The bacteria, uniformly dispersed, can be easily harvested in the centrifuge and the deposit resuspended in dextran–glucose solution.

A method is described for enumerating the viable cells with consistent results; the viable-cell content of different batches of vaccine can be accurately standardized.

A study of the keeping properties of the dried vaccine has shown that it has a life of at least twelve months when stored below 20° C.

The relationship between the viable-cell counts of various batches and the tuberculin conversion of guinea-pigs is shown. There is also a direct correlation between the viable-cell count and the size of local lesions in guinea-pigs after intradermal injection.

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ALDOSTERONE EXCRETION IN NORMAL AND TOXAEMIC PREGNANCIES*

BY

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An increase of sodium-retaining substances in the urine of women with toxaemia of pregnancy, determined by bio-assay, was first reported by Chart *et al.* (1951). Venning, Singer, and Simpson (1954) confirmed this, also using crude urine extracts for bio-assay. Greater release of sodium-retaining substances in urine can be obtained by allowing it to stand at *p*H 1 for twenty-four hours at room temperature (Axelrad *et al.*, 1954). Using *p*H 1 and β-glucuronidase hydrolysis prior to extracting the urine, Venning *et al.* (1955) reported an increase of sodium-retaining substances in toxaemia of pregnancy but little increase above normal in normal pregnancy.

Purification of urine extracts by chromatography before bio-assay has an obvious advantage. Such a technique has recently been reported by Barnes and Quilligan (1956), but without stating their evidence that the technique isolates aldosterone. They find a wide range of values in normal pregnancy, but the mean does not exceed the control value. In toxaemia they concluded that there is an increase in sodium-retaining substances, though their figures are all within the range of the non-toxaemic patients, and the mean values have a negative correlation with severity of toxaemia. Using a technique with single chromatography before bio-assay, Venning and Dyrenfurth (1956) reported that the aldosterone in seven normal pregnancies rose steadily from about 4 μg. per day to about 30 μg. per day at term, and that in toxaemic patients provisional results suggest that the aldosterone excretion is within the normal pregnancy range.

An alternative method of determining aldosterone by physico-chemical methods was reported by Neher and Wettstein (1955). This technique depends upon the alteration of relative positions of aldosterone and cortisone in two different chromatographic systems, whereby other steroids in the extracts are eliminated. In this way, the capriciousness of bio-assay is circumvented, but a new doubt is introduced about whether the technique is always capable of purifying the aldosterone completely.

Materials and Methods

The method used is a slight modification of that of Neher and Wettstein (1955). A twenty-four hour collection of urine, with chloroform as preservative, was brought to *p*H 1 and allowed to stand at room temperature for twenty-four hours. It was extracted three times with half volumes of chloroform, with the aid of a continuous stirrer. The chloroform was reduced to a smaller volume on a water-bath at 45° C. under reduced pressure and then washed twice with quarter volume of N/10 sodium hydroxide and twice with quarter volume of water, each wash being backwashed with chloroform. The extract was taken to dryness and then applied to paper which had been dipped in 20% propylene glycol in methanol, for chromatography in the Zaffaroni toluene/propylene-glycol system (Burton *et al.*, 1951). The width of paper used was determined by the amount of pigment in the extract and varied between 7.5 and 15 cm. A marker strip with cortisone and hydrocortisone was also run, and after three days it was developed with blue tetrazolium in sodium hydroxide. The cortisone region of the extract strip was eluted with a mixture of chloroform, ethyl acetate, and methanol in equal quantities. Water was added to the eluate to form two phases, the propylene glycol from the paper passing mostly into the aqueous methanol. The latter was extracted a second time with chloroform. The extract was taken to dryness and applied to paper 1.5 cm. wide and run in the Bush C system—toluene : ethyl acetate : methanol : water = 9 : 1 : 5 : 5 (Bush, 1952).

The first chromatography separates aldosterone and cortisone (which run together) from hydrocortisone. The second chromatography separates aldosterone (which then runs in the same position as hydrocortisone) from cortisone. The strip is developed with blue tetrazolium in sodium hydroxide so that substances with the $\Delta^4$3-ketone configuration, which are also reducing, can be determined. The amount of aldosterone was estimated by comparing the soda fluorescence with that of known amounts of cortisone and hydrocortisone.

Collections of urine were obtained from 55 normal pregnant women and from 20 with toxaemia of late pregnancy; in one normal and one toxaemic patient two determinations were made at different stages of pregnancy. The normal cases were out-patients but they were carefully instructed how to make the collections. The toxaemic patients were all in hospital for the urine collections. When the urine could not be extracted immediately, it was kept frozen at −15° C.

Criteria of Toxaemia of Pregnancy.—These were as follows: the development in a woman 28 weeks pregnant or over of a blood pressure of 140/90 or more, together with albuminuria and oedema. In this series there was only one woman with hypertension but no albuminuria, and she has been grouped among the normal pregnancies.

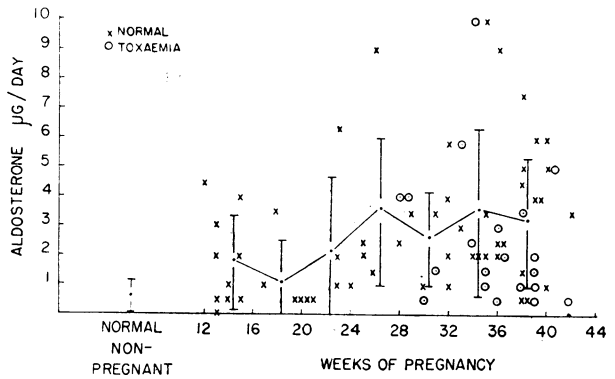
In the toxaemic group there were 10 primiparae and 10 multiparae; 21 of the normal group were primiparae and 34 were multiparae.

* Part of a thesis by I. H. M. for the degree of M.D. in the University of Cambridge.

Results

Recovery of Aldosterone.—After the addition of 6 μg. of pure aldosterone to urine there was a recovery of 55% on the final chromatogram.

Normal Range.—By this method the range of aldosterone excretion in normal men and non-pregnant women has been up to 2 μg. per 24 hours, with a mean and S.D. of 0.6±0.5 μg. per 24 hours.



Aldosterone excretion values for various stages of pregnancy.

The values for the various stages of pregnancy are shown in the Chart. As pregnancy progresses there is a tendency for the aldosterone values to increase and for the scatter to be greater. Of all the determinations, 35 are above the upper limit for normal persons. It is clear from the diagram that the values in toxaemia all fall within the range in normal pregnancy. The mean value in toxaemia for all weeks is 2.5±2.4 and the mean for normal pregnancies of 28 weeks and over is 3.6±2.5. These values are not significantly different.

Discussion

The high aldosterone excretion in normal pregnancy is a somewhat surprising finding. The mechanism for controlling the adrenals' secretion of aldosterone is not established. Axelrad *et al.* (1954) found that administration of corticotrophin gel to normal people did not increase aldosterone excretion. On the other hand, Singer and Stack-Dunne (1954) and Rosenfeld *et al.* (1956) have suggested that corticotrophin has some effect, and Farrell *et al.* (1956) have indicated that some pituitary factor may have an influence because in hypophysectomized dogs the aldosterone content of the adrenal vein blood is only 66% of that of control animals. That the central nervous system plays a part in the control of sodium metabolism is suggested by the fact that lesions in the brain may lead to a salt-losing state (Welt *et al.*, 1952) or to hypernatraemia. In the latter condition Cooper and Crevier (1952) report that there is usually damage in the region of the hypothalamus. Whether these states of abnormal sodium metabolism are mediated by aldosterone is unknown. In pregnancy the increased aldosterone excretion may be due (1) to alteration of the activity of the centre controlling sodium metabolism so that there is greater aldosterone secretion; (2) to the sodium excretory factors being raised and the aldosterone production being increased by the normal homoeostatic mechanism; or (3) to the minor role of the pituitary in affecting aldosterone secretion being increased during pregnancy.

The second of these possibilities seems the most probable. Pregnancy is associated with a large increase in progesterone production. Landau *et al.* (1955) reported that administration of progesterone in doses of 50 to 100 mg. per day caused a sharp rise in sodium excretion. The same effect is produced in patients with Addison's disease if they are maintained on either cortisone or deoxycortone but not if they have no replacement therapy. This suggests an antagonism between progesterone and adrenal cortical hormones. It may well be that during pregnancy the sodium-excreting effect of progesterone is counterbalanced by the rise in aldosterone secretion. The finding of Dieckmann and Pottinger (1956) that the intracellular sodium in muscle and skin is raised in normal pregnancy may well be connected with the increased aldosterone excretion.

The fact that the present study shows that the aldosterone excretion in toxaemia is no different from that in normal pregnancy requires some explanation. It was concluded by Chart *et al.* (1951) and by Venning, Singer, *et al.* (1954, 1955) that there was an increase of sodium-retaining substances in the urine in toxaemia of pregnancy. They used crude urine extracts and did not leave the urine at *p*H 1 for twenty-four hours to hydrolyse the conjugated aldosterone (Axelrad *et al.*, 1954). The free aldosterone to be found in urine is a very small fraction of an administered dose (Mills, 1954), but a greater percentage is excreted in a form released by prolonged acidification (Venning, Carballeira, and Dyrenfurther, 1954). Neher and Wettstein (1955) found the free aldosterone to be about 6% of the total after hydrolysis at *p*H 1 for twenty-four hours. It is to be assumed, therefore, that these crude extracts used in bio-assay contained only a small part of the aldosterone.

It is noteworthy that Chart *et al.* (1951), Venning, Singer, and Simpson (1954), and Barnes and Quilligan (1956), using bio-assay, found no increased sodium-retaining substances in the urine during normal pregnancy. This can be explained either by assuming, as Venning and Dyrenfurth (1956) have done, that more free aldosterone is excreted in toxaemia, or by postulating that another potent sodium-retaining substance is present in the latter cases. Purification of extracts of urine which have been subjected to hydrolysis at *p*H 1 and prior to bio-assay has been carried out in some instances. Barnes and Quilligan (1956) may have lost some aldosterone, using three consecutive chromatograms, because, as a group, their normal pregnant patients excreted no excess of sodium-retaining substances. Venning and Dyrenfurth (1956) have used the Bush (1952) B_5 system of chromatography to purify the extracts. There is some doubt about how effective single chromatography is in isolating any single steroid. Certainly the cortisone region in the toluene/propyleneglycol system, when eluted and run in the Bush C system, separates into at least five separate spots producing a typical soda fluorescence, though only rarely are all five spots in one extract. The association of one of these spots, which is not aldosterone, with water-retaining states is being further investigated.

The values in late pregnancy in the small series reported by Venning and Dyrenfurth (1956) are much higher than those of the present study. This may be due to greater losses in the double chromatographic technique, which involves more numerous manipulations. The single recovery figure of 55% suggests this may be so, and no correction has been made for this. The two techniques use entirely different methods of measurement, and estimating the amount of aldosterone by comparing its soda fluorescence with that of hydrocortisone gives unduly low values because in our hands the soda fluorescence of aldosterone is distinctly less than that of the same weight of hydrocortisone. On the other hand, in the Venning and Dyrenfurth method other sodium-retaining substances may be present in the region of the chromatogram which they elute.

"Secondary Aldosteronism"

An increased excretion of sodium-retaining substances has been reported in other diseases with fluid retention—for example, in nephrosis (Luetscher and Johnson, 1954; McCall and Singer, 1953; Neher and Wettstein, 1955), in cardiac failure (Deming and Luetscher, 1950; Singer and Wener, 1953; Neher and Wettstein, 1955; Axelrad *et al.*, 1955), and in cirrhosis of the liver (Chart and Shipley, 1953; Gordon *et al.*, 1954; Axelrad *et al.*, 1955). Only in nephrosis has it been proved that this substance is aldosterone, for in this instance it has been obtained in a crystalline state (Luetscher *et al.*, 1955). These conditions have been referred to as "secondary aldosteronism" (Conn, 1955) because

they differ greatly from the case described by Conn, in which there was excessive aldosterone production by an adrenal tumour (which he called " primary aldosteronism "). Further cases of this condition have been described by Foye and Feichtmeir (1955), Mader and Iseri (1955), Chalmers *et al.* (1956), Milne (1956, with previous evidence by Cope and Garcia-Llaurado (1954) concerning the aldosterone excretion in one of the cases), and Brooks *et al.* (1956). There is a striking difference between these latter cases, in which oedema is not present and there is a low serum potassium, and the so-called " secondary cases," in which the serum potassium is normal but there is water and salt retention.

Analysis of muscle and skin in toxaemic pregnancy (Dieckmann and Pottinger, 1956) shows that the intracellular sodium is down to non-pregnant levels. Dieckmann and Pottinger point out that the serum sodium and potassium levels are on average the same in toxaemia as in normal pregnancy but the total body sodium is increased. These findings are somewhat surprising, and it appears that the defect in toxaemia is primarily in the transfer of sodium either into the cells or into the plasma. The factors which control this transfer are complex (Conway and Hingerty, 1953), but they almost certainly include aldosterone (Hetzel *et al.*, 1956).

The glomerular filtration rate and renal plasma flow in toxaemia were found by Assali *et al.* (1953) to be 70–80% of those in normal pregnancy. This reduction is insufficient to make an appreciable difference to the relative aldosterone values in the two groups.

In view of the antagonism between progesterone and adrenal cortical hormones, as regards sodium excretion, the balance between these may be important in toxaemia of pregnancy. There has been some difference of opinion on whether the amount of excreted pregnanediol is normal or abnormal in toxaemia. de Watteville (1951) concluded that in toxaemic patients with normal foetuses the pregnanediol excretion was often within the normal range, whereas in patients with dead or maldeveloped foetuses the pregnanediol excretion was always below the mean for normal pregnancy of the same stage. If this antagonism is important, however, it is possible that small decreases in progesterone production might be significant, especially if there were a simultaneous slight rise in aldosterone secretion. It is possible that the urinary excretion of aldosterone does not reflect the plasma level of the steroid during pregnancy. This is the case with plasma hydrocortisone and its urinary metabolites (J. D. Martin and I. H. Mills, to be published), and with plasma 17-ketosteroids and the urinary excretion of them (Gardner, 1954). Both glucocorticoids and oestrogens can also influence salt and water excretion. It is probable that toxaemia of pregnancy is not to be explained by the absolute amount of aldosterone secreted but by the summation of the sodium-retaining factors relative to the sodium-excreting factors. It would not be surprising if there were more than one cause for toxaemia.

Summary

Aldosterone has been measured by a slight modification of the technique of Neher and Wettstein (1955) at various stages throughout pregnancy in 55 normal subjects and 20 cases of toxaemia.

The normal value of the reported technique is up to 2 μg. per day, with a mean and S.D. of 0.6 ± 0.5. In pregnancy the excretion is often above the upper normal limit. Values tended to be higher later in pregnancy and went up to 10 μg. per day.

In toxaemia the amounts excreted were within the range for normal pregnancy.

It is suggested that the increased aldosterone secretion in normal pregnancy may be to counteract the sodium-excreting effects of progesterone.

Toxaemia of pregnancy is probably due to disturbance of the balance between all the sodium-retaining substances and all the sodium-excreting substances. There may be more than one cause for toxaemia.

We wish to thank Professor F. T. G. Prunty for his helpful advice and criticism, and the consultant staff of the department of obstetrics for permission to use their patients for this investigation. We also express our appreciation of the painstaking technical assistance afforded by Miss F. Joy Wells.

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In a report on fly control in Israel Dr. H. SPEICH, medical consultant of the World Health Organization, has partially answered the question why flies are so much more of a nuisance and more difficult to control in hot climates than in temperate ones. During a recent visit to Israel Dr. Speich found that flies bred at the rate of 26 generations in one year, compared with 11 to 13 generations in central and northern Europe. Added to this, he found that the pupal development stage of flies in Israel lasts only three days instead of the usual 10 to 12 days in cooler climates. An experiment was performed in which a net was cast ten times an hour over a table in a large kitchen situated 18 m. from the nearest breeding-place, a cow-shed. The total number of flies trapped in three hours was 3,400. Taking into account the fact that about one-third of the flies present must have eluded each throw of the net, it was estimated that about 5,500 flies settled on the table during the three hours and that 1,800 fresh flies arrived every hour. Dr. Speich concluded his report by stating that sanitary measures, the well-planned use of correct insecticides, and the education of the public in such matters as the transmission of disease by flies and pollution of foodstuffs, are the only effective measures against the menace of flies in hot climates.