹ has suggested the name 'glomerulotropic hormone' or 'glomerulotropin' for the substance as it exercises its trophic action on the glomerulus. However, there is no experimental evidence to date that this material has any trophic activity as defined above, i.e., removal of the site of origin and

demonstration of zona glomerulosa atrophy in the survival preparation. At this preliminary stage, it might be better to name the postulated substance glomerulosa stimulating hormone (GSH).'

These general observations introduce the detailed sections which follow. Several excellent review articles which have appeared in the last two years^{29, 40, 37, 38, 39} cover most aspects of chronological development in the field so we will not recapitulate in detail here.

CHEMICAL STRUCTURE AND PROPERTIES OF ALDOSTERONE

Aldosterone is unique among the adrenal steroids in possessing an aldehyde grouping on position 18 (Fig. 3, A). In aqueous solution it exists largely as the 18 - o - 11 hemiacetal (Fig. 3, B).^{41, 42}

The provisional formula of a metabolite of aldosterone⁴³ which occurs in human urine in greater amounts than free aldosterone is shown (Fig. 3c). Steroid compounds structurally related to the cardiac glycosides and with the property of blocking the action of aldosterone on the renal tubule have been developed. The structure of two such compounds is shown (Fig. 3, D). In the steroid SC5233, R is CH₃, in steroid SC8109 (Spiroactone), R is H.⁴⁴⁻⁴⁷

Other compounds have the property of blocking the production of adrenal steroids, including aldosterone.^{48, 49} Examples are amphenone and 2-methyl-1,2-bis-(3-pyridyl)-1-propanone (BU4885), the latter having selective action on 11-beta-hydroxylation.⁵⁰

Action of Aldosterone

The biological actions of aldosterone cause a rise in Na⁺ and a fall in K⁺ in the extracellular fluid (E.C.F.). There is retention of Na⁺ and excretion of K⁺^{51, 52, 53} by the kidney, and a fall in the Na⁺/K⁺ ratio of saliva and faeces.¹ In acute experiments⁵⁴ it has been reported to cause a rise in Na(E.C.F.)/Na(I.C.F.) and a rise in K(I.C.F.)/K(E.C.F.) ratio in muscle and brain, although in chronic primary hyperaldosteronism the reverse occurs. This paradoxical situation is considered later.

Effect of Lack of Aldosterone

If aldosterone secretion is stopped or the peripheral action is blocked there are the following effects—an increase in urinary Na⁺ excretion and a decrease in K⁺ excretion, a rise in Na⁺/K⁺ ratio in sweat⁵⁵ and saliva^{56, 57}, a rise of intracellular Na⁺ and a fall of intracellular K⁺, and a fall of E.C.F. Na⁺/K⁺ ratio. Oedema and ascites associated with raised aldosterone production may be reduced greatly.^{58, 47} Clinical conditions of selective aldo-

sterone deficiency are being recognized, particularly following removal of adenomata in cases of Conn's syndrome.

The effect of deficiency of electrolyte-active secretion appears to differ between animal types, e.g. many adrenalectomized humans can be maintained on cortisone alone, whereas, with an adrenalectomized sheep on its normal diet, the withdrawal of electrolyte-active hormone causes death within four days.⁵⁷ In a sheep with severe adrenal insufficiency, aldosterone administration does not seem to be as effective as a life-saving procedure as DOCA. Swingle⁵⁹ found that aldosterone was ineffective in the resuscitation of the adrenally-insufficient dog.

Salt-losing Hormone

Adrenal 'salt-wasting' hormones 3-β-1-6-α-dihydroxy-pregnane-20-one and 3-β-1-6-α-dihydroxy-allopregnane-20-one have been isolated.⁶⁰ The former was recovered from the urine of patients with salt-losing adrenogenital syndrome. In this condition, a very low output of aldosterone occurs despite Na⁺ deficit.^{61, 62, 63}

Clinical Conditions which have been Reported to Cause Increased Aldosterone Excretion

These can be broadly sub-divided into eight categories:

- (i) Electrolyte disturbances (Na⁺ depletion, K⁺ loading).
- (ii) Conditions resulting in diminution of intravascular volume (haemorrhage, water depletion, sweating, surgery).
- (iii) Conditions associated with oedema or ascites (congestive cardiac failure, cirrhosis of the liver, nephrotic syndrome, hypoproteinaemia, idiopathic oedema).
- (iv) Conditions associated with disturbances of other endocrine systems (hyperthyroidism, hypothyroidism, pregnancy, insulin shock).
- (v) Adrenal cortical hyperplasia and tumours (Conn's syndrome).
- (vi) Conditions involving the C.N.S. (midbrain vascular accidents, cyclical insanity, anxiety states).
- (vii) Change of position and activity.
- (viii) Others including carcinoma of lung with or without adrenal metastases.

Relative Electrolyte Activity of Aldosterone Compared with other Steroids

Many naturally occurring steroids, e.g. cortisol, cortisone, corticosterone, testosterone, progesterone, if given in large doses cause Na⁺ retention. Desaulles^{63a} showed that aldosterone was 2,000 times as effective as corticosterone in causing

FIG. 4.—Basil, adrenalectomized and Na⁺ deficient. The effect on parotid salivary Na⁺/K⁺ ratio of intravenous infusion of unesterified d,l,aldosterone in isotonic saline at rates equivalent to 7.5, 15, and 30 μg. hr. of dextro isomer form. The arrows mark the time of cessation of infusion in each instance. The mean salivary K⁺ concentration at the plateau of response is recorded also.

FIG. 5.—George, adrenalectomized and Na⁺ deficient. The effect on parotid salivary Na⁺/K⁺ ratio of (i) intravenous injection of 20 mg. of hydrocortisone hemi-succinate followed by continuous infusion at the rate of 10 and then 20 mg. hr.; (ii) intramuscular injection of 5 mg. of DOCA.

urinary Na⁺ retention. We have found that i.v. aldosterone is over 1,000 times more potent than cortisol in causing a fall in salivary Na⁺/K⁺ ratio (Figs. 4 and 5). When given intramuscularly aldosterone is 40 times as effective as DOCA (Fig. 6).

The Normal Secretion Rate, Concentration in Peripheral Blood and Metabolism

The secretion rate of aldosterone in normal human subjects has been estimated by Ayres *et al.*⁶⁴ to be 170 to 190 γ/day, Ulick *et al.*⁹ found 150 to 300 γ/day, Peterson²³ 330 to 400 γ/day. This agrees approximately with an amount of

FIG. 6.—George, Na⁺ depleted and adrenalectomized. The effect on parotid salivary Na⁺/K⁺ ratio of (i) intramuscular injection of 1 mg. of d,l,aldosterone monoacetate; (ii) intramuscular injection of 20 mg. of desoxycorticosterone acetate.

250 γ reported by Mach *et al.*⁵¹ as the daily replacement dose for maintenance of patients with Addison's disease. The concentration of aldosterone in peripheral blood in normal subjects is 0.04 to 0.08 γ/100 ml.,²³ while Ayres *et al.*⁸ reported 0.03 γ/100 ml.

If tritium-labelled aldosterone is injected into normal subjects, approximately 70 per cent of the administered dose appears in the urine in the first 24 hours. The free steroids make up 4 to 8 per cent., metabolites conjugated as glucuronides 30 to 40 per cent., and those released after deconjugation, 10 to 20 per cent.²³ The tetrahydroaldosterone metabolite (Fig. 3, C) which occurs as a conjugate with glucuronic acid accounts for 10 to 15 per cent of the total radioactivity appearing in the urine.

Methods of Estimating Aldosterone Chromatography

The initial isolation of aldosterone was effected by the application of column and paper chromatographic techniques to the fractionation of adrenal extracts into their various adrenocortical components.⁴¹ As no specific chemical method is available which can reliably determine aldosterone in a mixture of corticosteroids, paper chromatographic separations have been used for the isolation of aldosterone from biological fluids in a form sufficiently pure to permit accurate quantitation by one or other of the non-specific methods.

The basic chromatographic systems generally used are those of Bush⁶⁵ and Zaffaroni.⁶⁶ Minor modifications have been introduced by many workers. There is no universal accord as to the most suitable combination of chromatographic systems to achieve the high degree of purity required, but the failure of one or two systems to give reliable resolution is well documented.^{35, 67, 68, 69} Probably the best system of chromatography and derivative formation to assist purification can be selected only in the light

considerable experience with the particular types of experimental animal, and the type of biological material being extracted.

Reports on the many chromatographic combinations employed have not always included comprehensive details of recovery experiments.^{29, 69} The standard method available to date appears to be the double isotope derivative assay and the double isotope dilution derivative assay of Kliman and Pearson.^{21, 22, 23, 70}

They summarize the method as follows: 'A double labelling technique which has proved useful in this laboratory for the assay of many steroids is applicable to the selective assay of aldosterone. Dried extracts of plasma or urine are acetylated with tritium-labelled acetic anhydride in the presence of pyridine and benzene to convert aldosterone quantitatively to the tritium-labelled diacetate. A measured amount of authentic aldosterone diacetate-C¹⁴ is added to each sample, and the double-labelled steroid purified by paper chromatography. After two chromatographies, the samples are treated with chromic acid to convert the diacetate to the monoacetate, which is then subjected to a final chromatography to separate the aldosterone from other tritium-labelled materials. The tritium and carbon-14 content of the purified steroid is determined by simultaneous counting in a liquid scintillation spectrometer. The aldosterone present in the original extract can be calculated from the determination of the amount of carbon-14 lost during the purification, the yield of tritium radioactivity, and the specific activity of the tritium-labelled acetic anhydride. An efficient assay can be done with 5 to 10 ml. of urine or 2 to 5 ml. adrenal vein plasma. This method will accurately determine 0.01 µg of aldosterone, and has the accuracy characteristic of isotope techniques.'

Recently it has been reported that, by using regenerated glass paper, a rapid and satisfactory separation of small quantities of steroids including aldosterone can be achieved.⁷¹ Possibly, in conjunction with one or other of the isotopic methods, much time could be saved provided the same high degree of purification can be achieved. Further progress along these lines will be of great interest.

Assay

The methods normally in use depend on aldosterone causing Na⁺ retention and increased K⁺ excretion by the kidney. The usual experimental animals have been adrenalectomized mice, rats and dogs.⁷²⁻⁷⁶ The material to be assayed is injected into animals recently adrenalectomized which are loaded with Na⁺. The alteration in Na⁺ and K⁺ excretion is estimated either by

flame photometry⁷⁷ or by radio-active isotope methods.⁷⁸ This change is compared with the effect of a standard dose of DOCA or authentic aldosterone.

The advantage of the use of the rat or mouse is that minute amounts of aldosterone may be assayed. These animals, however, excrete small volumes of urine, and the resultant change in electrolyte excretion due to aldosterone is not large. The method does not permit use of the animal as its own control, and depends on statistical analysis of difference between the control and the experimental group. Thorn *et al.*³⁴ state that because it is necessary to remove interfering steroids⁶⁹ before proceeding to bioassay there may be large losses of aldosterone which would obviously affect the reproducibility of the results. Difficulties of interpretation of results using a rat bioassay are evident in the paper of Orti *et al.*⁷⁶ in which the method was used to demonstrate the presence of an aldosterone stimulating substance in urine.

The effect of aldosterone on the parotid salivary Na⁺/K⁺ ratio of the sheep will be discussed more fully in a subsequent section. For bioassay purposes, an infusion can be made into the ipsilateral carotid artery loop. It has been shown that in the Na⁺ depleted adrenally insufficient sheep as little as 0.5 γ/hr. of aldosterone causes an unequivocal fall in salivary Na⁺/K⁺ ratio after a delay of 90 to 120 minutes. A vascular isolation procedure contriving that the ipsilateral carotid artery supplies the parotid gland only has been devised in the sheep. It is possible that this preparation will give reliable results with a smaller rate of aldosterone infusion. A possible objection to the use of ipsilateral intracarotid infusion for the purpose of comparing the biological activities of different pure steroids resides in the likelihood of very different degrees of protein binding. The time of transit between the tip of an intracarotid needle and the parotid gland is probably 1 to 2 sec.,¹⁰ and if this time were inadequate for the completion of binding, the procedure could give a physiologically erroneous estimate of the electrolyte activity of a steroid such as cortisol which is normally 80 to 95 per cent. bound.^{79-81, 23} The same objection does not hold for the use of the procedure for bioassay of electrolyte activity in whole blood.

Methods of Assessing Secretion of Aldosterone in Biological Experiments

Methods Depending on Direct Access to the Adrenal Gland

In vitro studies of steroid secretion have been made by many workers.⁸²⁻⁸⁶ Giroud *et al.*⁸⁷ have confirmed earlier evidence of Dean *et al.*³⁶ that electrolyte active corticosteroid is secreted princi-

pally by the zona glomerulosa. On the other hand, Lane and de Bodo^{88, 89} found in the dog that there is not the same atrophy of the fasciculata and preservation of zona glomerulosa after hypophysectomy as in the rat. The results obtained from *in vitro* preparations must be accepted with caution when they purport to yield information relating to normal physiological control of adrenal secretion. However, this method might provide useful information when 'screening' extracts of possible aldosterone-stimulating activity, provided that such extracts are subsequently tested in the whole animal under the conditions set out above (Factor 4).

Mulrow *et al.*⁸⁶ have summarized some explanations possible when crude extracts cause increased secretion of aldosterone with *in vitro* preparations. The action may be due to (a) a true physiological stimulus to the adrenal gland, (b) provision of an essential ingredient in the substrate, (c) the action of ACTH (which in turn could be either regulatory, or a standing condition necessary for some metabolic step under *in vivo* conditions, or (d) a non-specific effect.

In vivo methods are much to be preferred. Adrenal vein blood has been obtained under conditions of acute surgery, or recent surgical intervention.^{90-97, 22, 98-101} The direct effect of these procedures is to increase aldosterone output well above basal rate and if, in addition, amounts of blood are withdrawn which are large in proportion to the animal's circulating blood volume,¹⁰² the secretion of aldosterone will approach the maximum adrenal output. Hence, the main fact demonstrable in the latter instance is that an experimental procedure has reduced aldosterone secretion through interference with pathways which subserve the stimulus (trauma *plus* gross blood loss). However, the result may be difficult to interpret, as the blood loss may cause also progressive reduction of adrenal blood flow which, if severe, might limit aldosterone production. In Farrell's experiments on selective destruction of areas of the C.N.S. of cats,¹¹⁹ there is a suggestive relation between adrenal blood flow and aldosterone output. In the experiments on decerebration and bioassay of extracts the adrenal blood flow has not been reported.²⁸

Hume and Nelson²⁴ developed a method of chronic cannulation of the adrenolumbar vein in the dog which permits serial collection of blood at a sufficient interval after surgery to allow the assumption that the immediate effects have disappeared. However, they report values of 17-hydroxy corticosteroid output which are sporadically as high as with ACTH infusion, and this raises the question whether the animal is invariably undisturbed by the procedure of pulling the intra-

abdominal 'choker' which occludes the space between the adrenal and the vena cava. We have used Eik-Nes,²⁵ using Teflon prosthesis, removed surgical preparations which permit access to the adrenal vein in conscious animals. However, there is in this preparation no exclusive access to the arterial supply of the adrenal gland, and the samples obtained do not permit estimates of adrenal secretion rates, as the rate of blood flow cannot be measured.

Another interesting approach to the problem has been made by Mason¹⁰³ who has been able to insert a No. 7 or No. 8 catheter into the right adrenal vein in 9 out of 20 conscious human subjects.

In 1939, Levy and Blalock¹⁰⁴ made subcutaneous transplants of the intact adrenal gland into the neck of the dog. These animals survived for long periods and several had normal litters. From this one could conclude that the transplanted gland secreted normally, even under 'stress.' The transplants, however, allowed limited access only to the venous outflow from the gland, and no access to the arterial inflow.

To meet the desiderata outlined above and in particular to determine whether the adrenal is stimulated directly by the local changes of composition of arterial blood, an adrenal transplant preparation has been developed in this laboratory. The left adrenal gland of the Merino sheep is transplanted to a combined carotid artery-jugular vein skin loop in the neck.²⁰ The left adrenal usually receives a small artery from the renal artery and the adrenal vein drains into the left renal vein. The renal vessels are anastomosed end-to-end with the carotid and jugular vein. The kidney is discarded. Subsequently the right adrenal gland is removed. An animal with an adrenal transplant has the same time relations of paracrine response to changes of Na⁺ balance as one with both adrenals in their normal position. When a pneumatic cuff applied to the vascular loop cranial to the transplant is inflated to 300 mm. Hg., the carotid artery supplies essentially only the adrenal and simultaneous application caudal to the adrenal of a cuff inflated to 30 mm. Hg. contrives to drain the cannula in the jugular vein drains only adrenal vein blood. Thus the composition of adrenal arterial blood may be locally altered and the adrenal steroid output can be directly measured in the adrenal venous blood of the conscious undisturbed animal.

Methods Depending on the Concentration of Aldosterone in Peripheral Blood

Chromatography. Until the advent of radioactive isotope methods,⁶⁴ measurement of aldosterone in peripheral blood has not been a practical

procedure. With the Kliman and Peterson method²¹ aldosterone may be estimated in 50 to 75 ml. of peripheral plasma.²³ Peterson reports normal levels of 0.04 to 0.08 γ /100 ml.; 0.08 to 0.15 γ /100 ml. in patients with cirrhosis on low Na^+ diet. The biological half-time of aldosterone was 0.5 to 0.8 hr. for patients with Addison's disease, and normal men on low Na^+ intake, and 1.3 hr. for patients with liver cirrhosis. The normal 'turnover rate' was 330 to 400 γ /day, increasing to 560 γ /day on low Na^+ diet, and was 1000 γ /day for cirrhotic patients on low Na^+ diet.

Estimation by biological effects. Large changes of plasma Na^+/K^+ ratio may indicate deficiency or excess of electrolyte-active adrenal secretion. Conn discovered that DOCA reduced the Na^+/K^+ ratio in human sweat, and assumed that the low sweat Na^+/K^+ ratio observed during acclimatization to heat was due to increased production of electrolyte-active hormone by the adrenal gland. Frawley and Thorn⁵⁶ showed that DOCA reduced the Na^+/K^+ ratio in human mixed saliva. Simpson and Tait¹⁰⁵ and August, Nelson and Thorn¹ showed that aldosterone caused a lowering of human salivary Na^+/K^+ ratio. Observations on sweat and saliva have given information on peripheral aldosterone levels under varying physiological conditions and in disease states and, indeed, were largely responsible for Conn diagnosing confidently his early cases of primary hyperaldosteronism.

However, Thorn *et al.*³⁴ stated they found an absence of correlation between aldosterone excretion and salivary Na^+/K^+ ratio in man. The results show a large fluctuation in day to day aldosterone excretion, as against a smooth curve relating salivary Na^+/K^+ ratio to degree of Na^+ depletion. It is open to question which method of assessing change of aldosterone secretion is the more reliable. The acute effects of ACTH, aldosterone, sodium and potassium on parotid secretion were studied in the dog by Langley *et al.*¹⁰⁶ Intravenous injection of ACTH (80 U.) and aldosterone (75 γ) caused the pilocarpine stimulated salivary Na^+/K^+ ratio to rise. ACTH caused the secretion rate to rise, and aldosterone caused it to fall slightly. The ACTH effect may have been associated with the change in salivary secretion rate. The effect of aldosterone found was not large, and apparently the observations were made 60 minutes after injection. Ipsilateral intracarotid infusion of steroids in this laboratory has never had an effect on salivary Na^+/K^+ ratio under 60 min., and the full response may be delayed for as long as 240 minutes.¹⁰⁷ A similar time delay in the effect of aldosterone on urinary composition upon infusion into the renal artery

was reported by Barger *et al.*¹⁰⁸ Accordingly the results of Langley *et al.* have no significance.

The problem in using the salivary Na^+/K^+ in man is that it is a mixed secretion while concentrations of Na^+ and K^+ are not large and therefore the variation in composition in response to aldosterone is small. Further, the Na^+ concentration varies with secretion rate. These objections do not hold in the case of measurement of the parotid salivary Na^+/K^+ ratio in the ruminant.

Salivary secretion in a ruminant like the sheep is continuous, and 4 to 10 l. of a simple solution of inorganic ions are produced by the parotid glands each day. The salivary Na^+/K^+ ratio is normally about $180/6 = 30$, and as a result of aldosterone administration or Na^+ depletion it may fall as low as $20/160 = 0.12$. If a permanent unilateral parotid fistula is made, it is easy to collect serial samples of adequate volume for assay by flame photometry, and the scale of change induced is very large relative to any analytical error involved in photometry. The evidence justifying use of the parotid salivary Na^+/K^+ ratio as an index of aldosterone secretion follows:

1. There is no parotid response to Na^+ deficiency if a sheep is adrenalectomized and is adrenally insufficient, and very little response if it is given only sufficient DOCA to maintain it when in normal Na^+ balance. A normal parotid response does occur if the sheep is given a four- to eight fold increase of DOCA dosage.¹⁷

2. Bilateral adrenalectomy of an anaesthetized Na^+ depleted sheep causes the salivary Na^+/K^+ ratio to rise to normal after a latent period of 90 to 120 min.¹¹

3. Physiological adrenalectomy. In a Na^+ deficient sheep with an adrenal transplant the drainage of the total adrenal venous effluent causes the salivary Na^+/K^+ ratio to rise to normal after a latent period of 90 to 120 min. The re-injection of the adrenal venous effluent into the ipsilateral carotid artery loop or intravenously causes the salivary Na^+/K^+ to decrease to the level holding before the physiological adrenalectomy.^{20, 14}

The evidence that the effective component of the adrenal secretion is aldosterone is:

- (i) McDonald and Reich¹⁰⁹ have shown that in normal Na^+ balance the transplanted adrenal gland secretes 40 to 600 γ /hr. of cortisol and small amounts of corticosterone and cortisone. With this chromatographic method aldosterone was not detected in 75 ml. of adrenal vein plasma. In Na^+ deficiency aldosterone was always detected, being secreted in rates varying from 6 to 24 γ /hr. In moderate Na^+ deficiency there was no change of cortisol or corticosterone secretion, but in gross deficiency the rate of secretion of both was

moderately increased. Cortisol ranged from 330 γ /hr. to 1.2 mg./hr. and corticosterone from 5 γ /hr. to 73 γ /hr. The maximum values for cortisol and corticosterone were about one-third of those found during ACTH stimulation. Thus the significant consistent difference between normal Na^+ balance and Na^+ deficiency was the detection of aldosterone, and the results indicate that the salivary response was due to this steroid or some undetected steroid. In their studies on steroid output in the adrenal vein blood of dogs Rosnagle and Farrell¹¹⁰ found that Na^+ deficiency doubled aldosterone secretion, but did not affect cortisol. In man, Crabbe, Reddy, Ross and Thorn¹¹¹ have shown that Na^+ deprivation causes a consistent rise in urinary aldosterone secretion and has no effect on urinary cortisol secretion. In so far as urinary excretion of these compounds may be taken as reflecting adrenal secretion, the results indicate that the physiological adaptations to Na^+ deficiency in man attributable to electrolyte-active adrenal secretion are caused by increased aldosterone secretion and are unrelated to cortisol secretion.

(ii) Intravenous infusion of *authentic* aldosterone in Na^+ -deficient adrenally-insufficient sheep at rates corresponding to the range detected by chromatographic procedures causes a commensurate decrease in salivary Na^+/K^+ ratio to the extent observed in Na^+ deficiency (Fig. 4). Intravenous infusion of cortisol at rates 10 times greater than secretion rate detected by chromatographic analyses of adrenal venous blood during Na^+ deficiency has no effect on salivary Na^+/K^+ ratio (Fig. 5). This was examined also by observing the effect of intravenous infusion of these steroids during rapid correction of Na^+ deficiency by systemic administration of 260mM. of NaCl . It was found that infusion of aldosterone at 20 γ /hr. blocked completely the usual large rapid rise of salivary Na^+/K^+ ratio which occurs 120 min. after commencing correction of Na^+ deficiency. Aldosterone at 10 γ /hr. delayed and reduced the effect, whereas cortisol infusion at 12 mg./hr. caused little, if any, reduction of response. A factor contributing to these large differences in electrolyte activity, which indicate that it is unlikely that cortisol has any causal role in the salivary response to Na^+ deficiency, is that cortisol is 80 to 95 per cent. bound to protein in the peripheral plasma, whereas aldosterone is not significantly bound.¹¹²

In a sheep in normal Na^+ balance large fluctuations in secretion rate have very little influence on salivary Na^+/K^+ ratio, irrespective of whether the changes follow psychic stimuli or rumination or whether they are contrived by reduction of parotid blood flow.¹⁶ In a Na^+ -depleted sheep there is a

highly significant relation between parotid salivary Na^+/K^+ ratio and secretion rate. This effect has to be taken into account when using the salivary Na^+/K^+ ratio as an index of aldosterone secretion rate. This can be done satisfactorily in the main type of experimental context we have used for examining the control of adrenal secretion of aldosterone, viz. the adrenal response to rapid correction of Na^+ deficiency in a depleted animal.^{113, 11, 17, 14, 15} Prior to the experimental procedure there is a control period of 200 to 300 minutes during which the relation between secretion rate and salivary Na^+/K^+ ratio is determined. When the salivary Na^+/K^+ ratio or salivary K^+ or Na^+ concentration is graphed against an arithmetic ordinate scale there is a highly significant linear correlation with secretion rate. During the experiment (e.g. rapid correction of Na^+ deficiency or local alteration of the ionic composition of adrenal arterial blood) the position of each point relative to this line is determined. A sustained deviation from the control period line during the period of experiment is interpreted as reflecting a change of aldosterone secretion. In the case of the salivary Na^+/K^+ ratio, a rise above the line reflects an inhibition of secretion; a fall indicates stimulation. The results are graphed as parotid salivary Na^+/K^+ ratio 'corrected for secretion rate variation' and for reasons set out elsewhere¹⁴ the K^+ concentration 'corrected for secretion rate variation' is included also on the figure (Fig. 13). Whereas changes of Na^+ balance do not affect significantly the sensitivity of the biological indicator over the time scale of this type of experiment, the chronic effect of such changes on the sensitivity of the parotid to aldosterone have to be taken into account in other types of experiments. For example, a standard dose of aldosterone causes a much larger decrease of salivary Na^+/K^+ ratio in a Na^+ -depleted adrenalectomized sheep than it does in a sheep in normal Na^+ balance (Figs. 11 and 12). Reference to these figures indicates that the parotid salivary Na^+/K^+ ratio gives a relatively speedy reproducible index of the peripheral aldosterone level. The evidence from the results of stopping, starting and altering the rate of intravenous infusion of authentic aldosterone indicates that, provided due allowance is made for time of biological action, the salivary Na^+/K^+ ratio reflects commensurately changes of adrenal aldosterone secretion rate (Fig. 12). This would apply directly in experiments where the only change induced is local alteration in the ionic environment of the adrenal gland, but may require some quantitative qualification in short-term experiments where the volume over which aldosterone is distributed is rapidly changed.

Muscle biopsy. The Na^+/K^+ ratio or the Na^+

K^+ concentrations in muscle have been used as an index of aldosterone levels in peripheral blood. The results of Woodbury and Koch⁵⁴ have been mentioned previously. Gornall¹¹⁴ found increased aldosterone excretion was associated with a rise in Na (E.C.F.)/ Na (I.C.F.) and a fall of K (E.C.F.)/ K (I.C.F.) in women during the third trimester of pregnancy. However, there is some difficulty in reconciling these findings with those of Conn's syndrome, where measurement of intracellular electrolytes showed a rise in Na^+ and a fall in K^+ .^{2, 3, 115, 116} There are at least two possible causes of this difference:

(i) During the course of a prolonged surgical procedure, large electrolyte shifts occur— K^+ is lost from cells and Na^+ moves in.¹¹⁷ Hence it is probable that biopsy specimens be taken at the first opportunity during an operation.

(ii) If the rate of aldosterone secretion has been high for prolonged periods, urinary K^+ loss causes a deficit.¹¹⁸

Methods Depending on Excretion of Aldosterone

Urine. Nearly all the recent work published on the control of aldosterone secretion *in man*, has depended on the estimation of free aldosterone in 24 hr. specimens of urine. This has usually been carried out by chromatography, but some workers, notably Llauro,⁷⁷ have used bioassay procedures as well. The validity of the assumption that changes in urinary aldosterone output reflect changes in the rate of aldosterone secretion has been discussed previously^{29, 14} and will not be repeated in detail here. The main points are (i) only a small proportion of secreted aldosterone, ca 4 to 8 per cent, is detectable in the urine, (ii) small changes in renal tubular reabsorption of aldosterone would lead to large changes in aldosterone excretion, (iii) different methods of hydrolysis of conjugates yield different results. In order to separate aldosterone from interfering substances, great losses are incurred which must affect the reliability of the method. This applies particularly when it is intended to use bioassay procedures to estimate aldosterone which has been separated by chromatographic means.³⁴ Brenfurth and Venning⁶⁹ consider that four chromatographic systems are necessary for separation of aldosterone from human urine. Duncan, Middle and Bartter¹²⁰ have advanced reasons for assuming that change of urinary aldosterone excretion reflects change of plasma concentration. This may be legitimate under many conditions but might be questionable when there are acute alterations of renal haemodynamics or when tubular secretion changes are induced by pitressin or mercurial diuretics.

In an important paper by Ulick, Laragh and

Lieberman⁹ a new method is presented which overcomes some objections to urinary steroid estimations. The method is based on the discovery of a urinary metabolite (Fig. 3, C) of aldosterone⁴³ which occurs in larger amounts than free aldosterone. The method depends on the determination of the specific activity of this tetrahydro metabolite—a measure which indicates the dilution of an intravenous dose of 1μ of tritium-labelled aldosterone. The validity of the estimate of daily secretion rate depends on the following assumptions, (i) the labelled aldosterone mixes rapidly with the unlabelled aldosterone in the body, (ii) the subsequent fate of both forms of aldosterone is the same, (iii) the pool size of aldosterone remains constant during the study, i.e. rate of disappearance is equal to the rate of secretion, (iv) the proportion of aldosterone converted to the metabolite remains unchanged during the study, (v) the metabolite is not produced by the degradation of any other steroid. Variations in the rate of hepatic inactivation or of renal clearance do not affect the determination, unless they result in alterations of adrenal secretion rate. This promises to be a very useful method for clinical studies, and for some experimental work. It does not appear suitable, in its present form, for observation of changes of adrenal secretion rate over a timecourse which is much less than 24 hr.

Faeces. Orti, Ralli *et al.*⁷⁶ have reported experimental results depending on estimation of aldosterone in the faeces of rats. This was done on the basis that the main route of excretion in this species was via the faeces.

PHYSIOLOGICAL CONDITIONS WHICH INFLUENCE ALDOSTERONE SECRETION

Changes in Na^+ Balance

Na^+ deficiency causes increased aldosterone secretion in man and in all mammals investigated to date. In man the ability to respond to Na^+ depletion by increased urinary aldosterone excretion has been used as a test of normal aldosterone regulation.¹²¹

Replacement of Na^+ in the deficient animal or man decreases aldosterone secretion. With Na^+ deficient sheep, the rapid inhibition of adrenal aldosterone secretion which follows a systemic dose of Na^+ has provided an excellent physiological test system for the analysis of control of adrenal secretion. The important feature is that the 'inbuilt' biological indicator, the salivary Na^+/K^+ ratio, exhibits a large quantitative change over a short time scale, a situation analogous to the inhibition of an established water diuresis in the experimental animal which was used by Verney¹⁰ to estimate the anti-diuretic hormone released by intracarotid infusion.

In sheep, Na^+ deficiency due to loss of parotid saliva usually causes a fall of plasma Na^+ concentration. For example, 20 specimens of blood drawn from P.F.1 under control conditions with adequate Na^+ intake had a plasma Na^+ concentration of 148 ± 1.6 (S.D.) mEq/l. In eight external electrolyte balance studies in which free access to water was permitted and in which Na^+ deficit of 500 to 900 mEq. occurred, the Na^+ concentration of 41 specimens of blood was 143 ± 3.1 (S.D.) mEq/l. The Na^+ concentration of ten specimens drawn during the period 24 to 72 hr. following the replacement of Na^+ supplement was 150 ± 3.1 (S.D.) mEq/l. The plasma Na^+ concentration of 15 specimens taken from P.F.13, P.F.17 and P.F.33, whilst on adequate Na^+ supplement was 147 ± 3.7 (S.D.) mEq/l., of 21 specimens taken during Na^+ depletion 140 ± 5.8 (S.D.) mEq/l. and of ten specimens taken during the first 72 hr. of replacement of Na^+ supplement 152 ± 3.1 (S.D.) mEq/l. In gross Na^+ deficiency (700 to 1,000 mEq.) the plasma Na^+ may decrease to 120 mEq/l. or less. There is usually a rise of plasma K^+ concentration in Na^+ deficiency. The mean plasma K^+ concentration of 25 specimens of arterial blood taken from P.F.13, P.F.16 and P.F.33 whilst in normal Na^+ balance was 4.4 ± 0.4 (S.D.) mEq/l. Eleven specimens taken from these sheep during Na^+ deficiency had a K^+ concentration of 5.6 ± 0.8 (S.D.) mEq/l.

Many other changes occur, e.g. decrease in pH, plasma HCO_3^- , intracellular Na^+ concentration, plasma and extracellular volume, blood pressure and presumably right arterial pressure, cardiac output and total body water. There is a rise in haematocrit and blood viscosity.

When Na^+ deficiency is wholly or partly corrected rapidly by the standard procedure of systemic infusion of 60 to 70 ml. of 4 M NaCl during a period of 40 to 50 min., there is a 15 to 30 mEq/l. rise of plasma Na^+ concentration, a fall of 0.5 to 2.0 mEq/l. of K^+ concentration, as well as a substantial reversal towards normal of the deviation of haemodynamic conditions caused by Na^+ deficiency.

In considering the increased aldosterone secretion resulting from Na^+ depletion, the initial question is whether this is due to decrease of plasma Na^+ concentration. Though there is usually a fall as indicated above, in the course of a single balance experiment the plasma Na^+ concentration and salivary Na^+/K^+ ratio often do not show a correspondence suggestive of a commensurate cause and effect relation. The fall of salivary ratio is progressive, whereas the plasma Na^+ may fluctuate considerably, a finding reflecting concurrent operation of the osmo-regulating mechanism. The significant fall of plasma Na^+ in severe

FIG. 7.—P.F. 1. Comparison of the effect on (i) parotid salivary Na^+/K^+ ratio, (ii) parotid salivary Na^++K^+ concentration, (iii) plasma Na^+ concentration, (iv) external Na^+ balance of a standard episode of Na^+ depletion (O—O) with one in which water intake was severely restricted during the first 7 days of Na^+ depletion (X—X). Na^+ depletion commenced on day 0 in both instances, and was produced by withholding the daily Na^+ supplement of 595 m. equiv. of NaHCO_3 . On the morning of the 7th day the animal was permitted free access to water and drank 5 litres during the first 5 minutes.

FIG. 8.—Jack, Na⁺ depleted. The effect on parotid salivary Na⁺/K⁺ ratio (corrected for variation attributable to secretion rate) of intravenous infusion of 2 l. of 102 m. equiv./l. NaCl = 204 m. equiv. of Na⁺. The effect on plasma Na⁺ concentration, and the dosage of pitressin employed to prevent a water diuresis is shown also.

efficiency indicates 'accommodation' of the osmo-receptors, a process which is possibly dependent in their partaking in the changes of intracellular composition occurring in this state.

The following experiments are pertinent to this question:

A sheep was concurrently depleted of both Na⁺ and water, so that during Na⁺ deficiency there was a rise of plasma Na⁺ concentration instead of the usual fall. Nevertheless, the salivary Na⁺/K⁺ ratio fell, indicating increased aldosterone secretion.¹²² Fig. 7 shows this fall of salivary Na⁺/K⁺ ratio, the rise of plasma Na⁺ concentration during Na⁺ deficiency, and the fact that a large voluntary intake of water (3.5 l.) permitted on the morning of the seventh day, caused a large fall of plasma Na⁺ concentration, but little effect on the salivary Na⁺/K⁺ ratio.

In other experiments Na⁺ deficiency was corrected by the infusion of a hypotonic solution (2 l. of 102 mM/l. NaCl) and simultaneously the osmo-regulating system was prevented from causing a rise of plasma Na⁺ concentration by administration of pitressin. Though in this experiment the rise of salivary Na⁺/K⁺ ratio occurred at 180 min. instead of the usual 120 min., it is clear that adrenal secretion of aldosterone was inhibited, although no rise of plasma Na⁺ concentration occurred (Fig. 8).

In examining the possible role of increased plasma K⁺ concentration in causing aldosterone secretion sheep were given an artificial diet with very low K⁺ content. Na⁺ deficiency (600 to 700 mEq.) caused the salivary Na⁺/K⁺ ratio to fall, though there was a fall of plasma K⁺ concentration

instead of the usual rise.¹²² In these experiments there was also a rise of the plasma Na⁺/K⁺ ratio instead of the usual fall. The extent of decrease of salivary Na⁺/K⁺ ratio was not always as great as with uncomplicated Na⁺ deficiency, and investigation is proceeding to determine whether this results from a circumstance peculiar to this experiment, i.e. deficiency of K⁺ supply to the biological indicator, or from a smaller adrenal response than usual.

In the dog high K⁺ intake, if it leads to increased plasma K⁺ concentration, causes a rise in aldosterone excretion.¹²³ In man, if the dietary Na⁺ is reduced whilst on low K⁺ intake, the rise in aldosterone excretion may not be as great as that occurring with low Na⁺ intake alone.^{124, 120} Singer and Stack-Dunne⁹⁴ have shown that K⁺ deficiency reduces the amount of aldosterone detected in adrenal vein blood relative to control specimen collected under anaesthesia.

Observation of the blood pH and the plasma HCO₃⁻ concentration during the course of change in Na⁺ balance gives little reason to suppose that there is causal connection with the adrenal response,¹²² and it is a common finding that infusion of 4 M NaCl, which inhibits adrenal secretion in Na⁺ deficiency, does not produce any change in pH or plasma HCO₃⁻ over the time during which a full parotid response occurs.

These results as they stand, particularly as far as plasma Na⁺ and K⁺ concentration changes are concerned, might appear to suggest that these factors had little or no influence on adrenal secretion of aldosterone. They could be taken as evidence in favour of a hypothesis that change of volume of the extracellular or intravascular compartments or some consequence of it causes aldosterone secretion in Na⁺ deficiency and in number of other clinical conditions. Before considering the logical basis of this argument we wish to cite some further experiments.

In sheep, when adrenal secretion is inhibited by the standard procedure for correcting Na⁺ deficiency, some 60 to 70 ml. of 4 M NaCl are given. It is most unlikely that the effect produced is attributable to change of total body water and in any case, there is no effect if the same volume of 2.7 M glucose is given.¹¹ Hence, if the effect were attributable to volume changes, it would depend on redistribution of fluid between compartments, the hypertonic Na⁺ solution causing an expansion of extracellular and plasma volumes. If this is the causal mechanism, it is surprising that an equal volume of 2.7 M glucose has no effect, since, because of slow entry into cells, its osmotic effect would be quite large over the 120 min. following commencement of infusion. Gross water deficiency causing considerable reduction of plasma volume

FIG. 9.—P.F. 33. Concurrent depletion of both water and Na^+ . The effect on parotid salivary Na^+/K^+ ratio (corrected for variation attributable to secretion rate change), plasma Na^+ and HCO_3^- concentration, whole blood and plasma specific gravity and haematocrit of (i) permitting the animal to drink 2.42 l. of water, (ii) permitting the animal to drink 1.21 l. of a 434 m. equiv./l. NaHCO_3 solution — 525 m. equiv. Na^+ .

does not cause a parotid salivary response. The experiment shown in Fig. 9 confirms an earlier report on the absence of parotid salivary response to a large water intake after concurrent water and Na^+ deficiency. P.F.33 was depleted of both water and Na^+ and, when allowed access to water, drank 2.42 l. The decrease of whole blood and plasma specific gravity and haematocrit indicates the probability of considerable expansion of plasma and extracellular fluid volume. There was no effect on salivary Na^+/K^+ ratio. However, when, six hours later, the animal drank 1.21 l. of NaHCO_3 solution (525 mEq. Na^+), the salivary Na^+/K^+ rose rapidly towards normal after 120 min. In a sheep with an adrenal transplant it has been observed under similar conditions of concurrent water and Na^+ deficiency that one haemodynamic consequence of a voluntary intake of 3.46 l. of water was that the adrenal blood flow rose from 6.7 to 14.8 ml./min. over an interval of 4½ hr., yet there was no effect on salivary Na^+/K^+ ratio. Subsequent administration of 4 M NaCl caused a large rise of salivary Na^+/K^+ ratio and a further increase of adrenal flow to 18 to 19 ml./min. Experiments to determine the effect of expansion of plasma volume by injection of albumin are in progress. On two occasions injection of 200 ml.

of 25 per cent. sheep albumin (prepared by the Commonwealth Serum Laboratories) has caused a considerable rise of salivary Na^+/K^+ ratio in Na^+ -depleted sheep. In the one instance where it was measured the plasma volume rose from 2.1 l. to 2.73 l. as a result of the injection. There has been considerable investigation of this question by clinical experiment.^{125-128, 29} It appears that there is a close relation between adrenal response and change of Na^+ content of the body. However, it is not clear which, if any, of the measured physiological consequences of change of Na^+ balance act as a stimulus to the mechanism controlling aldosterone secretion.

In relation to analysis of this situation we draw attention again to the discussion above under the heading 'Plurality of Causes' and to an earlier discussion of the control of respiration where it has been considered that a number of causal factors may act algebraically in different circumstances.^{14, 129, 130} In such mechanisms there is the notion of a dominant receptor sensitive to variation in concentration of the material to be regulated. For such a role in the regulation of Na^+ control, one postulate is a Na^+ receptor responding to change of Na^+ load, e.g. a localized serially arranged transfer mechanism, where the amount of Na^+ reaching the distal units would be determined by the product of Na^+ concentration and blood flow. With concurrent Na^+ and water deficiency the haemodynamic effects would almost certainly cause a diminished Na^+ load despite raised Na^+ concentration. An albumin injection by increasing plasma volume and blood flow could result in an increased Na^+ load. On the other hand, the evidence, at least as far as adrenal blood flow is concerned, is that a large intake of water during concurrent water and Na^+ deficiency raises the blood flow to a far greater extent than plasma Na^+ is reduced, yet there is no effect on the parotid salivary Na^+/K^+ ratio, a finding against a 'load' receptor.

In Na^+ depletion there is a loss of Na^+ from intracellular fluid. There are cells which respond directly to specific ionic variation, e.g. respiratory control, taste buds, intestinal epithelium. It is possible that changes of intracellular Na^+ concentration might lead to activity of specialized cells. Though the changes of intracellular composition with concurrent water and Na^+ deficiency are probably complex, it seems likely that Na^+ deficit would cause loss of intracellular Na^+ . That there are mechanisms capable of registering Na^+ deficit as such is clearly indicated by selective drinking studies on sheep depleted of water, depleted of Na^+ , or depleted of both Na^+ and water. The first accurately selects the appropriate quantity of water, the second of salt and the third

both water and salt.¹⁴ There may be dominant receptors for each deficit which may act separately together, but the accuracy of ingestion clearly indicates that centres with 'memory' and 'coming' capacity would operate from such analysers. The aldosterone response is commensurate and continuous in relation to the absolute sodium deficit and in these respects is somewhat different from the ADH response, which is in proportion to the salt water ratio and which accommodates.

A further possibility not necessarily excluded by the existence of others is that the responsive tissue is not localized and that a metabolic consequence of depletion of a Na⁺ reservoir, such as bone, causes the release a chemical agent capable of specifically stimulating the adrenal glomerulosa.

Consideration of the difficulties of analysis led to the view that a decisive discrimination would result from devising a method permitting access to the arterial supply of the adrenal gland in the conscious animal. The primary object would be to determine whether or not local changes in the ionic composition of the arterial blood influenced aldosterone secretion. Also critical stipulations concerned in the establishment of a physiological response for a putative hormone might be met. The demonstration that a sheep with its sole adrenal gland transplanted to the neck has the same responses to alteration of Na⁺ balance as a normal animal has shown that the adrenal nerve supply is not essential for this physiological response.

During the past three years an extensive series of experiments has been made in this laboratory on the effect of local alterations of the ionic environment of the adrenal gland.^{14, 15} A full report will be published shortly. A summary of some aspects follows.

Four main types of experiments have been made: (i) *Local reduction of Na⁺ concentration and increase of K⁺ concentration of adrenal arterial blood.* An example of the main result of this series is shown on Fig. 10. Transplant 9 was in normal Na⁺ balance: the salivary secretion rate ranged between 1 and 4 ml./min. To ensure that the effect could be attributable to Na⁺ deficiency supervening in the course of the experiment the animal was given a large intake of Na⁺ via rumen during the experiment.

(i) Decreasing the Na⁺ concentration of adrenal arterial blood by 5 per cent. glucose infusion or infusion of 4 per cent. glucose with 10 mM./l. NaCl added did not affect the salivary Na⁺/K⁺ ratio.

(ii) Increasing the K⁺ concentration had little, if any, effect on the salivary Na⁺/K⁺ ratio.

(iii) The *concurrent* decrease of Na⁺ concentration and increase of K⁺ concentration of adrenal

FIG. 10.—Transplant 9. Normal Na⁺ balance. The effect on parotid salivary Na⁺/K⁺ ratio of the infusion into the adrenal arterial supply at 0 min. of the following solutions: (i) 5% glucose, (ii) 4% glucose with 10 mM. NaCl added, (iii) 141 mM. NaCl with 15 mM. KCl added, (iv) 4% glucose with 13.2 mM. KCl added. In each experiment the animal was given the doses of NaHCO₃ at the time shown to eliminate any possibility of Na⁺ depletion occurring during the course of the experiment.

arterial blood had an unequivocal effect on salivary Na⁺/K⁺ ratio within 120 minutes of commencing the intra-arterial infusion. The ratio fell to the range of 4 to 5. A second feature of the particular experiment shown on this figure is that the ratio rose again upon halving the rate of infusion. Fig. 11 records similar experiments. In this instance (middle section) the ratio commenced to fall 120 min. after beginning the intra-adrenal infusion and rose 60 to 70 min. after stopping it. The effect may be compared with that caused under similar experimental conditions by the intravenous infusion of 24 γ. hr. of authentic aldosterone. In the lower section of Fig. 11 an experiment is shown in which the parotid response has occurred more rapidly. The effect produced by halving the rate of adrenal arterial infusion is very interesting, and is suggestive of an 'undamped' system.

Fig. 12 is of considerable importance in that it shows the dose-response relation under these

FIG. 11.—Transplant 9. Normal Na⁺ balance. The effect on parotid salivary Na⁺/K⁺ ratio of (i) intravenous infusion of unesterified d,l,aldosterone at a rate equivalent to 24 µg./hr. of the dextro isomer form, (ii) infusion into the adrenal arterial blood supply of 4% glucose with 9 m. equiv./l. KCl added at 0.45—1.8 ml./min., (iii) infusion into the adrenal arterial blood supply of 4% glucose with 10 m. equiv./l. KCl added at 0.5—2.0 ml./min.

FIG. 12.—Transplant 9. Normal Na⁺ balance. The effect on parotid salivary Na⁺/K⁺ ratio of intravenous infusion of unesterified d,l,aldosterone at rates equivalent to 5.5, 11, and 16.5 µg./hr. of the dextro isomer form. The salivary K⁺ concentration at the plateau of response is shown also. The animal was given doses of NaHCO₃ at the times indicated to eliminate any possibility of Na⁺ deficiency occurring during the course of the experiment.

conditions of Na⁺ balance when the rate of intravenous infusion of authentic aldosterone was increased progressively.

Table 1 shows the results of two of a series of

TABLE 1.—THE EFFECT ON THE COMPOSITION OF ADRENAL VENOUS BLOOD OF INFUSION INTO THE ADRENAL ARTERIAL SUPPLY OF VARIOUS SOLUTIONS

Sheep and Date	Composition of Intra-arterial Infusion	Rate of Adrenal Infusion ml./min.	Volume of Adrenal Specimen	Rate of Adrenal		Haema- tocrit	Speci- men	Whole Blood			Plasma				
				Blood Flow ml./min.	Plasma Flow ml./min.			Na ⁺	K ⁺	Cl ⁻	S.G.	Na ⁺	K ⁺	Cl ⁻	HCO ₃ ⁻
T.P. 12 9/9/59	4% glucose with 14 mEq l. KCl added	1.71	34 ml.	12.0	9.6	25.5	Control Arterial	141	6.4	94	146	4.9	109	24.0	1.0288
						20.5	Adrenal Venous	121	6.5	82	122	6.0	89	20.0	1.0268
						20.2	Adrenal Venous	154	5.6		164	4.2	122	24.4	1.0275
T.P. 9 19/3/59	4% glucose with 18.8 mEq l. KCl added	1.54	33 ml.	13.3	9.0	32	Control Arterial	134	4.7		152	4.0	115		
						27	Adrenal Venous	121	6.0		131	6.3	100		

experiments in which the effect of such infusion on the composition of adrenal arterial blood has been measured by collection of the venous effluent from an indwelling polyethylene cannula. The change of plasma K^+ concentration produced was of the order observed often during Na^+ deficiency. Moreover, as shown on Fig. 11, infusion at half the rate with only 10 mM. l. KCl added to the fluid is effective. The decrease of plasma Na^+ concentration produced was large (21 to 24 mEq/l.) but it has been shown that the same salivary response follows if an infusion of 2 per cent. glucose with 75 mM. l. NaCl + 15 mM. l. KCl is given (cf. Fig. 11). This would reduce Na^+ concentration by approximately 10 to 12 mEq/l.

The specificity of the stimulation of adrenal secretion constitutes control of a number of conditions; e.g. the possibility that the effect is due to glucose, temperature changes, osmotic or oncotic effects is largely eliminated in that these are common to most of the procedures.

2. *Decrease of the Na^+ concentration and increase of K^+ concentration during rapid correction of Na^+ deficiency.* These experiments are complementary to those cited in Section 1. The aim was to prevent locally the rise of Na^+ concentration and decrease of K^+ concentration of adrenal arterial blood which occurs when Na^+ deficiency is rapidly corrected by systemic administration of 4 M NaCl. As indicated earlier (see Fig. 4 and 12) the parotid gland in Na^+ deficiency is more sensitive to aldosterone, and in this context better quantitative definition of the role of various ionic changes is possible. Confirming the results in Section 1, it has been shown that:

(i) Decreasing the Na^+ concentration of adrenal arterial blood (5 per cent. glucose infusion) does not affect the parotid response to correction of Na^+ deficiency.

(ii) Increasing the K^+ concentration of adrenal arterial blood (infusion of 136 mM. NaCl with 15 mM./l. KCl added) caused reduction of the parotid response.

(iii) Concurrent decrease of the Na^+ concentration and increase of K^+ concentration of adrenal arterial blood (infusion of 4 per cent. glucose with 10 to 15 mM./l. KCl added) caused a large reduction of parotid response, the effect being intermediate between that produced by intravenous infusion of authentic aldosterone at 10 and 20 μ /hr.

3. *Local increase of the Na^+ concentration of adrenal arterial blood during Na^+ deficiency.* The Na^+ concentration was increased progressively over the usual time interval of correction of Na^+ deficiency by systemic administration of 4 M NaCl (i.e. 40 to 60 min.) and then held at this plateau for 180 to 240 min. Increase of the plasma

Na^+ concentration also causes a decrease of plasma K^+ concentration (Table 1). The results of over 30 experiments in this series have shown that local increase of Na^+ concentration of adrenal blood by 10 to 15 mEq/l. has little if any effect on parotid salivary Na^+/K^+ ratio. Increase by 20 to 25 mEq/l. has a significant effect, but it is in no way comparable to that occurring when the same change of Na^+ concentration is caused by systemic dosage of Na^+ .

4. *Local increase of Na^+ concentration of adrenal arterial blood as Na^+ deficiency commences.* Ten to 15 hours after the last intake of Na^+ , the parotid salivary Na^+/K^+ ratio of a fistulated sheep which is secreting saliva at 100 to 200 ml./hr begins to decrease. Experiments have been made in which the Na^+ concentration of adrenal arterial blood has been increased at this time. The effect has been to prevent the usual fall of salivary Na^+/K^+ ratio. When the infusion is stopped, the ratio falls rapidly within 90 to 120 minutes. No effect occurs if the same infusion is given systemically. The investigation is still in progress. A particularly interesting feature emerging is that if the adrenal infusion is begun just after the salivary Na^+/K^+ ratio begins to fall the ratio rises to normal and remains normal whilst the infusion continues. If, however, the infusion is stopped and the parotid salivary Na^+/K^+ ratio falls, recommencement of the adrenal infusion some hours later does not cause the ratio to rise again. This result, consistent with the findings in Section 3, will, if confirmed in the necessary series of experiments, raise the interesting possibility that increased adrenal arterial Na^+ concentration can prevent the action upon the adrenal of another humoral factor at the onset of Na^+ deficiency, but once this factor has acted upon the adrenal for an adequate period, the effect cannot be reversed by increased adrenal arterial Na^+ concentration.

Summarizing, the results of the large series of experiments in Section 3, and those in Section 4, indicate formally by the method of difference that some factor or factors other than simple change of Na^+ and K^+ concentration act upon the adrenal gland to cause the response to Na^+ deficiency. As the transplanted gland is denervated, this factor is a humoral stimulus, and the evidence at present is against its being ACTH. The demonstration of adrenal response to concurrent decrease of plasma Na^+ and the increase of plasma K^+ concentration raises interesting questions. The extent of change necessary for this effect, i.e. approximately 10 mEq/l. rise of K^+ and 10 mEq/l. fall of Na^+ is well within the range of change observed in severe Na^+ deficiency. Investigations so far are suggestive that there is a threshold of ionic change for this effect. If this be so, it adds further weight

to the importance of the observation of many workers that there may be an adrenal response to Na^+ deficiency, though there is little or no change of plasma ionic composition. On the other hand, the response to local ionic change is quantitatively significant in terms of aldosterone equivalent and experiments are proceeding using the Kliman and Peterson assay to fulfil desideratum 7. It would seem to be of considerable clinical importance that the extent of concurrent deviation from normal of these two ions required to produce this effect is within the range of plasma change observed in patients when severe fluid loss results from diarrhoea, intestinal fistulae, intestinal suction, burns, and in patients with shock, renal failure, diabetic acidosis and other medical and surgical emergencies. In these situations the plasma ionic changes could be an important mechanism evoking maximal aldosterone secretion.

At this stage of the investigation of aldosterone control it is of great interest also to consider the problem in its general biological context, as this might be provocative of experiments which throw light on the mechanism of control in higher animals. As Chester Jones¹³² has pointed out, it has not yet been established at what point in the phylogenetic system adrenal secretion of aldosterone emerges. His recalculation of the data of Hartmann cited in his book, 'The Adrenal Cortex',¹³⁴ is suggestive that adrenalectomy in animals as primitive as elasmobranchs may cause a rise in plasma K^+ concentration. The characteristic effect of adrenalectomy (decrease of plasma Na^+/K^+ ratio) is seen in the toad. One might postulate that, at the earliest stage of emergence of aldosterone control, the evoking stimulus was decrease of plasma Na^+ and increase of plasma K^+ concentration acting directly on cortical tissue when these changes proceeded to an extent which threatened the characteristic differential of ionic composition between intracellular and extracellular fluid. With subsequent evolution a considerably more comprehensive system of control emerged which operates long before circulatory deterioration or other physiological effect causes severe functional embarrassment to such vital characteristics. This added mechanism, emerging in association with the great development of the nervous system, was functionally integrated with parallel development in animals of the capacity to manifest behaviour appropriate to the correction of specific chemical deficiencies.^{132, 14, 15} The functional end-result in the higher mammalian species is a specific hormonal control of aldosterone secretion^{14, 22, 29} and a specific and quantitatively accurate appetite for Na^+ salts. As a legacy of early phylogenetic development there remains also, as a second line mechanism, the capacity of

the adrenal cortex to respond directly to concurrent alteration of plasma Na^+ and K^+ concentration when the changes proceed to the extent seen in severe fluid loss, electrolyte depletion and circulatory deterioration.

Changes of Intravascular or Extracellular Volume

We have considered some experimental aspects of this problem in the previous section. A comprehensive presentation by Bartter and discussion of recent data was included in the 1959 edition of 'Recent Progress in Hormone Research'¹⁰⁰ and will not recapitulate here. Farrell's article 'Physiological Reviews'²⁹ summarizes the results of many of the experiments involving alteration of body fluid volume by administration of water and pitressin.

Constriction of the Inferior Vena Cava as a Stimulus to Aldosterone Secretion

Constriction of the thoracic inferior vena cava results in an increase of aldosterone secretion.^{5, 22, 135}

The following experiments throw some light on the physiological basis of this effect:

(a) In an earlier study by Ball and Davis¹³⁶ the diameter of the abdominal vena cava was constricted by 25 to 50 per cent. immediately above the renal vessels in order to determine whether increased adrenal and renal venous pressure would have the same effect. There was no change in aldosterone secretion and it was inferred that venous congestion of the liver was the cause of increased aldosterone secretion after caval constriction. Subsequently it was shown that if the inferior vena cava were constricted below the liver to an extent to cause femoral venous pressure to rise above 20 cm. of water increased aldosterone secretion occurred.⁹⁷ The effect of thoracic caval constriction was largely abolished if the animal were encased in an abdominal plaster cast.¹³⁶

(b) The infusion of dextran in amounts sufficient to maintain and increase circulating blood volume failed to prevent the rise of aldosterone secretion due to caval constriction.⁹⁷ On the other hand Bartter¹⁰⁰ reports that infusion of very large amounts of blood will reverse the effect of caval constriction.

Apart from loss of fluid from the vascular compartment, a possible explanation of the effect of thoracic caval constriction on aldosterone secretion is that the hepatic venous congestion might increase the rate of degradation of aldosterone and by reducing the peripheral feed-back on a regulatory centre, cause a stimulation of adrenal secretion. Alternatively, the congested liver may release a substance which acts directly or indirectly upon the adrenal gland.

Constriction of the Pulmonary Artery

is⁵ reported that experimental right-sided failure caused a six-fold increase in aldosterone secretion. In this situation, right atrial pressure is increased whereas with constriction of the inferior vena cava it is decreased, an observation which has to be considered in the light of the findings of Bartter¹³⁷ and Farrell *et al.*¹³⁸ that constricting the right atrium reduces aldosterone secretion.

General Consideration of Matters Relevant to Experiments on the Control of Aldosterone Secretion

It has already been shown that the adrenal glands respond by aldosterone secretion to concurrent decrease of Na^+ and increase of K^+ concentration in the environment. A considerable number of experiments has been reported dealing with the question of humoral stimulation of aldosterone secretion. At this stage it can be said that the effect of the Na^+ depleted adrenally-insufficient animal^{14, 15} and the blood of the dog with I.V.C. occlusion²² when used for cross circulation either in the survival preparation¹⁴ or the acute experiment²² stimulates the adrenal over the period of a few hours of the experiment.

Questions which arise are, (1) whether activation is by excitation or by release of inhibition, (2) whence arises the humoral agent and what is its nature, (3) by what mechanism is the secretion of the humoral agent governed.

Excitation or Release of Inhibition

The tissues are inactive unless excited whereas some are spontaneously active, e.g. sheep's adrenal, heart muscle. Inactivity in the latter tissues is due to inhibitory influences and activity arises by release of inhibition. It is theoretically possible that the adrenal is under the influence of an inhibitory material acting when the animal is in normal Na^+ and body fluid balance. The relative inactivity of *in vitro* adrenals makes this improbable, but aldosterone secretion in these circumstances is high compared with cortisol.⁸⁵ That total removal of the cranial contents, including the hypophysis, does not activate aldosterone secretion in sheep in normal Na^+ balance points in the same direction. For the present we may accept the proposition that aldosterone secretion arises from excitation of the adrenal.

Nature of the Excitation

The usual connotation of excitation is that activity commences at some relatively constant rate (latent period) after application of the excitatory stimulus and continues for the duration of application

FIG. 13.—Edgar—normal Na^+ balance. The effect on parotid salivary Na^+/K^+ ratio of spinomedullary decerebration and hypophysectomy. The parotid secretion rate is shown also.

FIG. 14.—Elmer—normal Na^+ balance. The effect on parotid salivary Na^+/K^+ ratio of spinomedullary decerebration and hypophysectomy. The parotid secretion rate is shown also. Approximately 5 hours before the decerebration, the sheep was given 150 m. equiv. of NaHCO_3 to ensure that Na^+ balance was normal.

(failure to do so is due to either fatigue or accommodation), and ceases at some fairly constant period after cessation of the application. There is a usual assumption, based on experience and its statement, e.g. in Muller's law, that the reacting tissue will have highest sensitivity to one particular environmental change (chemical in this instance), and that this substance arises locally or from specialized tissue which is usually grouped in an organ.

Removal of the site of origin should result in cessation of activation, with the appropriate time relations. Figs. 13 and 14 depict the results of

an experiment which indicate several considerations of importance in the analysis of these mechanisms. Sheep in normal Na^+ balance showed a variable reduction of parotid Na^+/K^+ ratio associated with anaesthesia and preparative surgery. Two hours after removal of the intracranial contents this Na^+/K^+ ratio began to rise and five hours later was at the top of the normal range. If the experiment had terminated then the result would indicate that the source of the excitation of the adrenal had been removed, but two to four hours later, without consistent trend in any physiological function being measured, the parotid Na^+/K^+ ratio began to fall again and in six hours had reached the previous low level. Presumably a series of changes followed each other but it seems likely that the adrenals were stimulated as a result of some change not involving activity of the encephalon or hypophysis. The last six hours of this experiment produced a result which makes clear that a simple interpretation at the 13 hours mark is not satisfactory.

The result contrasts with some of the findings in our series of experiments on *Na⁺ depleted sheep*,¹⁵ where de-encephalization and hypophysectomy did not cause the low salivary Na^+/K^+ ratio to rise. That the continued low level was due to activity of the adrenals was demonstrated by the appropriate rise after bilateral adrenalectomy. Other experiments demonstrated that this continued activity was not reversed by replacement of Na^+ to produce plasma Na^+ levels much in excess of normal. Indeed, these experiments, taken in conjunction with the failure of local increase of the Na^+ concentration in the adrenal artery during Na^+ deficiency to stop aldosterone secretion and Bartter's curious results showing failure of inhibition of the adrenal after caval deconstriction in vagotomized dogs, raise the possibility that a special mechanism is required to stop the adrenal activity once it has been started, or that the acute experimental procedures have started up a new mechanism activating the adrenal. This latter is clearly a possibility in experiments which produce pulmonary oedema and other dire results as recorded by Davis *et al.*⁹⁸ after cervical vagotomy.

The importance of temporary consequences of procedures in assessing the significance of short-term experiments applies also to more physiological procedures than those dealt with above. If to a sheep depleted of 700 mEq. of Na^+ 60 ml. of 4 M NaCl =240mEq. Na^+ are given intravenously during 40 min., the result is likely to be that after 3 to 4 hr. the Na^+/K^+ ratio is nearly normal, an indication that the adrenal activity receded to normal. But after 7 to 9 hr. the ratio may be reduced again—even to the original level—indicating reactivation of the adrenal, though there

had been only 50 mEq. Na^+ loss in this period. Apparently the rapid injection produced change at appropriate points which stopped the adrenal activity, but then perhaps redistribution of Na^+ occurred and brought about reactivation. Interpretation at the peak might have suggested that the Na^+ depletion was completely corrected but interpretation when stability was reached clearly indicated that replacement was not complete and that there were most interesting phases phenomena.

Having these general points under view, some principal experiments concerned with information about the sources of activating substances will be considered.

The Pituitary Gland

(a) *Hypophysectomy*. Data on the chronic effects of hypophysectomy indicate some reduction of aldosterone in adrenal vein blood of dogs²⁶ which is consistent with the results of Davis *et al.*¹⁴⁰ and Singer and Stack-Dunne⁹⁴ for rats. An important feature of the experiments of Davis *et al.* on dogs was that hypophysectomy was carried out six to eight weeks before caval constriction, which caused the usual rise of aldosterone excretion. Llauro¹⁴¹ had similar results for urinary aldosterone excretion in man. In acute experiments on dogs with chronic ascites due to caval constriction, hypophysectomy caused a large fall (76 to 97 per cent) of aldosterone in adrenal vein blood during the 2-hr. period of observation.¹⁴² In discussing this finding by Farrell *et al.*²⁶ that hypophysectomy reduced aldosterone by no more than 40 to 50 per cent., Davis *et al.* attributed the difference to the fact that in Farrell's experiments there was the additional factor of blood loss stimulating the adrenal, it being implicit that blood loss did not act via the pituitary. A number of reports of panhypopituitarism in man¹⁴³⁻¹⁴⁵ indicate variable urinary levels of aldosterone for patients not under Na^+ depletion, and even more variable responses to this, being approximately normal in some and almost absent in others.¹⁴⁶ Daily and Ganong¹⁴⁷ recorded normal response to Na^+ restriction in hypophysectomized dogs. McDonald (unpublished)¹⁴⁸ has shown that hypophysectomized sheep respond to sodium depletion by normal fall in salivary Na^+/K^+ ratio. Knobil and Greep¹⁴⁹ found that hypophysectomized monkeys tolerated Na^+ restriction as well as intact monkeys, though they did no specific studies on aldosterone.

ACTH

There can be little doubt that ACTH does not control the level of aldosterone production. Hernando *et al.*¹⁴⁴ refer to similar diurnal excretion rhythms for aldosterone and other cortical pro-

FIG. 15.—Normal sheep brain with pineal gland *in situ*.

FIG. 16.—Delilah—complete pinealectomy.

acts and Davis¹⁴² finds corticosterone and aldosterone varying approximately concomitantly after adrenalectomy, but there are many instances of independent variations.^{94, 150, 151, 88} Davis's¹⁴² results also show some non-correspondence of aldosterone and corticosterone. As previously mentioned,¹⁴ excessive doses of cortisol do not prevent normal salivary response to Na^+ depletion. Similar results came from Farrell, Banks and Letsky,¹⁵⁰ who showed that prolonged cortisone therapy suppressed adrenal secretion of cortisol

and corticosterone, but had little effect on aldosterone.

There are, however, reports which indicate that ACTH may play some part in aldosterone production. Hernando *et al.*¹⁴⁴ came to the conclusion that ACTH may enhance the secretion of aldosterone, but not initiate it. This is consistent with the results of McDonald and Reich¹⁵² on aldosterone output from the adrenal of Na^+ -deficient sheep. Singer and Stack-Dunne⁹⁴ got similar results in rats, and de Graeff¹⁴⁵ had similar reports

FIG. 17.—Freud—serial section of diencephalon and mid brain of sheep Removal of pineal and a section of the roof of the aqueduct of Sylvius.

for humans. Some of the differences between contradictory results may be due to contamination of ACTH preparations with aldosterone stimulating material, but the variation in the assay method is also probably important.

There are no reports clearly indicating that growth hormone or MSH have an effect on aldosterone secretion.

Pineal Gland

Farrell¹⁰¹ has presented data which suggest that the pineal gland may be the source of a humoral substance regulating aldosterone secretion. With this in view complete pinealectomy was done on two sheep with parotid fistulae. Fig. 15 shows a normal brain with the pineal gland *in situ* immediately anterior to the superior colliculi. Fig. 16 shows the same area at the post-mortem examination of Delilah. The pineal has been completely removed, a finding confirmed by microscopy of serial sections. Fig. 17 shows the posterior diencephalon and midbrain of Freud cut in coronal sections. The pineal has been removed and there has been destruction of an area along the roof of the aqueduct. Histological examination is proceeding. Three weeks after operation Delilah was tested and responded normally to Na^+ deficiency. After three days' depletion the animal was permitted to drink 600 mMol. of NaHCO_3 in 2 l. of water and the salivary Na^+/K^+ ratio rose from 0.9 to 28 over the next 4 hr. (Fig. 18). With Freud, as shown above, the lesion was much more extensive, and a significant feature of the post-operative condition was gross hyperextension of the neck. The sheep

FIG. 18.—Delilah—pinealectomized and Na^+ depleted. Freud—pinealectomized and Na^+ depleted. Effect on parotid salivary Na^+/K^+ ratio (corrected for secretion rate variation) and salivary K^+ concentration (corrected for secretion rate variation) of voluntary intake of 600 m. equiv. of NaHCO_3 dissolved in 2 l. of H_2O . In each instance the sheep drank all the solution immediately it was offered.

required considerable attention during the several weeks it was observed, and this included periods of hand feeding. Because it continually held its

head in this position accurate assessment of salivary loss was not possible. However, the salivary Na^+/K^+ ratio did decrease when the daily Na^+ supplement was withheld and, as shown on Fig. 18, the ratio rose at approximately the usual time when it was permitted to drink 600 m.M. of NaHCO_3 solution. Cardiovascular deterioration with Na^+ deficiency was greater than normal. Gross postural disturbance interfering with the voluntary intake was observed by Andersson (personal communication) in the course of his experiments examining Gilbert's¹⁵³ assertion that the subcommissural organ has a secretory role important in water and electrolyte metabolism.

It seems clear therefore that the pineal component of material extracted by Farrell *et al.*²⁸ which increased aldosterone production by the adrenals in his preparation is not essential for normal aldosterone responses to Na^+ deficiency. Nor does it appear that the decrease in aldosterone production resulting from pinealectomy in similar preparations¹⁵⁴ gives a cogent reason for regarding this structure as having other than its Cartesian function.

In the light of earlier consideration of the possibility of functional integration of aldosterone control and the neural mechanisms determining the selective appetite for Na^+ exhibited by deficient sheep,^{14, 15} a salt appetite study was conducted on Delilah. The animal demonstrated the usual capacity of sheep, losing approximately 400 mEq. Na^+/day from a parotid fistula, to adjust Na^+ intake to loss despite variation in the concentration of the NaHCO_3 solution (Fig. 19).

Extracts

There have been a number of papers which have reported an enhancement of aldosterone secretion by infusion of extracts. Almost every part of the diencephalon has been extracted^{101, 31, 14}

The widespread areas from which activity has been demonstrated raise the question as to specificity of the material used, and indicate the need for careful consideration of the criteria for hormone characterization set out in the introduction. Formal study of the response of the adrenal to pharmacologically active materials is required also. There are suggestive aspects of the studies but, to date, no specific tissue has been identified as the source of a substance causing physiological excitation of aldosterone secretion.

Nervous Mechanisms Concerned with Release of the Humoral Agent

The adrenal response to Na^+ depletion has been shown to be proportional to the absolute Na^+ deficit and irrespective of raised or lowered plasma Na^+ contrived by varying the relative body

FIG. 19.—Delilah. Study of selective appetite for Na^+ . The effect of variation of the concentration of NaHCO_3 solution offered on the voluntary intake of NaHCO_3 . The salivary Na^+/K^+ ratio and the average daily loss of saliva in each period is shown also. The cumulative Na^+ balance as calculated at the end of each 24-hour period is shown. Water was available continuously and the animal ate all of its constant ration of food each day throughout the experiment.

water content. There has been no systematic investigation of receptors concerned with this reaction. The response to bleeding is stated by Holzbauer *et al.*¹³⁹ to require intact splanchnic nerves as receptors. Mills, Casper and Bartter⁹⁵ indicated that intact vagus nerves were essential for cessation but not for initiation of the response to caval constriction. Davis *et al.*⁹⁸ have confirmed this latter proposition but did not release the caval constriction of any dogs following vagotomy. They did not confirm Bartter's¹⁰⁰ other proposition that the carotid sinus innervation plays a part in initiating aldosterone production. Rauschkolb and Farrell³⁰ report that cervical spinal transection, vagotomy, and section of the cervical sympathetic did not reduce the aldosterone production of dogs.

Removal of part of the nervous system has been widely practised. Farrell *et al.* indicated that

decapitation or mid-collicular decerebration, with noradrenaline support, greatly reduced the secretion of aldosterone in their dogs, whereas decortication and spinal transection did not.

In less extensive lesions, Farrell *et al.*^{102, 155} raise the possibility that there is an area in the mid-brain crucial to aldosterone secretion but methodological aspects of the evidence warrant caution.

Davis *et al.*¹⁵⁶ studied the effects of chronic lesions on the aldosterone excretion associated with caval constriction. He found that only those lesions involving the median eminence caused reduction of aldosterone secretion but the lesions did not cover the area most effective according to Farrell, nor do Farrell's lesions involve the median eminence.

In this laboratory we have investigated the effect of removing some parts of the nervous system on the parotid salivary Na^+/K^+ response in Na^+ -depleted and normal sheep. Earlier experiments involving de-encephalization and hypophysectomy in sheep in normal Na^+ balance were mentioned. It has not yet been possible to keep animals with de-encephalization alive long enough to see whether they respond to Na^+ depletion by change of parotid Na^+/K^+ ratio. It has been found that decerebration at the level of the anterior border of the superior colliculus does not cause a change in the parotid Na^+/K^+ of depleted sheep, but such animals respond to intravenous correction of Na^+ deficiency. When the sections passed through the inferior colliculus or were prepontine or spino-medullary, and there was anterior removal associated with hypophysectomy, in the majority there was little or no effect on the low parotid Na^+/K^+ and those animals did not respond normally to intravenous sodium replacement. In some animals there was a definite rise of Na^+/K^+ upon spino-medullary deencephalization, but in one instance this later reverted to the original level. That the low ratio was due to adrenal activity was shown formally in three such experiments by the ratio rising to 10 by 3 to 9 hr. after bilateral adrenalectomy. The results of the 31 experiments in this series to date tentatively suggest a significance in relation to Na^+ control of the region between the anterior border of the superior colliculus and the rostral border of the pons but much more convincing evidence, including survival experiments, is required.

It seems clear that there is a nervous level of organization required for reversal of response to Na^+ depletion in excess of that required to keep the reaction continuing despite Na^+ replacement. The hypophysis is not essential to keep the reaction going.

The present state of knowledge in regard to regulation of aldosterone secretion is thus far from

complete. Progress will depend upon closer recognition of the several sorts of circumstances which can elicit the adrenal response. It may be necessary to overcome the great difficulties inherent in demonstration by survival experiments in order to substantiate as physiologically valid suggestive evidence on aldosterone control arising from acute experimental preparations.

SUMMARY

The elucidation of complex clinical conditions involving disorder of aldosterone secretion requires identification by animal experiment of the main mechanisms which control aldosterone secretion. The introductory section of this paper is concerned with the methodological basis of such animal experiments. The principle approach adopted in the authors' laboratory has been that of step by step surgical preparation of an animal so that the function of anatomically deep seated, and otherwise inaccessible, structures can be studied in the conscious confident animal. It is important that the process of measurement of a physiological function should not itself introduce uncontrolled or undesired variables. The baseline levels are found in the conscious confident animal. A sheep with permanent unilateral parotid fistula and with the left adrenal gland transplanted to a combined carotid artery-jugular vein skin loop in the neck represents a very satisfactory preparation for experiments on aldosterone control. Using the Kliman and Peterson double isotope derivative assay for steroids, it is possible to have an accurate record, at frequent intervals, of adrenal secretion rates without sampling causing significant blood loss, and concurrent measurement of parotid salivary Na^+/K^+ ratio gives a continuous record of peripheral blood aldosterone concentration which, because of the short half-life of aldosterone, rapidly reflects changes of adrenal secretion rate. Difficulties in the interpretation of acute experiments involving surgical procedures under anaesthesia are discussed. The criteria for categorization of an extract which is active upon an effector organ as a hormone are considered.

Some of the principal chemical and biological methods for measuring aldosterone are described in the second section of the paper. In the third section, effects of variation of Na^+ and body fluid balance on aldosterone secretion are discussed. This includes a summary of experiments in this laboratory demonstrating that local concurrent reduction of Na^+ and increase of the K^+ concentrations in adrenal arterial blood causes increased aldosterone secretion. Some aspects of the evidence that there is a humoral agent other than ACTH which stimulates aldosterone secretion

discussed also. The final section of the paper is mainly concerned with experiments on the influence of the central nervous system on aldosterone secretion. Decerebration experiments in the authors' laboratory indicate central nervous control on aldosterone secretion and suggest an important localization in the midbrain, but the results emphasize the difficulties of interpretation of experiments involving acute nervous ablations. A rival experimental study of pinealectomized rats did not provide evidence of this structure playing a role in the adrenal response to changes in Na^+ balance.

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