SOME EFFECTS OF PHENOLIC ANTI-OXIDANTS ON SODIUM AND POTASSIUM BALANCE IN THE RABBIT

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THIS paper is part of a study of the biological activity of two commercial anti-oxidants, 2 : 6-di-*tert.*-butyl-4-methylphenol (BHT) and butylated hydroxyanisole (BHA), which is a mixture of two isomers, 85 per cent being 2-*tert.*-butyl-4-methoxyphenol and 15 per cent 3-*tert.*-butyl-4-methoxyphenol. These anti-oxidants are used for the stabilization of edible fats. In the course of metabolic studies of BHA (Dacre, Denz and Kennedy, 1956) and BHT (in progress), it was found that a series of daily doses of 1 g. to rabbits led to muscular weakness, inanition and death. Although it is well known that large doses of such phenolic materials are lethal, the mechanism of poisoning has been obscure. It will be shown here that large doses of BHA and BHT produce a gross disturbance of sodium, potassium and water balance in the rabbit.

METHODS

Animals

The rabbits were does of the New Zealand white strain weighing 1·3–2·0 kg. They were maintained on a standard pellet diet (wheat, 40 per cent ; pollard, 33 per cent ; bran, 27 per cent), and were kept singly in metabolism cages designed to permit the separate collection of urine and faeces. Except where otherwise indicated in the text, water and food were supplied *ad lib.* The daily intake of food and water and the urine volumes were measured. The faeces were generally dry and well-formed and were easily collected and weighed.

Materials and dosage

BHA (Sústane) was obtained from the Universal Oil Products Company, Riverside, Illinois, U.S.A., and BHT from the Shell Company of New Zealand, Ltd. Both BHA and BHT were dissolved in olive oil and 1 g. in 5 ml. of oil was administered by stomach tube to the rabbits each morning. Rabbits usually refused food during the 24 hr. after receiving BHA or BHT. Rabbits given 5 ml. of olive oil also showed a marked reduction in food intake.

Histology

Frozen sections of the adrenal were cut at 10 μ and stained with Oil Red O. Paraffin sections of tissues were stained with Harris's haematoxylin and eosin, Crossman's trichrome and Heidenhain's azan.

Sodium and potassium estimations

All estimations of Na and K were made by flame photometry.

In urine.—Direct determinations were made on appropriate dilutions of urine.

In serum.—Blood was collected from the marginal vein of the ear into silicone-coated tubes. The serum was separated by centrifuging the blood immediately after collection (cf. Cardus and Llaurado, 1955).

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In food and faeces.—About 1 g. of the material was weighed into a micro-Kjeldahl flask and digested with redistilled HNO_3 until the residue was colourless. This residue was taken up in 10 ml. of 0·1 N-HCl and the Na and K determined directly.

In tissues.—Rabbits were killed in a gas chamber and the muscles (tibialis anticus, soleus, biceps femoris), the diaphragm, heart and brain were dissected and samples with a wet weight of 0·3–1·0 g. were taken into weighed stoppered tubes. After reweighing, the tubes were placed in a deep-freeze cabinet for 2–4 hr. The tissues were dried from the frozen state. They were then weighed to give dry weight and the fat was extracted by shaking with 10 ml. of dry ether (dried by distillation from conc. H_2SO_4). A constant weight for extracted tissue was obtained for the biceps femoris, soleus, tibialis anticus and heart after one extraction, diaphragm after two and brain after three consecutive extractions with dry ether. The dry tissue was dissolved in 5 ml. concentrated HNO_3 (redistilled) and transferred to a micro-Kjeldahl flask and digested until the residue was white. The residue was taken up in 10 ml. of 0·1N-HCl for Na and K determinations.

Aldosterone assays

Urine produced by 3–4 rabbits in 24 hr. was pooled and 40 min. after acidification to pH 1 extracted 4 times with chloroform and then re-extracted 4 more times after 24 hr. The extract was evaporated to dryness at reduced pressure, taken up in a small volume of chloroform, dried in a stream of nitrogen, dissolved in 20 per cent ethanol in water and the concentrate assayed on adrenalectomized rats. The rats used in the bio-assay were injected with a portion of the extract and a load of Na and K. The activity of aldosterone was measured by the reduction of the Na/K ratio in the urine of the adrenalectomized rats and expressed as a percentage of the Na/K of a control group injected with solvent alone and given the same load of Na and K on the same day. The figures obtained as percentages have been converted to μg. of aldosterone by a calibration curve obtained previously with pure aldosterone. This method has been fully described by Llaurado (1956*a*, *b*).

Extracellular fluid volume

The thiocyanate method was used. The rabbit's bladder was emptied by suprapubic pressure. A 2 ml. sample of blood was taken from the left ear into a siliconed tube. A dose of 25 mg. of sodium thiocyanate in 2·5 ml. of water was injected slowly into the marginal vein of the right ear. Two hours later the rabbit's bladder was again emptied and a second sample of 2 ml. of blood taken from the left ear. Protein-free filtrates were obtained from the separated serum with 10 per cent trichloracetic acid. The thiocyanate in the protein-free filtrates of serum and in the urine were determined by measuring in a spectrophotometer the intensity of colour developed with ferric nitrate (see Elkinton and Taffel, 1942).

The extracellular fluid volumes are expressed as ml. Rabbits given BHA or BHT lose weight and this loss obscures the fall in extracellular fluid volume that occurs during treatment when the results are expressed as a percentage of body weight. Rabbits with similar initial weight (1·30–1·35 kg.) were chosen for this experiment. The mean value of the extracellular fluid volume of rabbits before dosing with BHA was 416 ml. or 31·5 per cent of body weight.

RESULTS

Increase in urinary sodium and potassium

When daily doses of 1 g. of BHA or BHT are given orally to rabbits there is a ten-fold increase in sodium excretion in the urine. At the same time there is a

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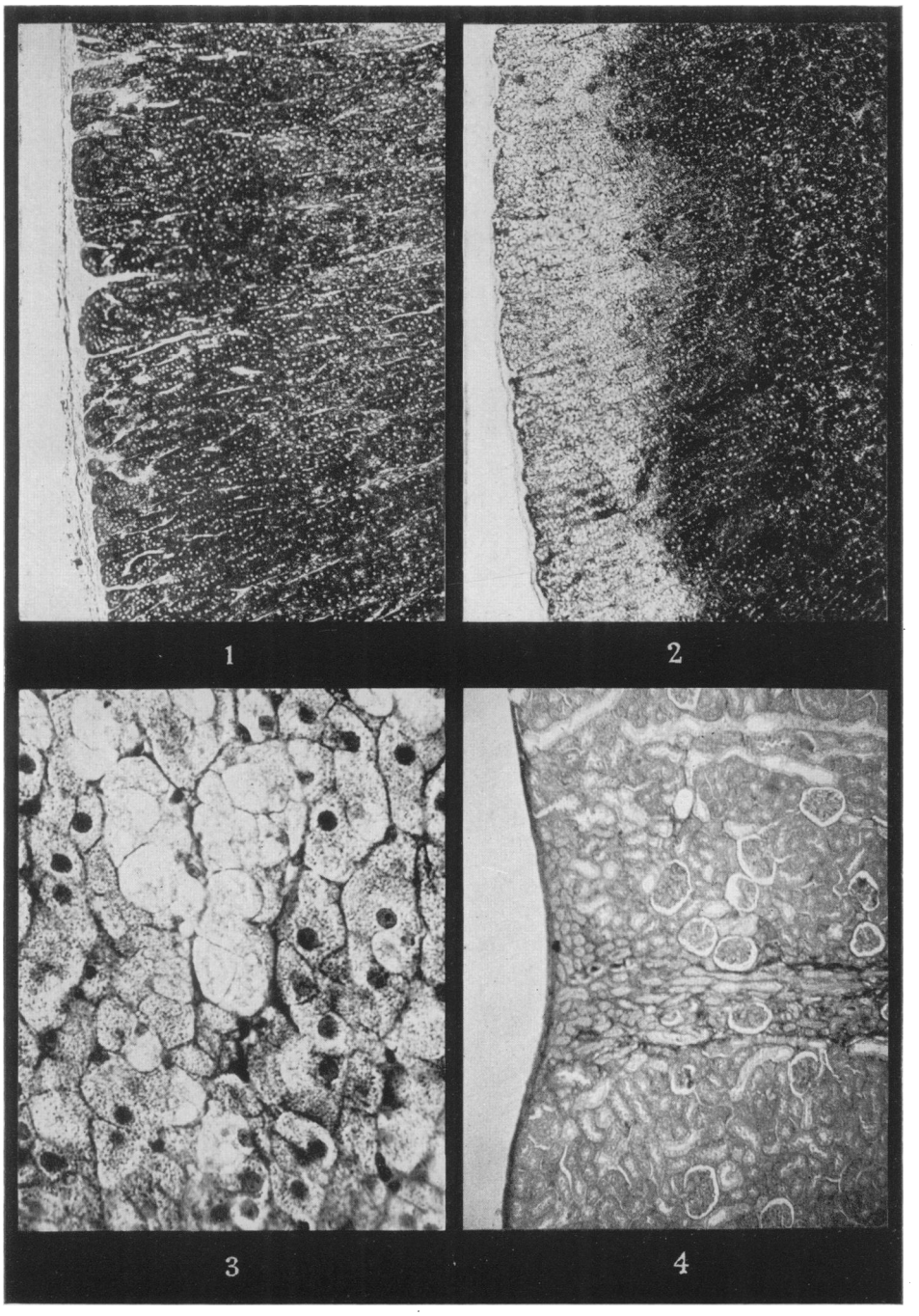
EXPLANATION OF PLATES

FIG. 1.—Z. glomerulosa and fasciculata of normal rabbit adrenal. Stained with Oil Red O.

FIG. 2.—Rabbit adrenal after five doses of BHA showing lipoid depletion in Z. glomerulosa. Stained with Oil Red O.

FIG. 3.—Rabbit adrenal after five doses of BHA. Inner fasciculata showing cells undergoing hydropic degeneration.

FIG. 4.—Kidney from control rabbit showing scarring and fibrosis.



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20 per cent increase in potassium output in the urine. The results obtained with BHA are given in Table I. Similar increases have been obtained with BHT.

TABLE I.—*Effect of Daily Dose of One Gram of BHA on Urinary Sodium and Potassium (as m-equiv./Day)*

	Basal level	Number of doses				
		1	2	3	4	5
Sodium	0·137	0·787	1·179	1·300	0·815	1·063
Potassium	5·394	6·690	5·847	6·058	5·130	5·260

Results are means for 13 animals.

Changes in the adrenal cortex

The normal appearance of the adrenal cortex of the rabbit when stained with Oil Red O is shown in Fig. 1. After a series of three to five doses of BHA or BHT there is a gross depletion of lipoid staining of the zona glomerulosa (Fig. 2). The intensity of staining of the other zones of the adrenal cortex is unaffected. Sections stained with haematoxylin and eosin do not show any cellular necrosis in the zona glomerulosa, but show some increase in cytoplasmic basophilia (cf. Symington and Davidson, 1956).

In sections stained with haematoxylin or by trichrome methods, single cells and small groups of cells in the zona fasciculata and especially in its deeper layers (the inner fasciculata of Nicander, 1952) are seen to be undergoing hydropic degeneration (Fig. 3). In some sections from control animals similar changes are seen occasionally in the zona reticularis but not in the zona fasciculata. In this paper no attempt will be made to evaluate the significance of the changes in the zona fasciculata.

Changes in aldosterone excretion

The remarkable increase in sodium excretion and the loss of lipoid staining in the zona glomerulosa, which seems to be the most likely site of the production of aldosterone (Deane, Shaw and Greep, 1948 ; Peschel and Race, 1954, and others), showed the need for data on aldosterone excretion in these rabbits.

A biological assay was used. For convenience of exposition the assays are expressed as μg. of aldosterone per day although no great weight is attached to the absolute figures owing to the difficulties that have been found in comparing results obtained in different laboratories (see Dodds, Garrod and Simpson, 1956). The changes in aldosterone level occurring with treatment are regarded as being more significant than the individual figures for aldosterone.

In rabbits treated with BHA or BHT there is a consistent rise in aldosterone excretion (Fig. 5). The average level of aldosterone excretion in normal rabbits was 0·027 μg. per day with a range of 0·003 to 0·061 μg. After dosing with BHA or BHT a high level of aldosterone excretion was maintained for periods up to one week. One group of four rabbits excreted an average per rabbit of 0·044 μg. on the seventh day of treatment compared with an initial level of 0·017 μg. The highest aldosterone levels were reached about the fourth day of treatment (levels of 0·15–0·19 μg.). The later samples showed some decrease in aldosterone but did not fall to pre-injection levels.

Renal changes

The association of a raised excretion of both sodium and aldosterone suggests that the increased sodium loss is due to changes in renal function. An examination of the kidneys of rabbits from our colony shows that all kidneys are affected by a chronic inflammatory process normally described as pyelonephritis (Fig. 4). The focal lesions of the kidney appear in the rabbits in the first three or four weeks of life before they are weaned. These lesions are sufficiently severe to prevent

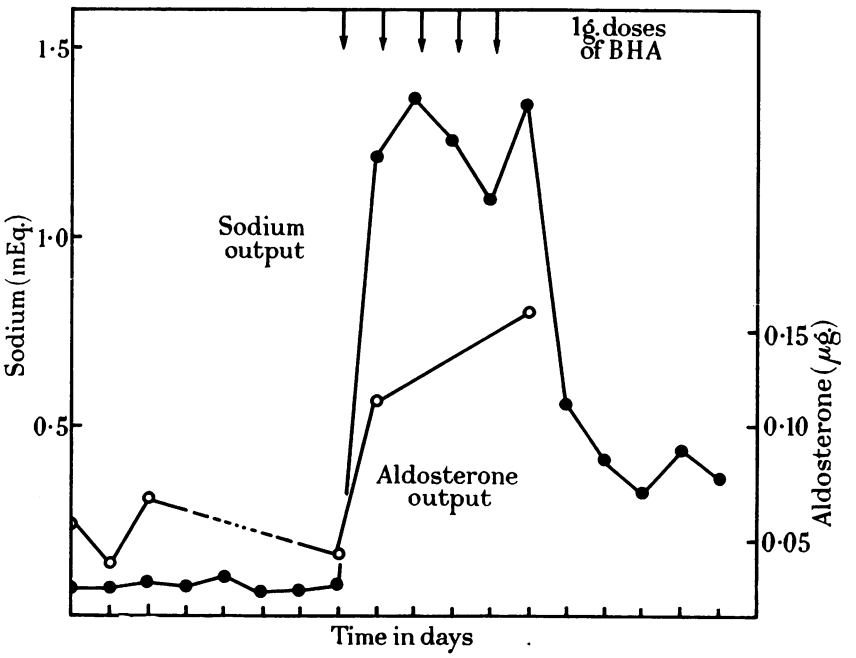

FIG. 5.—Effect of BHA on the Na and aldosterone excretion in the rabbit.

any detailed histochemical studies of the kidneys. In these acute experiments there was no evidence of vacuolation of the tubular epithelium which has been described in chronic potassium deficiency in man and some experimental animals (Conn and Johnson, 1956 ; Fourman, McCance and Parker, 1956, etc.).

Changes in serum sodium and potassium

The first three doses of BHA or BHT cause a slight rise in serum sodium. Only in the terminal stages is there any fall in the sodium level. Potassium, on the other hand, shows an immediate fall which is progressive and the level falls to about half the normal value after the sixth dose (Table II).

Sodium and potassium balance

The extent of potassium loss in treated animals determined by balance experiments is greater than might be expected from data on urine alone. Figures in Table I show that an increase of 2·01 m-equiv. of potassium above normal levels

TABLE II.—*Effect of Daily Doses of One Gram BHA on m. equiv./l-Sodium and Potassium in the Serum of Rabbits*

	Basal level	Number of doses 1	2	3	4	5	6	7
Sodium	146	148	150	150	145	145	142	126
Potassium	4·0	3·7	3·2	3·2	2·7	2·7	2·2	1·9

Each figure is the mean of four estimations.

was excreted in six days. This observation fails to take into account the fact that animals dosed with the anti-oxidants in olive oil ceased to eat. This reduces their potassium intake to zero and they are then in negative balance to the extent of the potassium in the urine and faeces. The effect on the potassium balance is shown in Fig. 6, where in the course of a series of six doses of BHA the rabbit

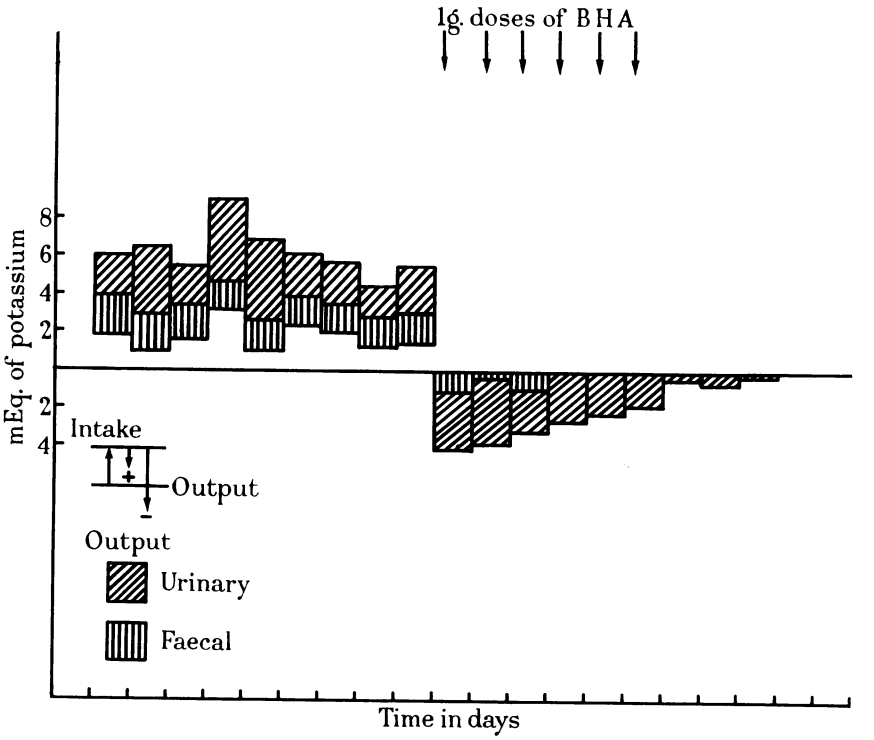

FIG. 6.—Effect of six daily doses of 1 g. of BHA on the K balance in the rabbit. Total K loss 36 m-equiv.

has lost 36 m-equiv. of potassium, which was about half of the potassium normally present in the body. Olive oil alone does not produce any significant potassium loss because the potassium output falls sharply with the decrease in food intake. The effect on sodium balance is less remarkable, and the corresponding sodium loss after 6 doses is 5·7 m-equiv.

The relative proportions of the daily losses of sodium and potassium to the amounts in the extracellular fluids and the cells is shown in Fig. 7. It can be

seen that the daily loss of sodium is unlikely to produce any marked effect on the sodium in the extracellular fluid, and therefore in the serum, for some days. The daily loss of potassium is grossly in excess of the amount in the extracellular fluid at any one time and can be maintained only by a continual withdrawal of potassium from the cells.

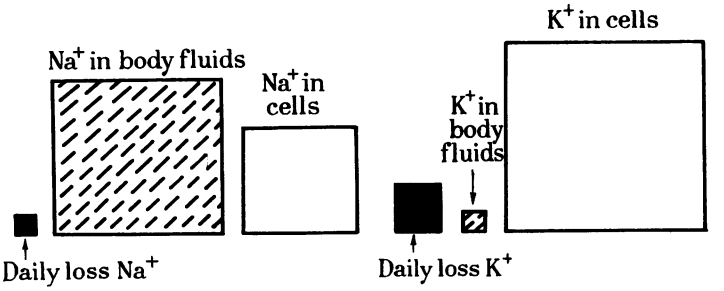

FIG. 7.—Comparison of daily loss of Na and K in treated rabbits with the total intracellular and extracellular Na and K.

Tissue sodium and potassium levels

The data for sodium and potassium are those obtained by direct analysis and no attempt has been made to divide them into extra- and intracellular levels within the tissue. The tissues chosen were white muscle (biceps femoris), red muscle (soleus), mixed red and white muscle (tibialis anticus), diaphragm, heart muscle and brain. These tissues show very different levels (Table III), and they respond differently to the loss of body potassium which occurs when either BHA or BHT is administered. In voluntary muscle, there is a decrease in muscle potassium and an increase in sodium. This change becomes accentuated after four or five days. The changes are shown for the tibialis anticus muscle in Fig. 8. On the other hand, the potassium and sodium levels of brain, heart and diaphragm show very little effect until the terminal stages when, about the sixth day, there is a fall in potassium and a rise in sodium. These effects are shown for heart muscle in Fig. 9. From these results it can be seen that the intracellular potassium is not drawn on uniformly at a time when potassium loss is considerable.

TABLE III.—*Sodium and Potassium in μ. equiv./g. Wet Tissue in Normal Rabbits*

Tissue	Biceps femoris	Soleus	Tibialis anticus	Diaphragm	Heart	Brain
Sodium	15·1±0·7	29·7±1·6	19·6±0·6	25·6±1·3	46·0±3·0	46·8±2·2
Potassium	88·0±4·5	82·2±4·1	94·3±1·2	63·2±2·5	62·9±1·2	71·4±2·5

Means and S.E.M. of 5 estimations.

Cause of death

The profound fall of serum potassium and the reduction in cellular potassium especially in the later stages of poisoning suggests that the rabbits are dying because of potassium deficiency. This conclusion is supported by the observation that the rabbits develop muscular weakness especially in the hind limbs. Rabbits can be kept alive by administering 1 per cent potassium chloride. Some of the

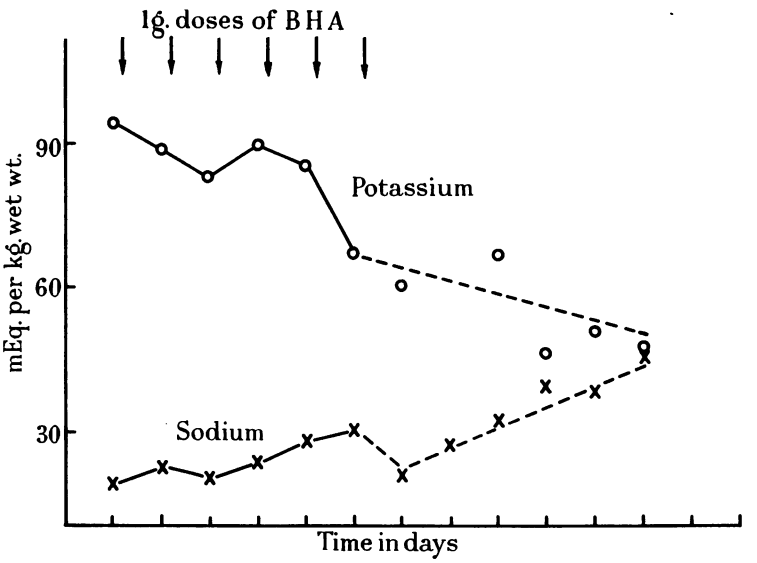

FIG. 8.—Effect of BHA on the Na and K levels in the tibialis anticus muscle. First six points on each curve are means of five animals, remaining points from single animals.

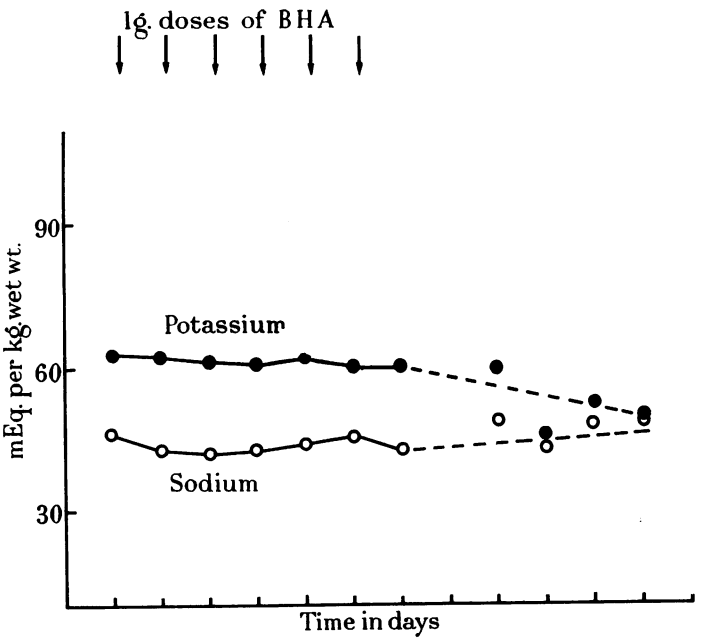

FIG. 9.—Effect of BHA on the Na and K levels in the heart muscle. First six points on each curve are means of five animals, remaining points from single animals.

affected animals will drink this solution. Others had to receive it by stomach tube.

These findings explain an earlier observation that rabbits which were fed cabbage survived many more doses of BHA or BHT than those supplied only with food cubes. It also explains the failure of sodium chloride therapy which was attempted when the sodium-losing effect was first detected. Replacement therapy with 9-α-fluorohydrocortisone acetate in an attempt to prevent the sodium loss was also tried before the aldosterone assays had been made. In view of the fact that aldosterone excretion was at a high level all the time and that death was due to potassium loss, it was not surprising that corticoid replacement therapy failed to keep the rabbits alive.

Extracellular fluid volume

It has been shown above that in treated animals there is an increased loss of sodium in the urine and an increase in the sodium content of muscle. This happens at a time when the sodium intake has been reduced almost to zero so

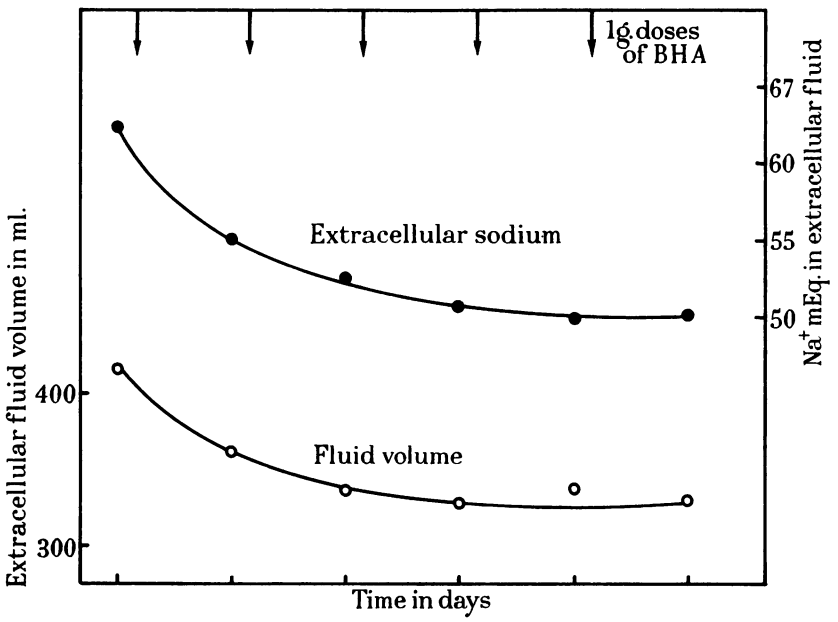

FIG. 10.—Effect of BHA on the extracellular fluid volume and the total extracellular sodium. Each point is a mean of four estimations.

that this movement can only happen at the expense of the sodium in the extracellular fluid. It has been shown that the concentration of sodium in the serum, and hence in the extracellular fluid, does not fall at least for the first five days. These observations are compatible only with a fall in extracellular fluid volume, and this has been found. In Fig. 10 the total amount of extracellular sodium is shown and it will be seen that there is an early fall in the amount of extracellular sodium that continues over the period of five days during which the animals are dosed with the anti-oxidant.

DISCUSSION

The death of rabbits given 1 g. daily of BHA or BHT is attributed to the loss in the urine of potassium which leads to a fall in the potassium in the muscle and other tissues.

The association of high aldosterone output with excessive potassium loss in man was first reported by Cope and Llaurado (1954), although the significance of this association was not properly understood until Conn (1955) showed that it is due to a hyperfunctional adrenal tumour, and established the clinical syndrome called primary aldosteronism. Subsequently it has been shown (Luetscher and Curtis 1955; Falbriard, Muller, Crabbé and Duckert-Maulbetsch, 1955) that a high aldosterone output can be also associated with excessive sodium loss in the so-called "sodium-losing" nephritis. In this case the primary lesion consists of an inability of the renal tubules to reabsorb sodium, and the hyperproduction of aldosterone is a compensatory mechanism to save body sodium. In the long run, however, this mechanism is detrimental because it may lead to potassium depletion.

In the experiments reported here the association of an increased excretion of sodium in the urine with an increased output of aldosterone suggests that the primary effect of the anti-oxidants is renal and the increased aldosterone secretion is secondary to the sodium loss. The histological picture of selective depletion of lipoid of the zona glomerulosa of the adrenal supports the current view that this is the site of aldosterone production. The relatively high level of aldosterone secretion even in the later stages of poisoning suggests that the loss of lipoid is not evidence of failure of the adrenal, but is a measure of the rapid production of aldosterone.

The underlying cause of the excessive loss of electrolytes in the urine has not been found. Both BHA and BHT are excreted mainly as glucuronides and ethereal sulphates. These materials are comparatively strongly acid and they may carry sodium and potassium with them into the urine. However there is very little increase in ammonium ion in the urine. There is the further possibility that BHA and BHT by their action as anti-oxidants may have a direct effect on the tubular epithelium of the kidney. Preliminary studies of the metabolism of BHT in the rabbit complementary to those already reported on BHA suggest that the metabolism of the compounds is very different. A striking finding in this paper has been the identical effects of these two compounds on the sodium, potassium and water balance in the rabbit.

SUMMARY

Two phenolic anti-oxidants when given to rabbits by stomach tube in doses of 1 g. per day produce similar effects on Na and K excretion in the urine: a ten-fold increase in Na and a 20 per cent increase in K.

The increase in urine Na has little effect on serum Na, and there is an early fall in extracellular fluid volume which parallels a similar fall in the total amount of extracellular Na.

The serum K falls steadily after five days of treatment, and this is associated with a fall in K and a rise of Na in voluntary muscle. Heart muscle shows little change in Na and K levels until the terminal stages.

The adrenal cortex shows a gross decrease in fat staining of the zona glomerulosa, the region believed to be the site of aldosterone production. At the same time there is an increased excretion of aldosterone in the urine.

A series of daily doses of either anti-oxidant leads to muscular weakness, inanition and death, which is attributed to the loss in the urine of K leading to a fall in the K in the muscle and other tissues.

It is suggested that the primary effect of the phenolic anti-oxidants is renal and that the increased aldosterone secretion is secondary to the Na loss. The underlying cause of the excessive loss of electrolytes in the urine has not been found.

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