A STUDY OF GASTRIC SECRETION AND KIDNEY FUNCTION IN CHRONIC FLUOROSIS IN EXPERIMENTAL ANIMALS.

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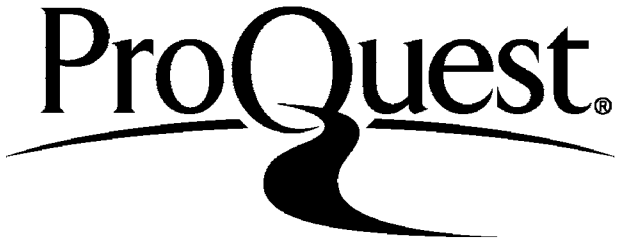

Reprints of supporting publications are inserted in a pocket on the back cover, viz:

"Kidney Function and Structure in Chronic Fluorosis" by Audrey M. Bond and Margaret M. Murray (1952), Brit. J. Exp. Path. 38, 168.

and "The Effect of Sodium Fluoride on the Output of Some Electrolytes from the Gastric Mucosa of Cats" by Audrey M. Bond and J.N. Hunt (1956), J. Physiol. 133, 317.

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ABSTRACT

Observations have been made on experimental animals subjected to short- and long-term ingestion of fluorides. These included renal function tests and the cytology and histochemistry of the kidney and gastric mucosa.

Rats given fluorides in the drinking-water showed albuminuria, glycosuria and a rise in urea output that increased steadily during the three weeks of the experiment. That fluorides interfere with tubular reabsorption was shown in an experiment in which rats on a sodium-free diet excreted sodium when given a fluoride (KF) solution to drink. Kidney clearance values were greatly reduced. Kidney lesions appeared, and activities of the enzymes succinic dehydrogenase and alkaline phosphatase were negligible. The gastric mucosa was similarly affected.

For long-term fluoride administration to rats NaF solution was mixed with the food. Within a few months kidney clearance values were reduced and the kidneys contained non-functioning renal parenchyma. The surviving renal epithelium showed normal succinic dehydrogenase activity. Casts noticed previously have been identified. The suggestion is made that it is the glomerulus which, primarily, is attacked by fluoride.

In chronic fluorosis the fundic mucosa of the rat stomach was found to be thin, due to the disappearance of many of the

parietal cells. Peptic cells were apparently unaffected and mucoid cells were stimulated. The histological changes confirm previous observations (Bowie, Darlow and Murray) that HCl secretion, but not pepsin secretion, is suppressed by fluoride.

Dogs, apart from reduced alkaline phosphatase activity in some kidneys, showed no kidney or stomach lesions after two years' fluoride feeding.

Rabbit kidneys and stomachs remained apparently normal after the animals had been drinking NaF solution for up to four years.

The results of some acute experiments included show that injection of NaF solution into rabbits raises the citric acid content of the kidney. In view of this, the possibility that it is due to the synthesis of a fluoro-fatty acid and provides a biochemical lesion (R.A. Peters) has been considered.

INTRODUCTION

THE OCCURRENCE OF FLUORINE COMPOUNDS.

Fluorine is present to the extent of 0·1% of the earth's crust. The characteristic ability of the naturally-occurring fluorine minerals to act as a flux gave rise to the name of the element. Fluorine, with an atomic weight of 19, occupies a position at the beginning of Group 7 of the Periodic Table and is the most electronegative element. Except under specially controlled conditions it cannot exist as fluorine, since its great reactivity causes instantaneous combination with other elements and compounds. With water fluorine forms hydrofluoric acid, and hence it can play no part in toxicology.

The fluorine-containing minerals include fluorite or fluorspar CaF_2, cryolite Na_3AlF_6, fluorapatite $3Ca_3(PO_4)_2.CaF_2$ and sedimentary phosphate rock, phosphorite. Much of the attention directed towards fluoride intoxication has come about because of the release of fluorine from these compounds during their purification or in the course of industrial processes in which they are used. Most waters contain some fluorine. Endemic fluorosis occurs through the use of waters with a high level of fluorine.

Fluorspar is used principally as a flux in metallurgical operations and is a raw material in the manufacture of hydrofluoric acid and aluminium fluoride.

Cryolite is important in the manufacture of aluminium by the Hall-Héroult process in which aluminium oxide (alumina) is dissolved in a bath of fused sodium fluoride-aluminium fluoride (or cryolite) and electrolysed between carbon electrodes. The alumina is dissociated into aluminium and oxygen, but part of the cryolite is also decomposed and fluorine is set free.

Apatites, which have the general formula $3Ca_3(PO_4)_2.CaX_2$ where X is a univalent anion, are common minerals. Hydroxy-apatite, $3Ca_3(PO_4)_2.Ca(OH)_2$, (the chief mineral constituent of bones and teeth) gives rise to fluor-apatite by exchange of $(OH)^-$ for F^-. Some fluor-apatite is always present in fresh bones and teeth, whilst in fossil material, constantly exposed to water containing soluble fluorides, far more is present. Carnot (1893) showed that fossil bones tend to acquire additional fluorine with age until a composition approaching that of apatite is reached. This fact, now that accurate methods for the quantitative estimation of fluorine are available, has recently provided archeologists with a reliable method for the "dating" of fossil material.

Phosphatic rocks always contain some proportion of fluorine, which is especially high in the secondary deposits. The secondary phosphate deposits are formed either from chlor- or hydroxy-apatite by partial conversion to fluor-apatite, or may be animal in origin, being composed of fossil shells, bones and teeth, or, as coprolite, of fossil excreta. The use of

phosphate rock in the preparation of superphosphate fertilisers, or fed to farm stock as a mineral supplement to the diet, is a common cause of animal fluorosis.

The presence of fluorine in certain coals is related to this property of phosphatic minerals to accumulate fluorine. Oily clays, such as the Jurassic Oxford clay valued for brick-making, have a high fluorine content due to their content of saurian and fish remains.

A correlation can be observed between the fluorine level of natural waters and the geological composition of the subsoils from which they arise. The presence of fluorine-bearing rock is not necessarily enough to cause the presence of a large amount of fluorine in the water. Calcium fluoride, for instance, is highly insoluble, and in Derbyshire, where fluorspar is particularly abundant, Sutton (1936) reported that only one out of forty-three waters examined had a fluorine content of more than one part per million (1 p.p.m.). Water from sources in phosphatic rocks or coals is often high in fluorine. This is due to the fact that phosphates, having acquired fluorine, yield it back to circulating water. Fluor-apatite is especially likely to release fluorine in areas where it is associated with sodium chloride (Bromehead, 1941) since then the very soluble salt, sodium fluoride, is produced by ion-exchange.

An additional cause of fluorine poisoning, which is of great historical interest, although important only occasionally

and in small areas, is volcanic eruption. The very toxic volatile compounds, hydrogen fluoride and silicon tetrafluoride, have been identified in volcanic emanations. Alkali and alkaline earth fluorides have been found also in the ash and lava from some volcanoes.

Fluorine has long been recognised as a poison, and this property has been made use of in the employment of fluorine compounds as preservatives (for food, wine, wood, etc.), disinfectants, rat and cockroach poisons and insecticides for the protection of animals and plants. As with all poisons, there are recorded instances of consumption of fluorine by man and other mammals and certain clearly-defined symptoms are noted in such cases.

Acute Fluorine Poisoning.

Acute fluorine poisoning has occurred when high concentrations of volatile fluorine compounds have been inhaled. In man, a more usual cause of acute fluorosis is the swallowing of soluble fluorine compounds in cases of attempted suicide or murder, or the frequently-encountered confusion of one household substance with another. Fluorine has been administered to laboratory animals in volatile form as well as by mouth and by injection.

In general, the symptoms of the intoxication may be classified into two groups. One shows that the effect of fluorine on the central nervous system and musculature is both

excitatory and paralytic. (In fatal cases there is marked cyanosis and death appears to be from respiratory failure.) Other symptoms reveal an acute local irritation of the gastro-intestinal tract, marked by vomiting, abdominal pain and diarrhoea. At post-mortem, acute inflammatory conditions with haemorrhage of the alimentary tract and hyperaemia of other abdominal organs are often seen. In addition, thirst, salivation, diuresis and sweating may be noted, and signs of a toxic nephritis have been observed. Inflammatory conditions of the lungs follow inhalation of the volatile fluorides, and where the poison has been taken in the form of HF or H_2SiF_6 there is corrosion of mucous membranes. Roholm's classical monograph (1937) contains a review of published work on acute fluorosis in man and animals.

In the case of laboratory animals death follows the administration of doses of the order of 0·5 g fluorine/Kg body weight by mouth, or 0·08 - 0·15 g fluorine/Kg body weight by injection. Man is more sensitive: less than 5g NaF have caused death in a few hours. The toxic and lethal doses of various fluorine compounds are to be related to their solubilities (and therefore to their rates of absorption) as well as to their fluorine content. The effective doses vary also with the species of animal to which they are administered, and the age, diet and general condition of the individual concerned.

Chronic Fluorine Poisoning.

While experiments on acute fluorine poisoning have been relatively few and were mostly carried out early in the history of the aetiology of fluorosis, a vast body of data pertaining to chronic fluorine poisoning has accumulated. Chronic fluorosis has been studied in man in areas where the supply of drinking water was high in fluorine, in farm stock fed on phosphatic rock or exposed to industrial hazard and in experimental animals.

Industrial Fluorosis. Dusts containing fluorides have been identified as the cause of many, though not all, of the various outbreaks of fluorosis on farms close to certain factories and industrial undertakings. Bartolucci (1912) published an account of a disease among cattle on a farm near an Italian superphosphate factory. Cattle that had spent 4-5 months on the farm became thin, and their coats were poor and dry. They moved slowly and apparently painfully, and had tender swellings at the joints and thickened bones of the ribs and head. Bartolucci stressed the similarity of this then-unknown disease with osteomalacia. The cattle were found to be consuming water contaminated with factory effluent, and when the water-supply was changed the disease was no longer apparent.

Cristiani (1925), in Switzerland, and Slagsvold (1934), in Norway, described a very similar condition among cattle near aluminium works. In Britain, Bosworth, Green and Murray (1941) and Blakemore (1942) investigated the aetiology of a severe

osteodystrophy in cattle grazing within a mile of a Bedfordshire brickworks which used a marine clay high in fluorine. This, in 1941, was the first recognised outbreak in Britain.

In 1943 there was a well-recognised incidence of fluorosis in sheep grazing in the environs of the aluminium works at Fort William, and as a result of this, in 1945, a Fluorosis Committee was appointed by the Medical Research Council to study the effects of exposure to fluorine compounds on people working in or living near the factory. Bone changes recognised as due to fluorosis were seen in some of the older work people, although none of the workers examined was found to show clinical disability (Medical Research Council Memorandum No. 22).

Murray and Wilson (1946) described a Lincolnshire farm which was affected by fluorine-containing fumes and dust from local ironstone-burning dumps. Grain yields were poor; horses, cows and poultry died; sheep were lame and the human inhabitants of the farm complained of limb pains, cough and gastric disorders The windows of the farm house were found to be etched by the fluorine fumes.

Endemic Fluorosis. The disease known since 1100 A.D. in Iceland as "gaddur", and recognised to be among the sequelae to to volcanic eruptions, has been identified (Roholm, 1937) with fluorosis. Written accounts are available describing fully the illness among farm animals which followed the eruption of Mount Hekla in 1845, and museum specimens of bones and jaws have

been preserved. Animals near the volcano at the time of the eruption died, showing the symptoms of acute fluorosis. In the following year the survivors were observed to be weak and emaciated, with a decreased milk yield, impaired limb movement, thickened joints and exostoses on the long bones and the jaws. The teeth of the young animals affected showed selective abrasion of molars, and there was pitting of incisors.

Not unlike gaddur is the disease "le darmous" endemic in Morocco and other parts of N. Africa where there are deposits of fluorine-bearing phosphatic rock. Le darmous is known primarily as a dental condition. Velu (1922, 1923) published the first description of this disorder which is seen in the permanent teeth of individuals living in the affected areas during the period of development of the teeth. Sheep are especially vulnerable. In mild cases the dental enamel is chalk-white and without translucence; if the degree of fluorosis is more severe, patches or bands of brown or black discolouration are seen. The surface of the enamel is rough and pitted. Such teeth show reduction in hardness and are brittle and friable, and so wear irregularly and unduly rapidly. Teeth worn into sharp points may enter the gingiva of the opposing jaw. The tooth affection, which is a developmental defect, is usually accompanied by a bone disorder, seen especially in the mandible and characterised by thickening due to a newly-formed and irregular layer of porous bone. The marrow becomes porous and deficient in cells. Spontaneous bone fractures are common.

The fluorine hazard to grazing animals, which consume earth and dust-covered vegetation, is naturally far greater than it is to man. Endemic fluorosis is seen, however, in the human population of regions where the fluorine content of the water is unusually high and is revealed by dental changes. Osteosclerosis may be apparent in elderly people, but the general symptoms of osteomalacia are not seen in man. Dental fluorosis preceded osteosclerosis in the aetiology of the disease in man, and it has been studied since 1916 when Black and McKay investigated a little-known disorder "mottled teeth" in Colorado. Black and McKay were unable to establish the cause of mottled teeth, but Churchill (1931, 1932), who analysed a number of water samples from various regions of the U.S., noted that where mottled teeth were endemic the water contained 2 p.p.m. or more of fluorine. Meanwhile, Smith, Lantz and Smith (1931) were reproducing mottled enamel in rats by giving them a ten-fold concentration of the drinking water from an affected area.

In the milder form, and in the early stages of the more severe form, the teeth are mottled with spots and patches of a dead-white opaque character. Later, pigmentation may occur and penetrate some distance into the enamel, which, in fluorosis, is more than normally permeable.

Black and McKay (1916) commented that caries was rarely seen in the mottled teeth in Colorado, and the work of many authors, including Ainsworth (1933), Armstrong and Brekhus (1938), Dean, Jay, Arnold, McClure and Elvove (1939 & 1941), and Weaver (1944), established that the presence of 0·5 - 1·0 p.p.m. F in the drinking water taken during the period of development of the teeth would afford protection against dental caries without causing hypoplasia of the enamel. Many surveys in the U.S.A. and England have been carried out and much attention directed of recent years towards the question of the beneficial or harmful effects of fluoride consumption.

Bone changes in man recognised as due to fluorosis are not so common as in animals. The occurrence of human fluorosis does not of necessity imply a high level of fluorine in the drinking water, although this is sometimes the case; in areas where the standard of nutrition is low, the toxic effect of a quite small intake of fluorine is exaggerated (Murray & Wilson, 1948).

Shortt, McRobert, Barnard and Nayar (1937) described chronic fluorosis among the inhabitants of a mottled-enamel area in India Their findings included calcification of ligaments, tendons and fasciae, and the presence of osteophytic outgrowths. There was synostosis of joints and especially of the vertebral column. Endemic fluorosis in South Africa was studied by Ockerse (1946). Districts with more than 3 p.p.m. F were common, and mottled

enamel was frequently encountered. On one farm in Pretoria Ockerse (1941) found native African workers to be using a bore-hole water containing nearly 12 p.p.m. F. Six men who had spent eighteen years at the farm were available for examination, and all showed a general clinical picture of stiffness of the spine and pain in the joints. The most advanced case had a curved "bamboo" spine, i.e. practically all the vertebrae were calcified and he was unable to stand erect. Linsman and McMurray (1943) recorded similar instances in a high-fluorine area in Texas, U.S.A., in a well-nourished population. Long-continued residence in a high-fluorine area is very likely to lead to some degree of osteosclerosis, and there can be no doubt that many cases of the disorder exist unrecognised. Osteo-sclerosis, unaccompanied by dental fluorosis, has been shown to occur among long-term workers in many industries which use fluorine compounds. Its occurrence among cryolite workers in Greenland and Denmark was investigated by Roholm (1937).

Freedom from the obvious symptoms of osteomalacia does not mean that man can endure chronic fluorosis without some damage to the soft tissues. The cryolite workers questioned by Roholm (1937) complained of dyspepsia (80%), shortness of breath (43%) and cough. Arthritic and rheumatic symptoms were mentioned and were reflected in doctors' reports. The gastro-intestinal symptoms, including lack of appetite, nausea, vomiting and constipation or diarrhoea, were seen to be definitely

associated with the work, for they were noticed on beginning work and returning to it after holidays.

The Fort William investigation (1949) revealed that although no worker complained of dyspnoea on exertion, and there was apparently no excess incidence of pain in the back in even the most exposed workers, some 15% of this group suffered from dyspepsia compared with 5% of the local population.

Experimental Fluorosis. Tooth changes and the osteomalacia-like condition have been many times reproduced experimentally. Rodents are popular experimental animals when it is desired to record tooth changes, for their incisor teeth grow constantly from a persistent pulp and changes due to fluorine ingestion may be seen at any stage in the life of the individual. In other animals fluorine only affects the structure of teeth when it is absorbed during their development.

The first description of dental fluorosis in rats was provided by McCollum, Simmonds, Becker and Bunting (1925); 0·0226% F as NaF was added to the diet. Changes noted subsequently in the teeth included enlarged and overgrown upper incisors, with the normal orange pigment absent and a dull, chalky appearance. The teeth were brittle and of poor quality. The tooth changes provide a ready means of assessment of fluorosis in the rat, and it has been claimed (Smith and Leverton, 1934) that the addition of 0·0014% fluorine to the diet produces demonstrable hypoplasia. When low-level fluoride feeding is

stopped, the newly-developed enamel is normal, so that by alternately feeding fluoride and discontinuing its use a banded effect may be achieved. The abnormality is seen histologically (Schour and Smith, 1934) to consist of defective formation and calcification of both the interprismatic substance and the prisms of the enamel.

In general, animals having a suitable intake of fluorine develop the osteomalacia-like condition. Clinically, the disease is characterised by a tendency to form exostoses, either as isolated outgrowths or as a complete superficial covering of porous white bone of high F content and deficient calcification. Dental changes are apparent in rodents and in young mammals of all species. A general condition of unthriftiness, with anorexia and diminished growth in young animals, is seen. There are signs of poor food utilisation and a consequent loss of weight. The coat becomes coarse and the skin scaly. In severe cases photophobia and a discharge from the conjunctivae are observed. Thirst, polyuria and diarrhoea are commonly reported. Hypochromic anaemia may be present. Work done on chronic fluorosis in animals prior to 1937 was collated and reviewed by Roholm (1937), who included in his monograph studies made by himself on rats, dogs, calves and pigs. Both Roholm and the American group of workers, Kick, Bethke, Edgington, Wilder, Record, Wilder, Hill and Chase (1935), noted degenerative changes in the kidneys of pigs fed 15 mg fluorine/Kg body weight per day as NaF, Na_2SiF_6 or Na_3AlF_6 (Roholm) or 1% or more

phosphatic rock in the diet (Kick et al). A comprehensive review of the literature on fluorine poisoning was published by Greenwood (1940).

Excretion of Fluorine. It is significant that the modifications in bone structure in response to chronic fluoride ingestion are slow to develop. The results of many experiments show that the mammal has a tremendous capacity for eliminating fluorine via the kidneys, and that in spite of the affinity of the bone mineral (hydroxyapatite) for fluoride, the halide is only retained in dangerous amounts when sufficient is absorbed to exceed the renal capacity to excrete it. Rabbits' kidneys are particularly efficient in this respect. In this laboratory rabbits have been given a solution of 0·05% NaF in the place of drinking water, and when killed after two to four years they displayed little or no evidence of fluorosis.

McClure and co-workers, whilst engaged in studies designed to test the desirability or otherwise of fluoridating public water supplies, have made apparent the efficiency of the human renal system in eliminating fluorine. McClure and Kinser (1944), in a non-quantitative survey, saw a general correlation of urinary fluoride with domestic water fluoride through the range 0·5-4·5 p.p.m. F. Zipkin, Likins, McClure and Steere (1956) found that within a week of adding 1 p.p.m. F to a water-supply previously lacking fluorine, the adult section of the population concerned was excreting urine containing 1 p.p.m. F. Adaptation

required a longer time in children, but was as complete.

Fluorine is excreted also in the faeces, especially where it has been consumed in a form not readily absorbed, and it has been shown that it may be present in sweat and perspiration (McClure, Mitchell, Hamilton and Kinser, 1945).

That fluorine in excess of the excretory capacity is deposited in bones and, to a lesser extent, in teeth was confirmed by, among others, Sonntag (1917) and Bethke, Kick, Edgington and Wilder (1930). Large amounts of fluorine may be retained in the bones. Blakemore, Bosworth and Green (1948) found that the ashed bones of cattle grazed near certain brick-kilns in Bedfordshire contained 1·0-2·7% F, as compared with 0·05-0·08% F for animals on uncontaminated pastures. These workers removed a cow from the fluorosis area to Cambridge, and observed that over a period of eight months its urinary fluorine content fell from 28 p.p.m. to 13 p.p.m., and the fluorine content of ashed rib-bones fell from 1·30% to 0·68%. The animal quickly regained condition and showed no clinical signs of fluorosis after a few weeks. Blakemore et al. noted that the rate of excretion of fluoride was most rapid in the first month, declined during the second month and remained nearly constant during the remaining six months of the experiment. They suggested that fluorine is not easily given up by the skeleton, and that, under conditions of "defluoridation", fluorosed bone is replaced most readily where it has been most recently laid

down, or, alternatively, that those parts of the skeleton most necessary to bear stresses and strains are re-habilitated first and most quickly.

Several authors, including Largent and Heyroth (1949), Savchuck and Armstrong (1951), have confirmed the withdrawal of fluoride from the bones. Glock, Lowater and Murray (1941) fed young rats on a diet containing 4·7 p.p.m. F for up to 46 weeks and noticed an accumulation, rapid at first and then more gradual, in the bones. After 46 weeks NaF was withdrawn from the diet and elimination of fluorine was seen following a course similar to the uptake curve. In the same paper, the authors commented on a phenomenon which emphasizes the affinity of bone salts for fluorine. A spectrographic examination of normal human rib-bone, taken post mortem from residents of London (where the water-supply contains less than 0·5 p.p.m. F) showed a general increase of fluoride content with increasing age. The values found were from 0·02% F to 0·3% F in fat-free bone.

THE EFFECT OF FLUORINE ON KIDNEY STRUCTURE AND FUNCTION.

It seems not illogical to expect the kidney tissues to show signs of damage in instances where they are exposed continually to the highly toxic fluoride ion. The luminal aspect of the tubule cells would appear to be the most vulnerable part of the kidney because of direct communication with the fluoride solution. When fluoride is being absorbed, the alimentary tract and liver are in contact with the poison, but the other soft

tissues are protected at the expense of the kidney. Only if dangerously high levels of fluoride are being taken into the body in the drinking water, so that the fluoride content of the blood becomes and remains high, is there appreciable risk of injury to the other soft tissues. Whether or not the thyroid (which has a very large blood flow) is affected by fluoride is disputed, and the adrenal cortex has been mentioned in connection with a similarity seen between fluorosis and the scorbutic syndrome (Phillips, Stare and Elvehjhem, 1934). In cases where a high rate of fluorine ingestion is suddenly discontinued the danger to the digestive organs ceases; mobilisation of fluoride from the skeleton, however, maintains for a time the situation vis-a-vis the kidney.

That the kidney does, in effect, respond to fluorine ingestion by changes in the gross or minute structure or by functional disturbances is made clear from many observations. Gottlieb and Grant (1932) found that injection of 1% NaF into dogs was followed by diuresis, with increased chloride and nitrogen excretion and a rise in the pH of the urine from acid to alkaline. Mild hyperaemia of the kidneys was seen in a dog subjected to such daily injections for six weeks. Chronic fluorosis in rabbits (Muehlberger, 1930) was revealed as an intense congestion of the glomeruli.

Kick, Bethke and Edgington (1933), in the course of a comprehensive study on the role of fluorine in animal nutrition,

observed diuresis and thirst in pigs. These animals were given phosphatic rock at the rate of 908 g/100 lb. diet for 140 days. Other pigs, having half this amount of phosphate rock for two years, were found at autopsy to have kidneys that were "firm, pale in colour and presented a 'hob-nailed' appearance". Destruction of the epithelium of the convoluted tubules was noted and a marked infiltration of fibrous tissue. In two animals the degeneration had progressed so far as to be the apparent cause of death. These authors were disinclined to attribute the kidney changes to fluorine, since feeding up to 100 g NaF/100 lb. diet for 140 days, or up to 60 g NaF/100 lb. diet for two years, produced no alterations in function or appearance of the kidneys of their pigs. The kidneys of rats appeared normal when the animals were maintained on a diet including 2% rock phosphate, 0·05% NaF or 0·16% CaF_2.

Kidney changes were also recorded four years later by Roholm (1937). Animals of four species were given cryolite or sodium fluoride in various concentrations for long periods, and detailed post-mortem examinations were carried out. The kidneys of rats which received up to 0·15% cryolite, or 0·18% NaF, were affected in nearly every case. Signs of a chronic, mostly interstitial, nephritis of uniform character were seen. The surface of the kidneys was contracted and uneven. Histological examination showed a hyaline degeneration of some glomeruli, and an irregular dilatation of the lumina of the tubules. Cystic

areas and some serous content were seen in the latter. The epithelium of the tubules was low but in a good state of preservation. Connective tissue was abundant. The renal tissue in general showed hyperaemia and scattered round-cell infiltration, but there was no evidence that any other tissue was affected.

Pigs were made to consume 15 mg. F/Kg. body weight/day as NaF, Na_2SiF_6 or cryolite for 171 days. After only four weeks, thirst and a profuse diuresis was observed. As with the rat, no abnormality was seen at post-mortem examination in any organ but the kidneys. These appeared to be smaller and more dense than those of the control pigs, pale and with an uneven surface. Radial section showed the cortex to be narrow. There were signs of chronic nephritis: replacement of renal tissue by connective tissue was frequent, giving an appearance of radial striping, and many tubules had irregular or dilated lumina. The tubule-cells were low but well-preserved.

A somewhat different picture was presented by two calves, to which either 20·4 mg. F as NaF/Kg. body weight/day, or 60 mg. F as cryolite/Kg. body weight/day was administered, on average, for 195 days. The kidneys of these animals were not remarkable. No macroscopic changes were noted in any organ, but most organs showed certain general parenchymatous degenerative changes, and these were most marked in the kidney, liver, heart muscle and central nervous system. Nuclei in the affected tissue were

apparently normal, but the cytoplasm was swollen and poorly-staining, and either granular or vesicular.

Dogs reacted to fluorine feeding (78 mg. F as cryolite/Kg. body weight/day or 13·8 mg. F as NaF/Kg. body weight/day for about 600 days) by developing both types of abnormality mentioned above.

In addition to these experiments, Roholm was able to carry out autopsies on two cryolite workers who had died from pneumonia following radical herniotomy in one case and syphilitic heart disease in the other. No functional renal abnormalities had been detected in either case. The kidneys of one,who had been employed in the factory under moderately dusty conditions for eight years,was normal, but evidence of some slight renal nephritis was seen in the other man who had worked with cryolite for twenty-four years.

On the other hand, clinical, radiological and biochemical investigations carried out by Shortt et al.(1937) on the human population of an Indian mottled-enamel area disclosed not only severe osteosclerosis but, in most cases, impaired kidney function. Siddiqui (1955) applied kidney clearance tests to fourteen subjects with endemic fluorosis in the Hyderabad-Deccan, India, and found reduced efficiency in each case.

Several years before Siddiqui's publication, Bond and Murray (1952), impressed by the immediate polyuria and polydipsia

caused by fluoride administration to rats and desirous of finding some means of detection of the early stages of fluorosis in man and animals, carried out an examination of the structure and function of the kidneys of rats on a fluorine-containing diet. The rats were given 0·05 - 0·1% NaF in the diet (amounting in the case of an adult rat to 15 mg. NaF/day) for at least ten months. A strikingly high nitrogen excretion was found, and it was suggested that this was linked with the emaciation typical of animals with chronic fluorosis. In particular, the urea-nitrogen was raised to two or three times the control level. The rats showed a mild glycosuria which became appreciable when their blood sugar was raised to 125 mg/100 ml.

Autopsy findings suggested a vascular, glomerular and tubular degeneration, leading to an interstitial fibrosis of the kidney. The kidneys were dark, shrunken and nodulated. The "radial striping" of the cortex, described by Roholm (1937) in pigs, was exemplified here in histological material which had been treated to demonstrate activity of the enzyme alkaline phosphatase. Elongated fibrotic strands of tissue, roughly cone-like in shape and corresponding in position to the medullary rays of the normal kidney, radiated outwards from the medulla. These areas were devoid of phosphatase activity and consisted of degenerating glomeruli and tubules with connective tissue replacing the normal renal parenchyma. Radial sections showed that the fibrotic tissue was responsible for the "hob-

nailed" appearance of the kidney, for the course of the straight connective tissue fibres from medulla and capsule was less than the normal width of the cortex. This caused wedges of unaffected tissue surrounded by connective tissue strands to bulge, to all appearances, into the capsule surrounding the kidney. The normal tissue showed an alkaline phosphatase activity more intense than in the control tissue, and presumably this accounted for the slight degree of glycosuria observed with resting blood-sugar levels.

Detailed examination of sections of the kidneys showed that glomeruli, tubules and blood vessels were affected. The Malpighian corpuscles exhibited dilatation of the sub-capsular space and thickened, ill-defined capillaries, but there was no haemorrhage. The cytoplasm of the cells lining the tubules was granular or vacuolated; the free borders were ragged and the brush borders obliterated. Nuclei were absent in places. The lumina of some tubules were dilated and contained cellular debris. Arterioles near the glomeruli or collecting tubules showed thickened walls. Groups of darkly-staining, rounded nuclei with no evidence of surrounding cytoplasm were seen and were equated with nuclei displaced from the epithelial lining of the tubules rather than with infiltrating cells. The reticular "framework" of argyrophil fibres round the renal cells was coarser and more abundant than in the control kidneys.

It was upon this work that the experiments to be presented in this thesis were based. In the current experiments emphasis has been laid on kidney clearance tests and possible enzyme inhibition.

Pindborg (1957) carried out an experiment designed to demonstrate the initial stages of the kidney lesion in chronic fluorosis. Rats were maintained on a diet including 0·05% NaF for up to 35 days. No kidney changes were noted in rats killed before 21-28 days of the diet. The pathological processes seen at 21-28 days were principally in the medulla, and were manifested as a pronounced dilatation of the loop of Henle in the juxtacortical region. Very soon afterwards they were followed by dilatation of the convoluted tubules of the cortex. Pindborg suggested that the essential pathological mechanism in fluorine poisoning of the kidney is some kind of "stop" in the loops of Henle.

THE EFFECT OF FLUORIDE ON THE GASTRO-INTESTINAL TRACT.

The condition of the gastro-intestinal tract in fluorosis has received less attention than has that of the kidney, as judged by the relative amounts of work published on the two organs. Damage to the stomach and intestine has been reported in cases of accidental poisoning by soluble fluorides, and in acute experiments. Dalla Volta (1924) described a microscopic examination of haemorrhagic gastro-enteritis in cats and rabbits after ingestion of 2% NaF by stomach tube.

Marconi (1930) described hyperaemia of the gastric and intestinal membranes of guinea pigs subjected to fluoride ingestion for the period of one month, and Hauck, Steenbock and Parsons (1933) reported minor haemorrhages in the pylorus of rats and duodenum of chickens following consumption of a diet including sodium fluoride.

Gastro-intestinal dysfunction is implied in reports on the state of health of cryolite workers (Roholm, 1937), among whom depression of appetite, nausea and diarrhoea or constipation were common complaints. Depression of appetite and loss of weight are almost invariably mentioned in connection with chronic fluorosis. Lantz and Smith (1934) commented that when rats were put on to a diet containing 0·1% NaF, they scattered their food and lost weight rapidly. Roholm (1937) wrote of difficulty in inducing the two calves, which he used in his experiments on chronic fluorosis, to take the full quantity of diet allowed, and at one stage the ingestion of fluoride had to be suspended for three days in order not to lose the animals.

It is noteworthy that reluctance to eat food containing fluoride is apparent in experimental animals long before the teeth become brittle or abraded and eating may be assumed to be difficult or painful; nor is it necessarily due to the animals being able to detect fluorine compounds and finding them unpalatable. Lawrenz, Mitchell and Ruth (1939) found that administration of cryolite at the rate of 10 p.p.m. F in the drinking water depressed the appetite of rats. These workers

noticed that rats would retain a higher proportion of a dose-level of fluoride if the halide was given in water than if it was included in the food supplied, and they explained this on the basis of impairment of absorption of food. The presence of calcium in the alimentary tract is of significance in the absorption of fluoride, since the nearly-insoluble CaF_2 is readily formed from ions in solution. Dermal changes and alopecia observed by Spira (1949) to develop in rats in chronic fluorine poisoning could be reversed at will by substituting a diet lacking fluoride and rich in calcium carbonate.

It is probable that reduced ability to digest and absorb food would, under some conditions, lead to the development of deficiency disease (e.g. avitaminoses) which could exacerbate the symptoms of fluorosis or be themselves mistaken for aspects of fluorosis. Murray and Wilson (1948) studied dental fluorosis in a phosphate-rock mining district of Morocco where the domestic water-supplies contained about 1 p.p.m. F. They observed that whilst the teeth of European children born and reared in the district on a dietary satisfactory in amount and quality were of excellent structure, the poorly-nourished children of the indigenous population had severe dental fluorosis with pitting, erosion and marked staining of the enamel, together with certain skin alterations indicative of deficiency of the vitamin B_2 group.

Acting on a suggestion of Murray's, Spira (1950) investigated the effects of supplementing with B vitamins the diet of rats

given increasing quantities of NaF. Grave skin lesions and loss of hair, which he had found previously to be characteristic of rats ingesting NaF for long periods under his experimental conditions, failed to appear in the presence of vitamin B complex and the animals were enabled to withstand larger amounts of fluoride. Other workers have noticed an analogy between fluorosis and the scorbutic syndrome (Phillips, Stare and Elvehjhem, 1934). Raghavachari and Venkataramanan (1940) saw in a survey of the population in part of S. India that up to 75% of the people consuming water which contained the small amount of 1 - 3 p.p.m. F suffered from some degree of osteosclerosis. They believed nutritional factors to be involved, principally vitamin C.

Gastric Secretion. A direct action of fluoride on the isolated gastric mucosa of the frog was shown by Crane, Davies and Longmuir (1946). A concentration of 0.006M NaF decreased the normal activity (including potential difference, resting acid secretion, acid secretion in response to histamine and rate of aerobic glycolysis). Patterson and Stetten (1949) established that fluoride also inhibited mammalian gastric mucosa: the resting acid secretion of excised rat stomach was inhibited by 0.01M NaF.

A direct investigation into possible inhibition of acid secretion in the cat's stomach in vivo when exposed to fluoride

was conducted by Bowie, Darlow and Murray (1953). Dilute HCl or NaF solutions were placed in the stomachs of the anaesthetised cats, with the pyloric sphincters tied, and the gastric contents withdrawn at suitable intervals by means of a stomach tube. It was found that the normal response of the gastric mucosa to intravenous infusions of histamine or gastrin (i.e. the profuse secretion of HCl) was depressed or abolished by NaF at a minimal concentration of 0·005M. In some instances, even the slight resting acid secretion appeared to be absent if the mucosa was bathed with the fluoride solution. The effect was readily reversible on removing the fluoride, and the tissues exhibited no macroscopic evidence of damage at the end of the experiment. The secretion of pepsin was seen to be uninhibited by NaF.

Some evidence of the mechanism by which the HCl content of the stomach is lessened in the presence of fluorine was provided by Bond and Hunt (1956). They showed that an instillation of 0·005M NaF into the cat's stomach (secreting in response to histamine) produced not only a reduction in the concentration of H^+ in the stomach, but also in the concentrations of Cl^- and K^+. The reduction in the amount of Cl^- was markedly less than that of H^+; electrical neutrality was achieved by an equivalent rise in the amount of Na^+ present. In addition, H^+ was lost from HCl instilled into the unstimulated stomach if the mucosa had been treated with fluoride solution immediately beforehand. In

this experiment the Cl^- concentration remained constant and there was a rise in the concentration of Na^+ parallel with the loss of H^+. These findings, and a further observation that the rate of efflux into the blood of ^{24}Na instilled into the gastric cavity was increased forty-fold when the mucosa was exposed to fluoride, led the authors to suggest that the action of fluoride on the gastric mucosa is not only to depress HCl production. Fluoride may make the mucosal cells abnormally permeable to positive ions by affecting some enzymatic reaction, and hence reduce the acidity of the stomach contents still further by permitting H^+ to "leak" into the blood in exchange for Na^+.

MODE OF ACTION OF FLUORINE.

Fluorine has a general or specialised effect on certain enzyme systems responsible for processes such as fermentation, glycolysis, gastric secretion and the ionic balance of red blood corpuscles.

It was known in the nineteenth century that fluoride in dilute solution would kill bacteria and prevent cell-division in yeasts. Many investigations on different organisms have made it clear that glycolysis is more fluoride-sensitive than respiration, and anaerobic glycolysis more sensitive than aerobic glycolysis. Lipmann (1929) has shown that the greater fluoride-sensitivity of glycolysis is not due either to injury of the tissue consequent on anaerobic conditions or to protection by oxygen.

Fluorine has been used of recent years as an "antimetabolite", i.e. as a tool to block the cycle of enzyme reactions in an isolated tissue so that a part only of the system may be studied. Among the first to use NaF to produce biochemical lesions were Peters, Rydin and Thompson (1935), who found that it reduced the oxygen-uptake of pigeons' brain tissue.

Fluoride and the Enzymes of Calcification. Developing bone is a tissue that is readily viable when removed from the body, and the ossification of hypertrophic cartilage takes place, under suitable conditions, in vitro.

A state of "supersaturation" with Ca^{++} and $PO_4^{\equiv}$, i.e. a concentration of those ions greatly in excess of their occurrence in plasma, is necessary to bring about calcification in vitro unless a phosphoric ester, such as calcium hexose monophosphate, is present (Robison, 1923; Robison and Soames, 1924). This indicates that a phosphatase is present in the tissue, which, by splitting the ester, brings about a local condition of "supersaturation" (Robison and Soames, 1924).

The application of selective inhibitors led Robison, Macleod and Rosenheim (1930) to postulate that, in vivo, calcification is dependent on two mechanisms. The first of these is a phosphatase-phosphoric ester system which supplies Ca^{++} and $PO_4^{\equiv}$, and the second is a system utilising the ions for the orderly deposition of bone salt. Robison et al.(1930) found that if the tissue were desiccated or treated with organic solvents,

calcification did not take place although the first mechanism was unaffected. Similarly, the presence of 0·00001M NaF or 0·0001M sodium iodoacetate inhibited calcification without inhibiting the phosphatase (Robison and Rosenheim, 1934). A "second mechanism", depending upon an enzyme system inhibitable by fluoride, was postulated to explain these findings.

Glock (1940) observed that calcification of the bones of young rats subjected both pre- and post-natally to the influence of fluoride was retarded, and that the amount of glycogen present in the ossifying cartilage cells in these rats was depressed considerably below the high level normally found. She suggested that glycogen is the primary source of the phosphoric esters utilised in the process of calcification and that, therefore, the inhibitory effect of fluoride on bone calcification might be attributed to the diminished amount of glycogen.

Fluoride and Phosphatases. Certain other phosphatases, in addition to the alkaline bone phosphatase studied by Robison et al., resist inhibition by small amounts of fluorine. The literature suggests (Massart and Dufait, 1939 & 1942; Cloetens, 1940) that neutral phosphatases tend to be more readily inhibited than acid or alkaline ones, and show a striking analogy with enolase in their unvarying inhibition by fluoride and activation by Mg^{++}.

Cloetens (1940) claimed that the alkaline phosphatase of kidney is not inhibitable by NaF, in contradistinction to the

highly sensitive liver alkaline phosphatase. Bond and Murray (1952) showed that, in chronic fluorosis, alkaline phosphatase is absent from the rat kidney only when fibrotic tissue has replaced the renal parenchyma, and that in the convoluted tubules remaining the activity of the phosphatase is unusually high.

Fluoride and Glycolysis. The comprehensive monograph of Borei (1945) lists several different kinds of fluoride action, theoretically possible, which might account for the physiological effects of the halide. These include a calcium-precipitating effect, resulting in the formation of CaF_2. A relatively higher concentration of fluoride is required to bring about the precipitation of the corresponding salt with magnesium, since MgF_2 is more soluble than CaF_2.

The ability of fluoride to react with calcium and magnesium to form salts is related to its ability to "block" the reactions of these metals when they lie on the surface of the enzyme and perform a function in the prosthetic group. A complex is found which leads to inactivation of the enzyme. The enzyme enolase (which acts in fermentation and glycolysis) is inhibited by fluoride in this way, although it was many years before this was proved to be the case.

Lipmann (1928) observed that when glycolysis in minced frog muscle was inhibited by NaF, the amount of inorganic phosphate split off from phosphoglyceric acid was diminished, and he interpreted this to mean that NaF prevented dephosphorylation.

Davenport and Cotonio (1927) found that fluoride caused a decrease not only in free phosphate but also in total carbohydrate content. The latter effect was shown by Meyerhof and Lohmann (1927) and Lipmann (1930) to be due to inhibition of lactic acid formation. Lohmann (1930) and Nilsson (1930) both reported that the end-point of the glycolytic system poisoned by fluoride is a difficultly-hydrolysable phosphate ester. This substance was isolated by Nilsson (1930) and characterised as phosphoglyceric acid ester.

A series of complementary investigations by Embden, Deuticke and Kraft (1933) showed that fluoride-poisoned frog muscle would form lactic acid from pyruvic acid. Lohmann and Meyerhof (1933) extended this work and isolated the fluoride-sensitive reaction as being between phosphoglyceric acid and phospho-enol-pyruvic acid (that is, not a dephosphorylation mechanism as had been believed) and dependent on the enzyme enolase. Warburg and Christian (1942) succeeded in crystallising enolase, and it was then made clear that the inhibition is due to replacement of Mg^{++} on the enzyme surface by magnesium fluorophosphate.

The experiments showed that the reaction is as follows:

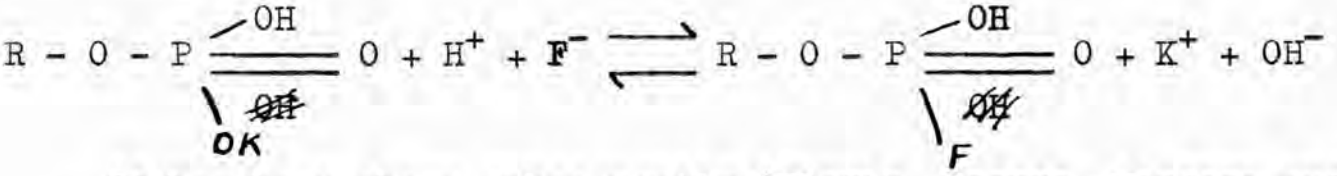
Mg fluorophosphate + Mg enolase $\rightleftharpoons$ Mg fluorophosphenolase + Mg salt

and that, where c represents concentration of ions,

$$c\ Mg^{++} \times c\ PO_4^{\equiv} \times c^2F^- \times \frac{\text{residual activity}}{\text{inhibition}} = \text{constant.}$$

Fluoride inhibition, therefore, increases not only with increasing fluoride concentration but with increasing amounts of magnesium and phosphate. A relationship between phosphate concentration and inhibition by fluoride has been noticed in later work involving other enzyme systems, and several suggestions have been put forward in explanation.

Malm (1947) postulated the inhibition of the enzyme system of the respiratory chain by the formation of a magnesium-fluoride phosphate-enzyme complex analogous with that formed by enolase. Malm further suggested that an early stage in the inhibition was the production of a monofluorophosphate in the cell from free phosphate and HF (in which form fluoride has been shown to enter the cell; Malm, 1940)



It has been shown subsequently (Flavin, Castro-Mendoza and Ochoa, 1956) that monofluorophosphate can be formed under physiological conditions. In the course of studies on the mechanism of carboxylation in propionate metabolism, a bicarbonate and fluoride-requiring enzymic cleavage of ATP was discovered which yielded ADP and monofluorophosphate ($PO_3F^{\equiv}$) as products.

Inhibition of glycolysis by interference with enolase leads, necessarily, to a diminished production of ATP and a failure in

the energy supply to the system. Of recent years, ATP has been regarded as the energy transmitter between glycolysis and the active transport of cations. Straub (1953) stated that human erythrocytes accumulate K^+ by using the energy of ATP. It has been known since 1951 that human erythrocytes (and those of certain other mammalian species which show a high concentration of K^+ within the erythrocyte) depend upon glycolysis for the maintenance of concentration gradients across the cell membranes (Harris, 1941; Danowski, 1941).

The concentration gradient is not maintained in erythrocytes that are exposed to fluoride: K^+ is then lost and Na^+ diffuses into the erythrocyte. In an experiment conducted by Davson (1941) rabbit erythrocytes lost 90% of their K^+ in three hours in the presence of 0·04M F at 38·5°C. Such a high rate of extrusion of K^+ implies a permeability constant of the erythrocyte membrane greater than could exist under normal conditions, for it exceeds that for which the erythrocytes' capacity to "pump" Na^+ could compensate. Indeed, Eckel (1953) postulated that entirely different mechanisms are responsible for the accumulation of K^+ against a concentration gradient and the rapid efflux of K^+ from the erythrocyte in which glycolysis is inhibited.

Whittam (1958) showed that the rates of fall in K^+ influx and ATP concentration are parallel in glucose-starved human erythrocytes and that both states are reversed on incubation

with glucose. He concluded from his experiments that ATP is at least one of the links in the coupling between glycolysis and active transport of K^+ in human erythrocytes, and that a membrane carrier utilises ATP in the transport of K^+ across the membrane. It is conceivable that the influx of Na^+ into the lumen of the cat stomach previously filled with a fluoride solution, as reported by Bond and Hunt (1956), can be explained by a similar hypothesis.

Fluoride and Tissue Respiration. Borei's monograph (1945) includes not only a detailed survey of work done on enzyme systems poisoned by fluoride, but also gives an account of experiments of his own, designed to localise the fluoride-sensitive link in the respiratory chain. Borei recognised succinic dehydrogenase to be inhibited by fluoride, but he found the enzyme alone to be less fluorine-sensitive than the whole succinic oxidase system including the cytochromes. From experiments involving the yeast system, he concluded that the effective fluorine-sensitive factor was in the cytochrome system and that it was, in fact, cytochrome C.

Evidence that cytochrome C is not implicated in the inhibition of tissue respiration by fluoride was furnished by Slater (1949, 1950). Slater and Bonner (1952) explained the effect on the basis of the inhibition of succinic dehydrogenase by fluoride in the presence of phosphate. The evidence of

their experiments, in which heart-muscle preparation was used as the enzyme system, indicates that the reaction is between equal numbers of molecules of fluoride, phosphate and enzyme.

Fluoroacetate and the Synthesis of Fluorocitric Acid.

Considering the parallelism between the metabolic pathways in yeast, muscle and developing bone, and in view of the fact that the fluoride ion in low concentration has a profound effect on all of these, the new field of investigation into the natural occurrence of and toxicity to animals of fluoroacetic acid opened up by R.A. Peters is of significance.

Peters inaugurated studies on the phenomenon of poisoning by fluoroacetate, the toxic principle of the S. African plant Dichapetalum cymosum, which possesses the property of being highly poisonous to the living animal and yet has no action on isolated enzyme systems. The effect cannot be due to the liberation of F^-, since the bond CF is sufficiently stable to resist the action of hot concentrated sulphuric aicd (Saunders and Stacey, 1948) and no enzyme has been found which can break it.

Liébecq and Peters (1948) showed that the toxicity is due to a lethal synthesis. Like acetate, fluoroacetate is converted to an "active C_2 compound" and then combines with oxaloacetate to form a tricarboxylic acid. Under normal conditions citric acid is formed, which, by the agency of the enzyme aconitase, undergoes rearrangement in preparation for its subsequent

decarboxylation. In the presence of the poison, however, fluorocitrate is produced and acts as a competitive inhibitor of aconitase (Lotspeich, Peters and Wilson, 1952). The tricarboxylic cycle is blocked, the animal's energy supply fails and citric acid accumulates in the poisoned tissue. Not every tissue carries out the synthesis: an intracranial injection of fluoroacetate is without effect on the pigeon, whereas an intraperitoneal injection is followed by death in convulsions induced by interference with brain metabolism. The accumulation of citric acid is considerable in heart muscle, and particularly so in the kidney (Buffa and Peters, 1949). The amount of citric acid in the soft tissues (excluding body fluids) of normal animals is, as a rule, low. Dickens (1941) has shown that up to 70% of the total citric acid content of the body is in the bones. In tumour tissue the citric acid content is frequently, though not invariably, high (Dickens, 1941).

There is no evidence as yet that animals synthesise fluorofatty acids as does Dichapetalum cymosum and certain other plants, but this possibility has been considered in the work to be reported.

EXPERIMENTAL SECTION

PLAN OF EXPERIMENTS

Kidney Function. The experiments to be presented include an extension of the preliminary work published (Bond and Murray, 1952) on alterations in the structure and function of the rat kidney in response to fluorine ingestion. Kidney clearance tests were carried out on control rats, rats which had been consuming small amounts of fluoride for long periods and rats to which larger amounts of fluoride had been administered for short periods only. The distribution of the enzymes succinic dehydrogenase and alkaline phosphatase were studied in kidney slices taken from rats of the three groups.

It has been shown (Bond and Hunt, 1956) that one effect of fluoride on the stomach of the cat is an increased permeability with respect to sodium ions. In order to test the possibility that the renal epithelium of the rat shared this particular property, a short-term experiment was devised which required the administration of fluoride combined with some substance other than sodium. KF was chosen as a suitable fluoride compound. Adult rats were placed on a diet free, as far as possible, from sodium, to make full use of the capacity of the tubular epithelium for re-absorbing Na^+. When the level of excreted Na^+ had fallen to a low, constant value KF was given either by injection or by its inclusion in the drinking-water, and the urine subsequently voided was analysed for sodium.

As well as sodium, urea was estimated in the urine of the rats so treated. A raised level of urea-nitrogen in the urine of rats subjected to fluoride-feeding for long periods has already been noted (Bond and Murray, 1952), but the earlier experiments gave no indication as to whether the effect appeared with the introduction of fluoride into the diet or only after prolonged exposure to the poison. Observations on thirst and appetite were made, and tests for glycosuria and albuminuria were carried out. Toxic properties are associated with the potassium ion, and so, to avoid attributing to fluoride any effects induced by potassium, the experiment was repeated using other rats to which either KCl, providing a potassium level equivalent to that of the KF feeding, or NaF, providing a fluoride level equivalent to that of the KF feeding, was given.

On the histological side, staining techniques additional to those used in the earlier experiments were employed to identify tissue constituents.

Gastric Secretion. With regard to the stomach, the effect of fluoride in inhibiting hydrochloric acid secretion in acute experiments is well known. Bowie, Darlow and Murray (1953) showed this effect to be reversible if dilute fluoride solutions are used, so that it does not depend upon destruction of the parietal cells and, moreover, that pepsin secretion (in the cat) appears to be uninhibited in the presence of fluoride.

A histological survey was carried out to establish whether the prolonged administration of fluoride would affect the condition or the numbers of parietal and peptic cells. Various stains, specific for different types of cell, were used, and the distribution of the enzyme succinic dehydrogenase was followed. Succinic dehydrogenase activity is associated with mitochondria with which the granules of the parietal cells have been identified.

The "redox theory" of Conway and Brady (1948) postulated that the H ions of gastric juice are derived from metabolic H atoms by the agency of a catalyst operating across the parietal cell membrane in a redox cycle. Inhibition of an enzyme of the metabolic cycle, e.g. succinic dehydrogenase, would necessarily lower the amount of HCl that could be produced.

Histochemical and cytological observations were made on the stomachs and kidneys of dogs and rabbits which had been receiving fluoride for periods ranging from three months to four years. In addition, the details are included of an experiment designed to test the hypothesis that fluoride injected into an animal may interfere with enzymes of the citric acid cycle, as does fluoroacetate, and cause an accumulation of citric acid.

METHODS

Animal Procedure

Diet. The rats used were of an inbred hooded strain and were fed on the stock diet (Coward, 1938) described previously (Bond and Murray, 1952). The diet was mixed with an equal weight of tap water for the control rats or 0·1% NaF in long term fluoride feeding. Tap water was supplied to drink. When a sodium-free regimen was required, salt-free preparations of casein and yeast were used in the diet, and sodium chloride, milk and green vegetables were excluded from it. Rats received the sodium-free diet (with which only distilled water was supplied) for at least two months before being given KF. Early attempts to administer KF in the food under observed conditions failed because, after the first meal, the rats refused to eat. In all but one of the experiments recorded the substance administered was given in the drinking water for 2-3 weeks. KF was given as 0·15% solution (equivalent to 1·0 mg. K^+/ml. and 0·5 mg. F^-/ml.), NaF was given as 0·10% solution (equivalent to 0·5 mg. F^-/ml.) and KCl as 0·2% solution (equivalent to 1·0 mg. K^+/ml.). In one experiment KF was given by means of a single intraperitoneal injection of 4 ml. 0·31% solution.

The dogs, the stomachs and kidneys of which were examined histologically, were maintained on a good mixed diet. The test animals received 20 mg. F as NaF/day, and were killed after

three months or after 22 months.

Rabbits, in one experiment, were given 6 mg. NaF/100 g. body weight/day by mouth for three months. In another experiment a group of three rabbits was given a solution of 0·05% NaF to drink for up to four years.

Urine Collection. Rats were kept in individual metabolism cages for periods of four to seventeen days. Urine was collected under toluene into a vessel changed every 24 hours.

Kidney Clearances. Inulin and p-aminohippuric acid (PAH) clearances were carried out as follows. The rat, its bladder emptied by supra-pubic pressure, was injected intraperitoneally with 25 mg. PAH (as the sodium salt)/100 g. body weight in about 3 ml. 2% Na_2SO_4, and 33 mg. inulin/100 g. body weight in 2-4 ml. isotonic saline. The rat was then put into a metabolism cage equipped for the collection of urine. After 60 minutes the bladder was emptied and the rat removed and killed by a blow. Blood was obtained from an incision into the heart through the thorax and collected into a heparinised centrifuge tube. The floor of the metabolism cage and the funnel were rinsed with distilled water, the washings being added to the urine in the collecting cylinder, and the volume was made up accurately to 50 ml.

Chemical Procedure

Sodium in urine was determined by means of the flame photometer.

Urea in urine was estimated by the urease method of Scott, which uses boric acid solution to absorb ammonia, and urea in blood by the method of Conway (1935).

For albumin in urine the sulphosalicylic acid test was applied, confirmed, where a positive result was obtained, by boiling.

Benedict's test (Cole, 1944), modified by halving the volume of protein-free urine, was used to test for the presence of glucose in urine, since it was found that this procedure gave results identical with those of the more elaborate Cole's test (Cole, 1944).

Inulin in plasma and urine was estimated by means of a slight modification of the resorcinol method of Roe, Epstein and Goldstein (1949).

PAH in plasma and urine was determined by the diazo-reaction according to the method of Bratton and Marshall (1939) for sulphonamide derivatives.

Histological Procedure

Preparation of Tissue. Thin radial or longitudinal slices of the kidney of rat, dog and rabbit were cut, close to, but not including, the centre of the organ. As far as possible comparable positions in the fundus of the stomach were selected for excision, but in the cases of the dog and rabbit this could only be approximate. In the rat, however, it is possible to be precise, and the sections examined were cut from a strip of

tissue running parallel with, and immediately posterior to, the junction of the fundus with the thin-walled, non-secretory portion of the stomach. The pieces of tissue were fixed in neutral formalin saline, Regaud's fixative or ice-cold acetone as required.

Succinic Dehydrogenase. The method followed was that of Seligman, Gofstein and Rutenberg (1949). According to this procedure, sections of fresh-frozen tissue are incubated with succinate and neotetrazolium chloride in a phosphate buffer at pH 7·6. The hydrogen removed from the substrate reduces the neotetrazolium first to a pink monoformazan pigment and then to blue diformazan precipitated at the sites of enzyme activity. In most of the experiments the modification to the method suggested by Rosa and Velardo (1954), which increases the speed of the reaction by introducing cyanide into the incubation mixture, was adopted. Sections of kidney and stomach were cut at 20μ or 40μ, and incubated for 10, 20 or 30 minutes. The sections were fixed in buffered formalin solution and mounted, without counterstaining, in Apathy's mountant.

In order to determine the type of effect produced by fluoride on succinic dehydrogenase action under these conditions, kidney sections from a series of control rats were treated as above and compared with corresponding sections incubated in the presence of 0·05M NaF.

The procedure followed is not well suited to quantitative work, and small differences in enzyme activity are likely to be masked by factors inherent in the method. For instance, the true thickness of a section of frozen tissue must depend to some extent on the temperature during cutting. Moreover, any agitation of the sections during incubation increases the rate of the reaction. Where the differences between test and control specimens were considerable, however, and providing that all the sections of the same specimen similarly treated compared closely with one another, it was considered permissible to record a difference.

Alkaline Phosphatase. The method of Gomori (1946) was applied to kidney sections 5μ in thickness. In this procedure sections are incubated in a buffered glycerophosphate solution containing calcium ions. Calcium phosphate is laid down at sites of enzyme activity, and the precipitate is converted successively to cobalt phosphate and to black cobalt sulphide. The sections were incubated for 10, 20 or 30 minutes, and the nuclei stained with neutral red.

Staining Techniques. The general-purpose stains haematoxylin and eosin were used with representative sections of all kidney and stomach preparations. The stomach sections were stained also with acid eosin and toluidine blue, which gives a brighter picture and differentiates between parietal cells (pink) and peptic cells (blue). Mucoid and epithelial cells appear mauve and pepsinogen granules brown-red.

A specific stain for pepsinogen was employed on part of the stomach material. This was the ethylviolet-Biebrich scarlet stain of Bowie (1924) which characterises pepsinogen as violet granules and stains all other tissues pink.

Alcian blue, introduced by Steedman (1950) as a specific stain for mucins, was used for both kidney and stomach material. The procedure followed was that described by Gurr (1956) which includes staining with haematoxylin and chlorantine fast red. By this technique nuclei are stained purple-blue, acid muco-polysaccharides of epithelial and connective tissue mucin blue-green, cytoplasm pale yellow and collagen fibres cherry-red.

Mallory's connective tissue stain and the methyl violet stain for amyloid material were employed on a few kidney sections.

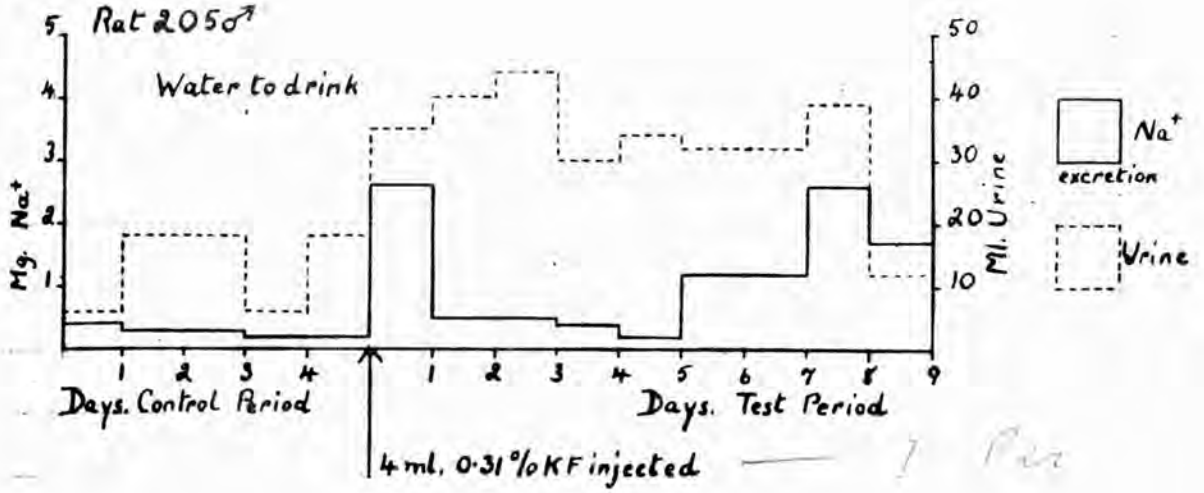
RESULTS

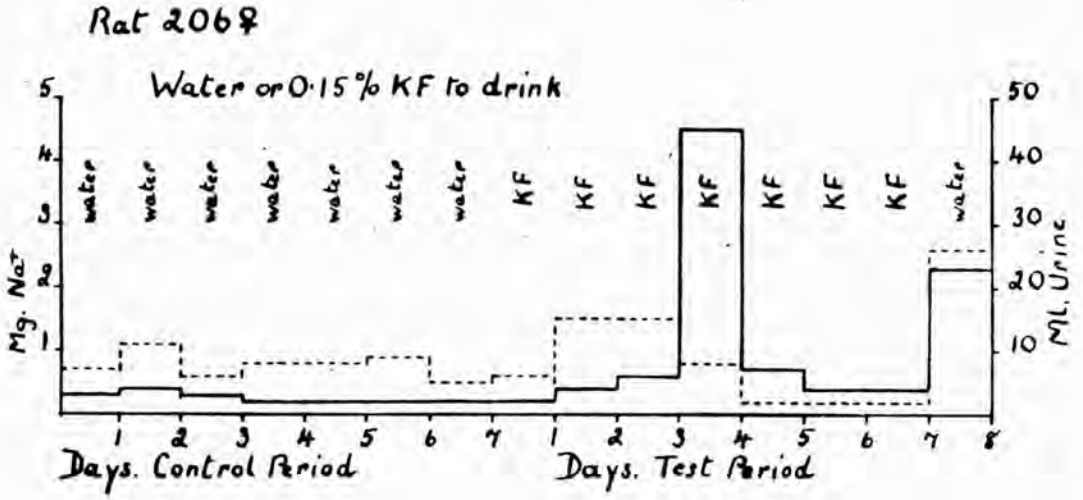
Experiments with Rats

Tests were carried out on rats which had been subjected to fluoride feeding, either by inclusion of NaF in the diet for long periods or by being given a solution of KF or NaF to drink for two or three weeks. A close correlation was found between the responses of any individual rat to the various tests.

General Observations. Rats severely affected by fluoride were thin and listless, with a harsh coat scanty in patches. They lost weight, and post-mortem examinations revealed that only very little subcutaneous fat was present. The muscle layers of the alimentary canal were characteristically thin, appearing sometimes to be almost transparent. In the most extreme condition there was a tendency for sores to develop on the tail and punctate haemorrhages to occur on the paws. Such rats showed reduced renal clearances and histological evidence of gross damage to the kidneys and stomach.

It is evident that individual rats vary widely in their ability to withstand the toxic effects of fluoride, and that this characteristic is unrelated to sex. Of the rats given KF in the drinking-water, one, 206, showed severe fluorosis after only six days. On the other hand, a litter-mate, 207, drank the fluoride solution for twelve days and remained in all respects indistinguishable from a control animal.





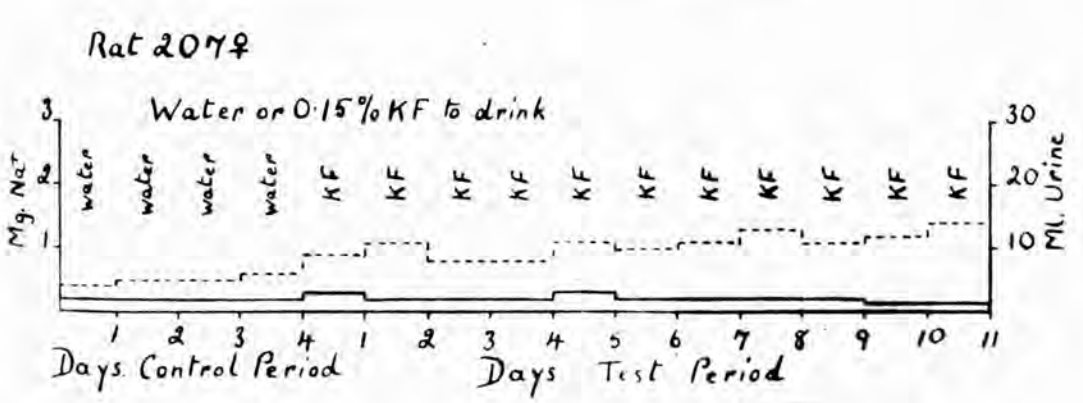


Fig. 1. The influence of KF administration on the excretion of sodium in urine and the urine output in three rats.

Probably the effect of fluoride on rat 206 was increased by the animal's refusal to take food. Several rats, to which either KF or NaF was given in the drinking-water, suffered no loss of appetite and these animals showed little indication of fluoride poisoning. For the most part, however, the rats left their food untouched as soon as the drinking-water was replaced with a fluoride solution. Rats allowed to survive for more than about a week did begin to eat again, but only to a limited extent and they continued to lose weight. Temporary loss of appetite was seen also in young rats when they were given the NaF-containing diet.

Young rats put on to the NaF-containing diet showed, almost immediately, polydipsia and polyuria. The rats given fluoride solutions to drink did not show this response: they drank no more liquid than they had been accustomed to do during the control period when water was supplied. In two cases out of three, however, where water was offered after several days of KF administration, it was accepted avidly and the output of urine showed a marked rise (Figs. 1 & 2). The rat which received KF by injection showed persistent polydipsia and polyuria (Fig. 1).

Introduction of KF or NaF into the drinking-water was in every case followed by immediate albuminuria. Some of the older rats showed mild albuminuria in the control period, and in their cases the condition was intensified when fluoride

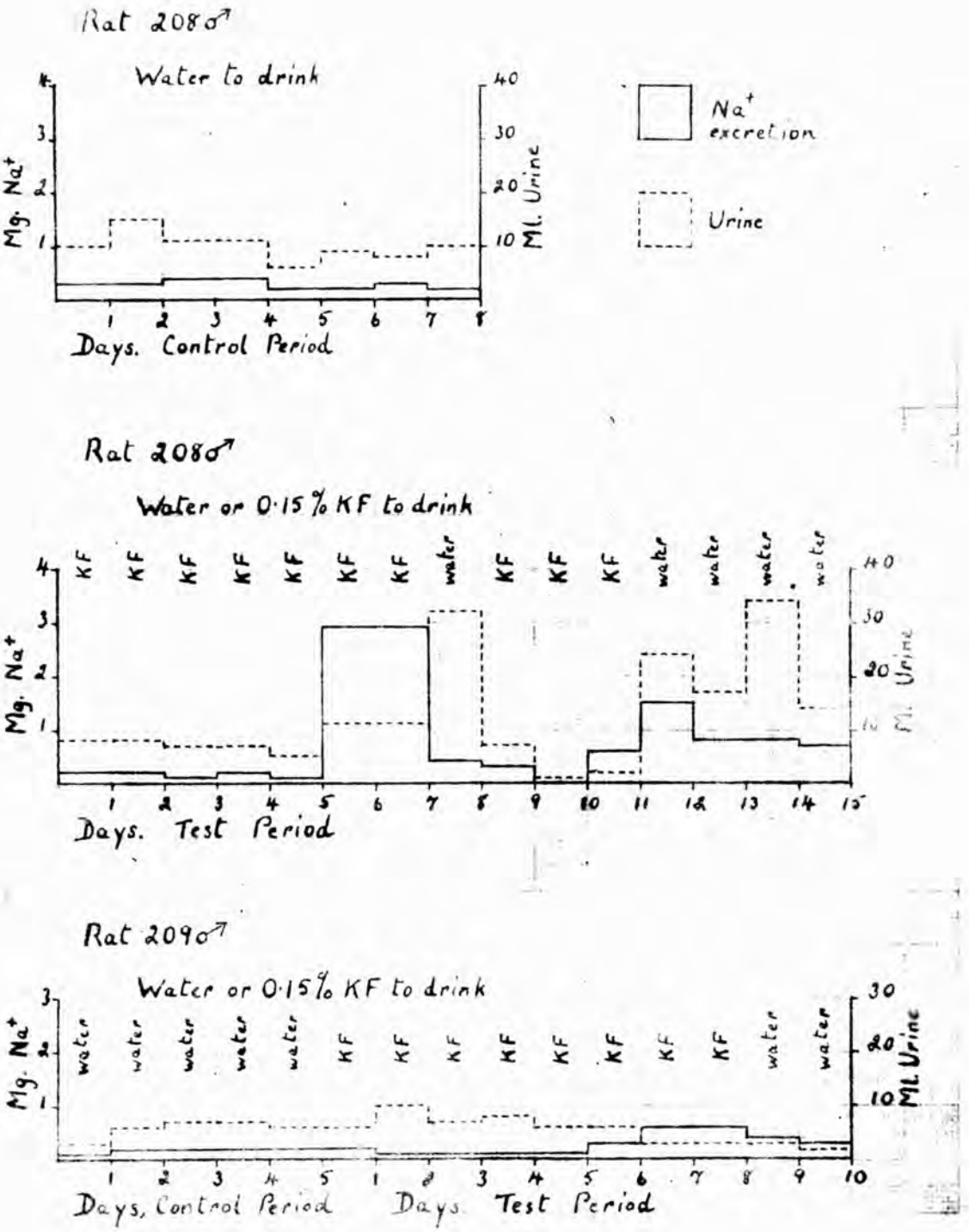


Fig. 2. The influence of KF administration on the excretion of sodium in urine and the urine output in two rats.

consumption began. Albuminuria usually persisted during the first two weeks of the administration of KF or NaF in the drinking-water but diminished, and in two cases disappeared, in the more fluoride-resistant rats by the end of three weeks. Albumin was not found in the urine of rats which had received NaF in the diet for several months (Bond and Murray, 1952).

The first urine specimen to be collected after the drinking-water had been replaced with a fluoride solution invariably contained glucose. Thereafter the glucose test gave positive results intermittently, but without any definite pattern being discernible.

Sodium Excretion. The urinary output of sodium was studied in five rats before and during the administration of KF. In two instances (rats 205 and 206, Fig. 1) giving KF was followed closely by the excretion of urine containing more than ten times as much sodium per diem as had appeared in the samples during the control period. The high rate of sodium-loss was not maintained; it declined to the control level for several days and rose again subsequently. Rat 208 (Fig. 2) excreted no sodium in excess of its control level until the sixth day of fluoride administration, when the pattern demonstrated by the two rats previously mentioned began to be followed. Rats 207 and 209 (Figs. 1 & 2), given KF for ten days, failed to excrete more sodium than they had been excreting during the control period. These two rats suffered no loss of appetite and, in

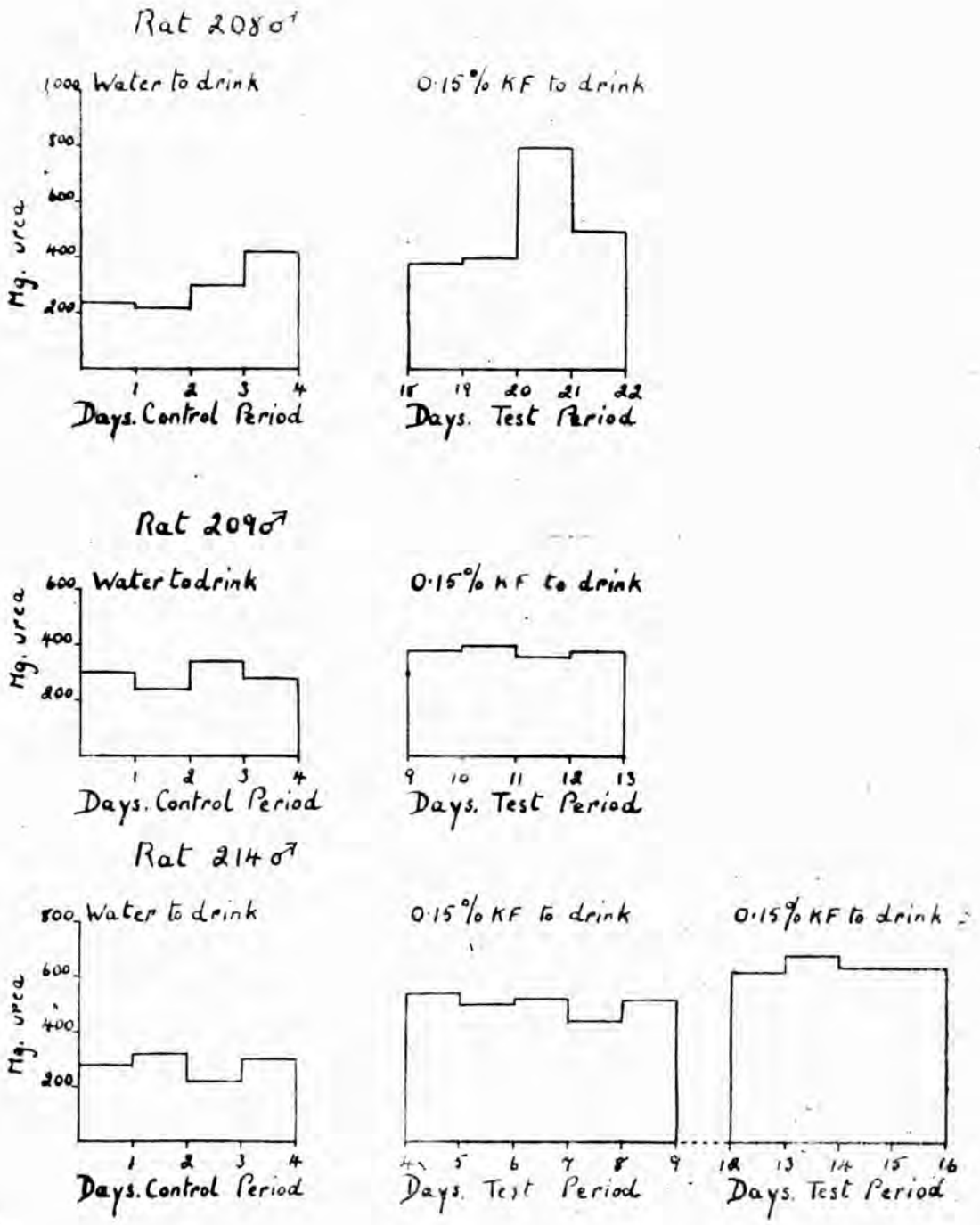


Fig. 3. The influence of KF administration on the excretion of urea in three rats.

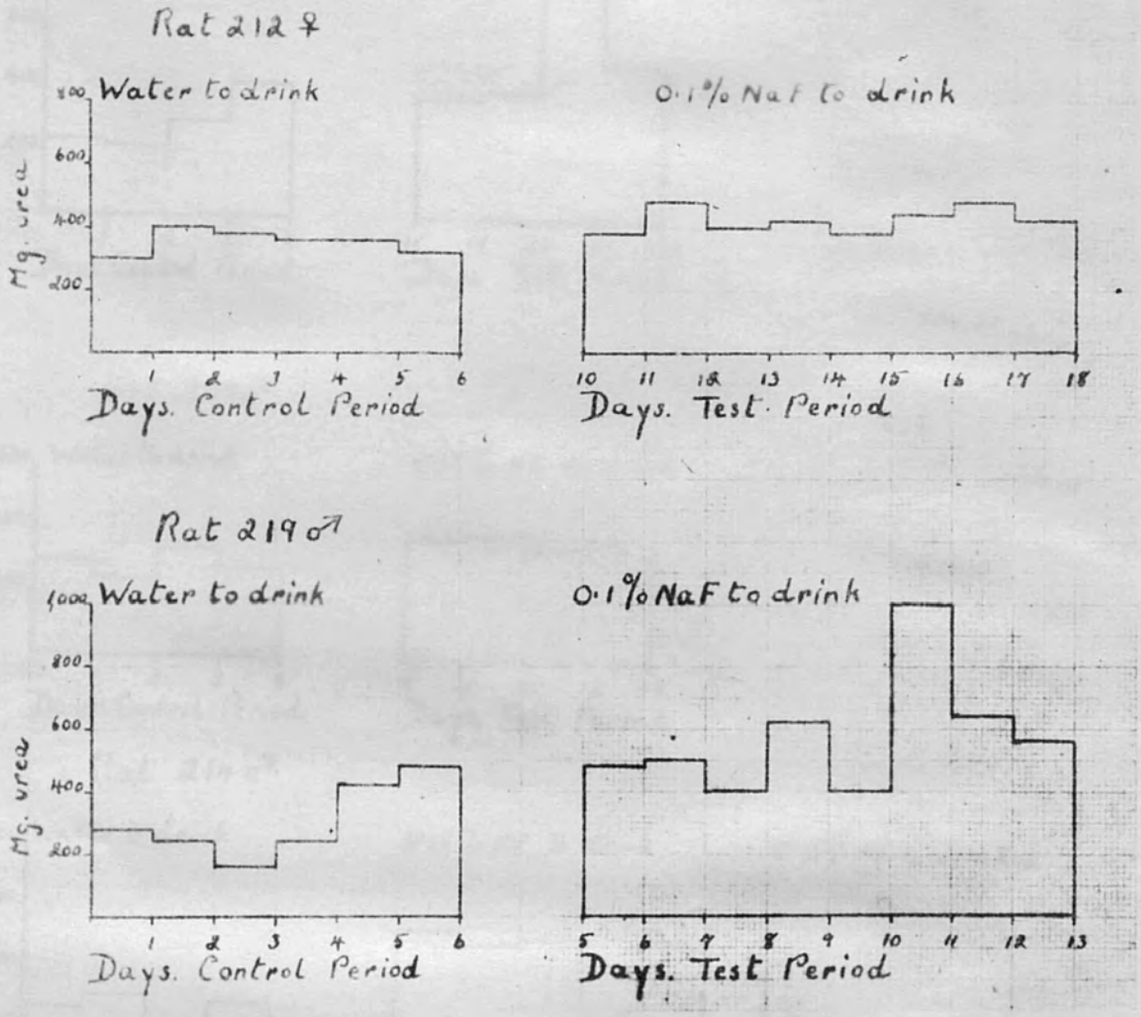


Fig. 4. The influence of NaF administration on the excretion of urea in two rats.

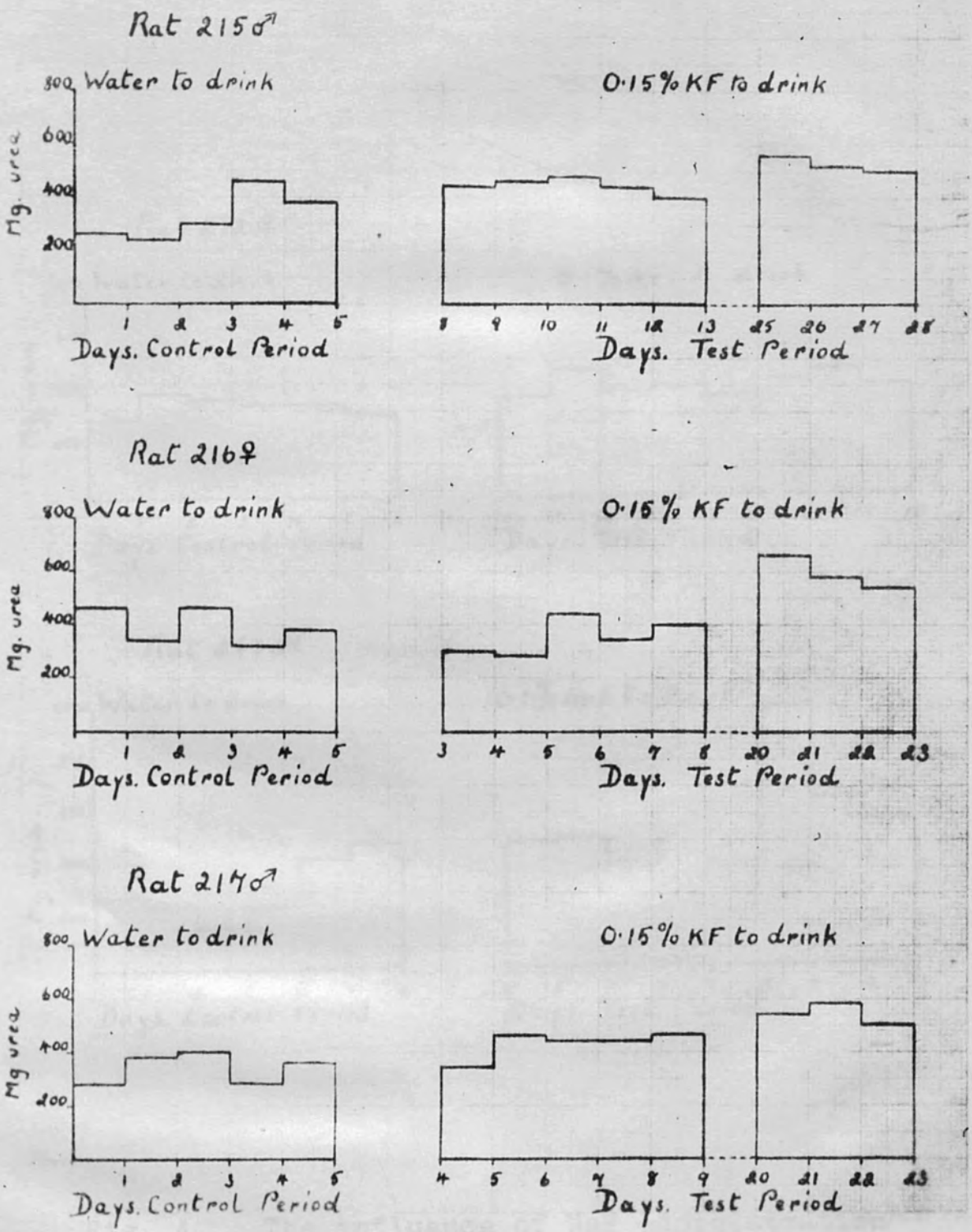


Fig. 5. The influence of KF administration on the excretion of urea in three rats.

all respects observed, they appeared to be entirely unaffected by fluoride. Figs. 1 & 2 show that the amount of sodium excreted in a 24-hour period is independent of the volume of urine.

Urea Excretion. The results of the eight experiments set out in Figs. 3, 4 & 5 show that, despite considerable individual variations in the output of urea, a rise was always recorded within a few days of replacing the drinking-water with KF or NaF solutions. The figures include only a short control period for each rat, but the values given are representative of those found in several such control periods at intervals of three or four weeks. A high rate of nitrogen (particularly urea-N) excretion has been found in rats fed on the NaF-containing diet for ten months or more (Bond and Murray, 1952). The current experiments were not continued for more than 28 days, and it may be seen (Figs. 3, 4 & 5) that within that period the rate of excretion of urea rose with increasing time of fluoride administration. It is noteworthy that in most cases the introduction of KF or NaF into the drinking-water caused a very marked decrease in food consumption, a factor which would be expected to lower the urea output.

One rat, 220 (not included in the figures), failed to show any significant increase in urea excretion during the ten days of KF administration to which it was subjected. It was killed on the eleventh day and its blood analysed for urea. A value of 620 mg/100 ml. blood was obtained, which compared with 32 mg/100 ml. for blood taken from a control rat.

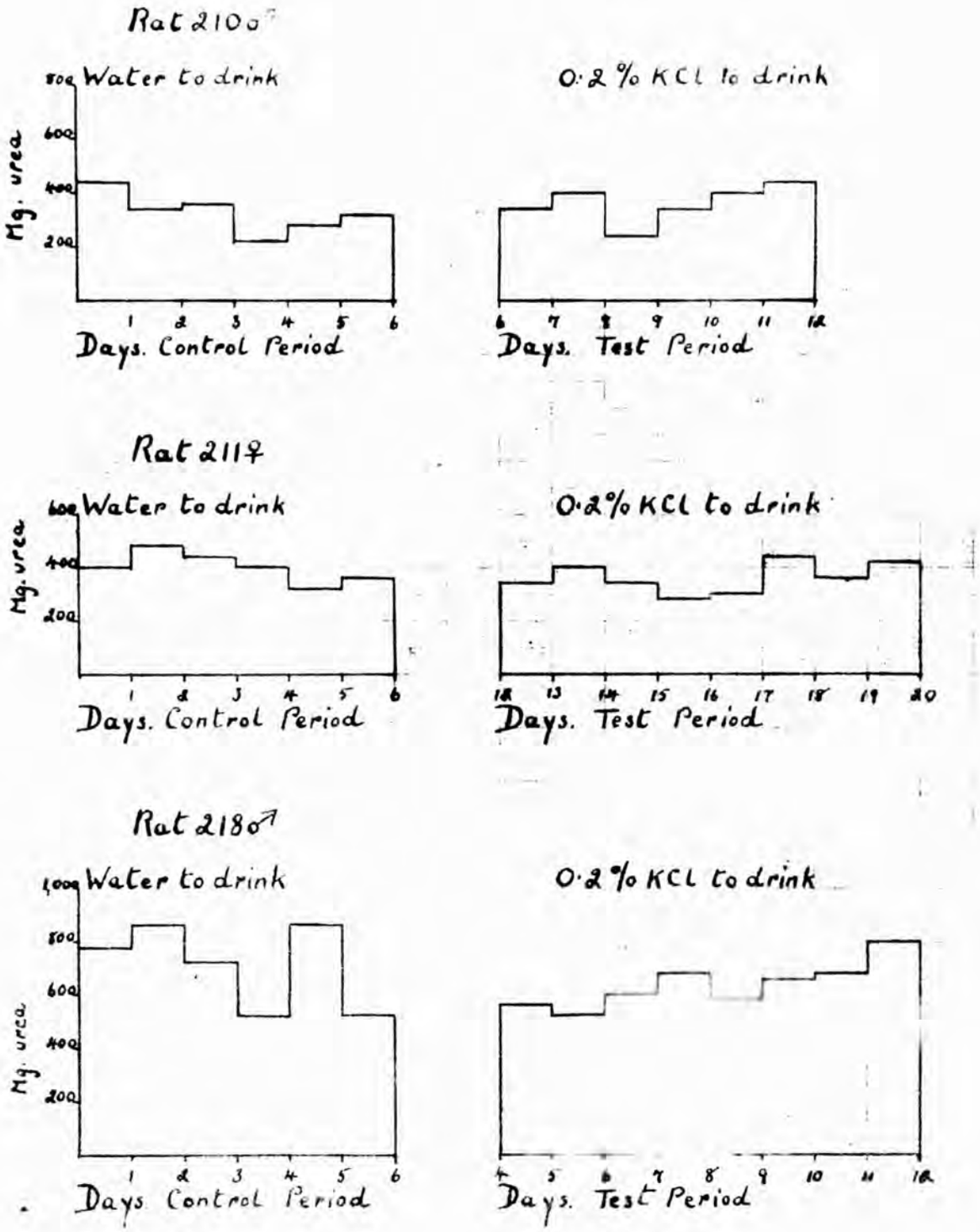


Fig. 6. The influence of KCl administration on the excretion of urea in three rats.

Fig. 6 shows that KCl administration was not followed by an increase in urea output.

Renal Clearances. Four rats which had received NaF in the diet for twelve months or more were all seriously affected and showed reduced renal clearances of inulin and p-aminohippuric acid. The values found for the renal clearances in eleven other rats, however, emphasize the ability of some individual rats to withstand for a time an intake of fluoride such as severely affects other rats. Rats 149 and 150, for instance, had renal clearance values falling within the normal range after consuming NaF for eight months, whilst rats 143 and 148 had reduced inulin clearances and rat 146 had reduced clearances for both inulin and PAH after only six months of the fluoride diet.

It appears that fluoride poisoning reduces the inulin clearance (which represents the glomerular filtration rate) more readily than the PAH clearance (which represents the renal plasma flow). In every case a normal inulin clearance in a "fluoride" rat was accompanied by a PAH clearance falling within the range of the values found for control animals (TABLE I). Mildly-fluorosed rats tended to show a reduced inulin clearance with a normal PAH clearance (TABLE II) and, invariably, where the PAH clearance fell below the control range there was also a much-reduced inulin clearance (TABLES II & III).

TABLE I

Renal clearances of inulin and p-amino-hippuric acid in control rats.

Rat No.	Age in months	Clearance IN (ml plasma/ 100g rat/min)	Clearance PAH (ml plasma/ 100g rat/min)
51 ♂	12	0·25	4·51
52 ♂	22	0·29	1·84
53 ♂	24	0·21	1·45
54 ♀	24	0·26	1·60
55 ♀	18	0·33	1·19
56 ♂	24	0·22	3·01
57 ♀	6	0·36	3·79
58 ♂	8	0·38	1·40
59 ♀	12	0·42	2·73

Clearance IN	Mean 0·302	S.D. ± 0·095
Clearance PAH	Mean 2·39	S.D. ± 0·97

TABLE II

Renal clearances of inulin and p-amino-hippuric acid in rats receiving 0·05% NaF in the diet.

Rat No.	Period of NaF diet (months)	Clearance IN (ml plasma/ 100g rat/min)	Clearance PAH (ml plasma/ 100g rat/min)
142 ♀	3	0·34	3·01
143 ♂	6	0·08	1·89
144 ♂	6	0·33	2·40
145 ♂	6	0·20	1·20
146 ♀	6	0·14	0·58
147 ♀	6	0·32	2·81
148 ♀	6	0·09	2·00
149 ♂	8	0·35	2·50
150 ♀	8	0·33	1·42
151 ♂	9	0·14	0·80
152 ♀	9	0·12	1·41
153 ♀	12	0·06	0·45
154 ♂	12	0·08	0·20
155 ♂	24	0·005	0·36
156 ♀	24	0·014	0·27

TABLE III

Renal clearances of inulin and p-amino-hippuric acid in rats given solutions of KF, NaF or KCl to drink.

Rat No.	Solution given	Period of Administration (days)	Clearance IN (ml plasma/ 100g rat/min)	Clearance PAH (ml plasma/ 100g rat/min)
206 ♀	0·15% KF	6	0·0005	0·05
208 ♂	0·15% KF	12	0·16	1·41
209 ♂	0·15% KF	12	0·32	2·70
210 ♂	0·2% KCl	12	0·26	3·21
211 ♀	0·2% KCl	14	0·45	3·83
212 ♂	0·1% NaF	18	0·18	1·42
205 ♂	0·15% KF (by single injection)	-	0·003	0·05

The lowest renal clearances found (TABLE III) were from two rats (205 and 206) which showed signs of severe toxicosis after the administration of KF. The other clearance values obtained from the rats which had been given solutions of fluoride or KCl to drink also confirm the impression gained from the experiments mentioned previously. Rat 208, which did not respond at once to KF, showed a reduced clearance of inulin only, as did rat 212 which received NaF in the drinking-water. Rat 209, which successfully resisted the effects of KF, had normal clearance values, and those obtained from two rats given KCl solution to drink were indistinguishable from the control findings.

Histology and Histochemistry of the Rat Kidney.

General Structure. The staining technique which employs alcian blue and chlorantine red reveals that connective tissue may be stained with either agent according to its composition. A section of the kidney of a control rat shows a number of fine crimson-stained collagen fibres surrounding arteries and veins, but otherwise collagen is not present. The delicate connective tissue covering the capillaries of the glomeruli stains blue-green, as does the connective tissue supporting the medullary tubules. The renal cortex in the control rat is composed of closely-packed glomeruli and tubules, between which connective tissue cannot be differentiated with these stains, although Mallory's stain and a silver impregnation technique reveal that a fine framework of reticular fibres is present.

The administration of fluoride to a rat appears to cause an alteration in the distribution of connective tissue in the kidney to take place. The amount of collagen found is nearly always less than would have been expected. This was particularly evident in the rats to which fluoride solutions were given to drink. In these animals collagen disappeared almost completely from around the renal arteries and veins. In most of the rats given NaF in the diet for long periods perivascular collagen was present to only a limited extent. The "fibrotic" parts of the kidneys of rats suffering from chronic fluorosis do contain collagen fibres, but these are more sparse than are generally

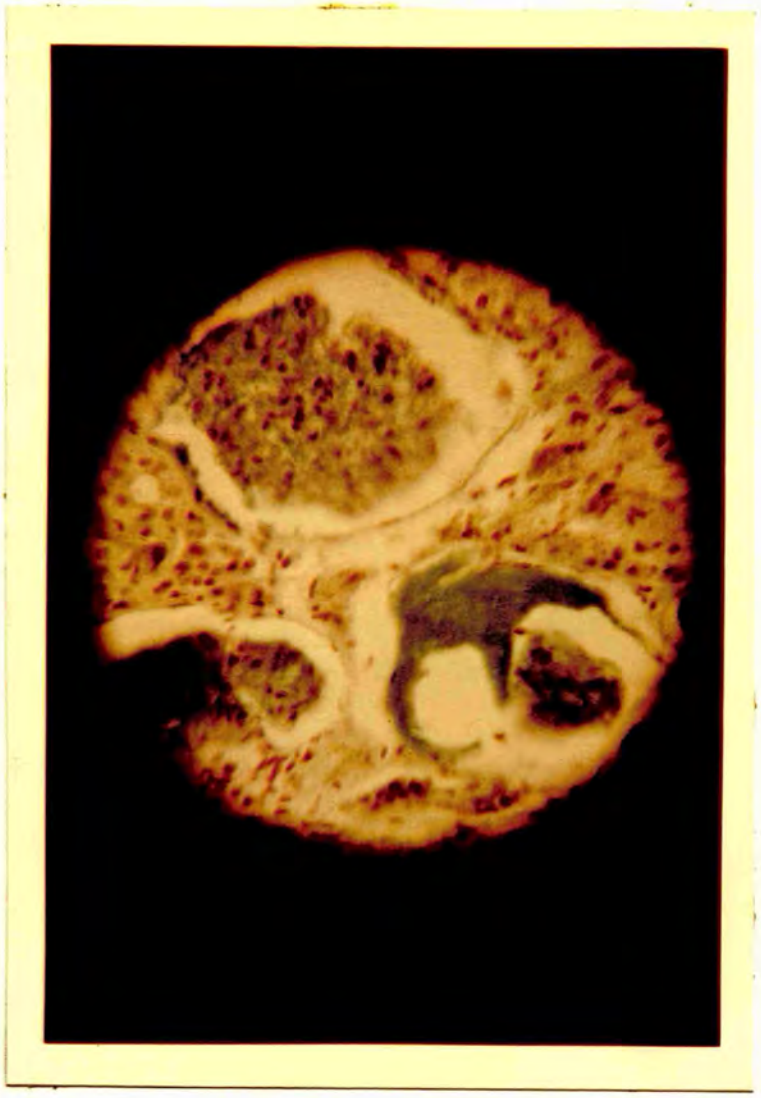


PLATE 1. Photomicrograph x 420 of kidney of rat 170 (given NaF in diet for 12 months). Alcian blue and chlorantine red. Shows disintegrating glomeruli and development of "amyloid" substance in capsules.

encountered in the nephritic animal. The kidney of a cat, killed during recovery from an infective respiratory disease, showed replacement of large areas of the renal epithelium by parallel bundles of collagen fibres. The wedge-shaped regions in the cortex of the fluoride-fed rat where the renal epithelium is no longer functional are composed of degenerating tubules compressed together, dilated tubules and a few collagen fibres. Only a narrow bundle of collagen fibres radiates from the medulla into the "wedge".

The acid-muco-polysaccharide-containing connective tissue, however, which stains blue-green with alcian blue, is increased in amount in the medulla in chronic fluorosis and sometimes extends into the juxta-medullary region of the cortex also. Small inter-tubular patches of alcian blue-staining tissue were found in the cortex in some of the sections examined.

Alcian blue staining is found to be an obvious feature of sections of the kidneys of rats with fluorosis, as it distinguishes, specifically, one of the three kinds of casts found in the tubules. A study of a number of sections indicates that this particular material is formed from the connective tissue covering the capillaries of the glomerulus and lining the capsule. Frequently, a capsule is seen which contains the remnants of a glomerulus and also a finely-fibrillar mass, stained with alcian blue (PLATE 1). Casts of a similar substance are to be seen in the cortical tubules and in the medulla. Usually, as the lower end of the nephron is approached the fibrils disappear,

leaving a clear structureless mass staining with a greater intensity. This material is coloured blue with Mallory's stain, confirming its connective-tissue origin, and satisfies the chemical requirements of the methyl violet stain specific for "amyloid". Occasionally the wall of an artery or vein is seen to be infiltrated with the "amyloid" material.

Another type of cast in the kidney of the "fluoride" rat is found to be hyaline in whatever part of the kidney, cortex or medulla, it appears. In the alcian blue-chlorantine red stained sections this material takes up no dye. With Mallory's stain the "hyaline" casts appear yellow, they stain strongly with eosin and take up carbol-fuchsin (a stain for fibrin and the hyaline substance in degenerating tissues).

Many of the casts in the tubules are readily recognisable from a haematoxylin and eosin stained section as cellular debris. This appears as a particulate mass, staining lightly with eosin, in which nuclei are embedded. Without doubt these casts are related to certain groups of tubules which have a low, feebly-staining epithelium. This response to fluoride was seen particularly clearly in two rats which received KF in the drinking water. Kidney sections from these animals showed that tubules with epithelium of normal height were rare. For two or three days before death the rats excreted a urine dense with cellular material. At post mortem their kidneys were found to be yellow in colour and grossly enlarged.

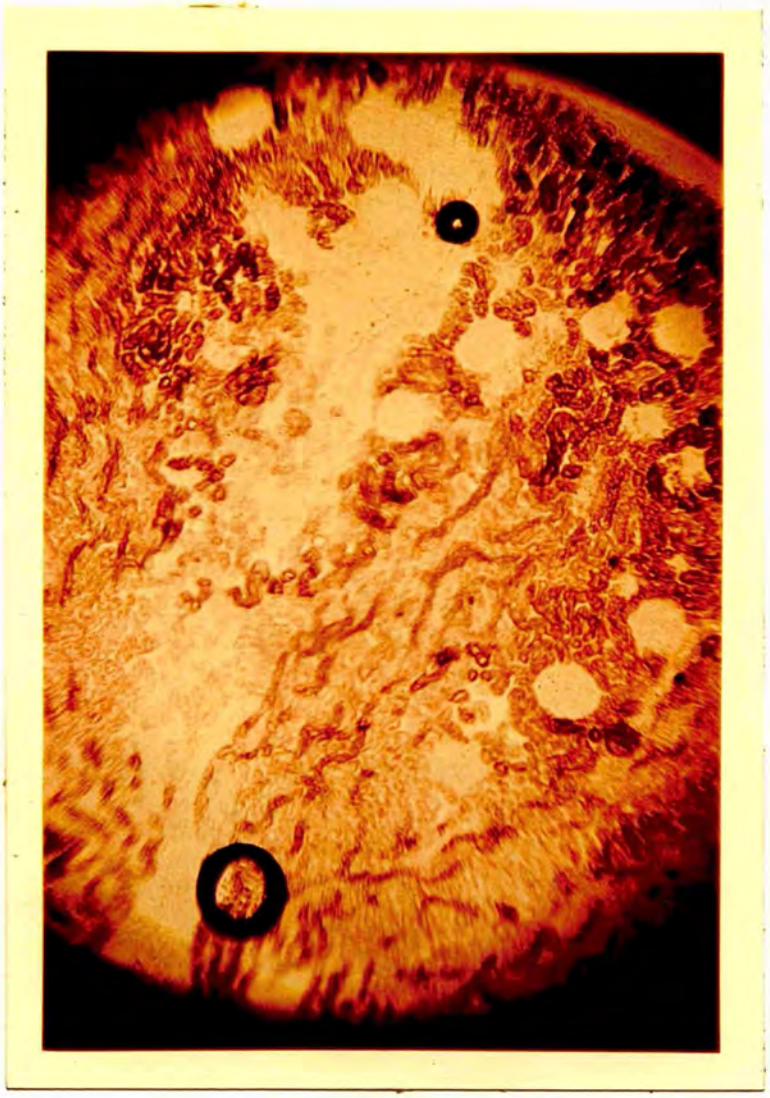


PLATE 2. Photomicrograph x 55 of kidney of rat 175 (given NaF in diet for 8 months). Demonstration of succinic dehydrogenase activity. Shows activity in tubules and absence of enzyme in glomeruli and in an area of replacement tissue.

Succinic Dehydrogenase. Succinic dehydrogenase is an important enzyme in the energy-giving processes of the cell, and its inhibition by fluoride has been held to be sufficient to account for the inhibitory effect of fluoride on an isolated cytochrome oxidase system (Slater and Bonner, 1952). It is to be expected that fluoride interferes with the histochemical reaction for succinic dehydrogenase in tissue slices. In the method used, sites of high enzyme activity are shown by the precipitation of a purple-blue diformazan pigment. A diffuse pink colouration, monoformazan, precedes the deposition of diformazan, and if still present after prolonged incubation indicates sites of low enzyme activity.

Succinic dehydrogenase activity in the kidney is confined to the cortex. In the control rat evidence of intense activity can be seen in the convoluted tubules and in the thick straight segments of the loop of Henle. The glomeruli, thin segments of the loop of Henle and collecting tubules are devoid of activity.

Inhibition of the enzyme by fluoride took different forms according to the treatment given to the animal or tissue slice concerned. Sections from the kidneys of rats with chronic fluorosis showed that in the surviving renal epithelium the activity of succinic dehydrogenase was equivalent to that of a control rat. The "wedges" of replacement tissue were, however, without activity (PLATE 2).

Very pronounced inhibition of the enzyme was seen in the kidneys of two rats which received KF in the drinking water for a short period and were severely affected by it. Kidney sections from these animals showed practically no diformazan after thirty minutes incubation, and less monoformazan than is produced by a section from a control rat kidney in ten minutes incubation.

In another experiment the kidneys of fourteen control rats were used to determine whether NaF exerted an inhibitory effect on succinic dehydrogenase tissue slices in vitro. Several of the sections cut from each kidney were incubated in the ordinary way and the remainder were incubated in the presence of 0·05 M NaF. The NaF-treated sections were found to differ from the corresponding controls, not in the depth of colour of the deposit but in the smaller amount of precipitate formed. These results indicate that fluoride does not retard the reaction with neotetrazolium, but decreases the number of sites of succinic dehydrogenase activity. Possibly there is complete abolition of the activity of the enzyme where fluoride is in contact with the cut surface of a cell, but no effect upon the undamaged cell less readily penetrated by fluoride.

Alkaline Phosphatase. It was the early results on the distribution of this enzyme which prompted an investigation into the nature of the kidney damage in the fluorotic rat. The appearance of a kidney section from such an animal is striking:

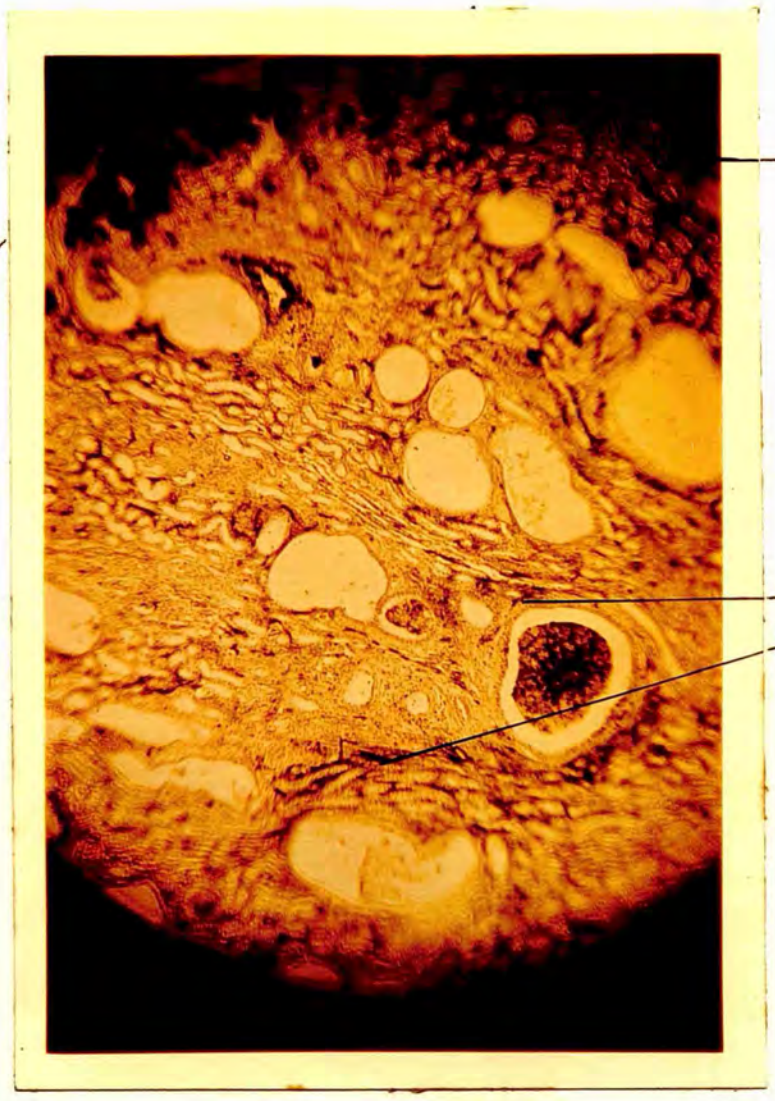


PLATE 3. Photomicrograph x 55 of kidney of rat 174 (given NaF in diet for 10 months). Demonstration of alkaline phosphatase activity: shows intense activity in juxta-medullary cortex and low activity in medulla, as black deposits.

"wedges" of replacement tissue devoid of alkaline phosphatase activity alternate with areas of surviving renal epithelium in which the activity of the enzyme is increased beyond that of a normal rat (Bond and Murray, 1952). It is likely that such a picture represents successful adaptation to a prolonged intake of fluorine, for there is a broad correlation between the degree of enzymic activity and the general health of the animal. Absence of alkaline phosphatase, or reduced activity of the enzyme in parts of the cortex, was seen in rats that had been consuming the fluoride diet for a short time only, and patches of renal epithelium distinguished by subnormal, normal and supra-normal alkaline phosphatase activity were seen to co-exist in the same kidney section.

In the kidneys of control rats alkaline phosphatase activity is seen only in the proximal convoluted tubules and in the tunica adventitia of the small arteries and veins. The amount of the cobalt sulphide precipitate formed is, of course, related to the length of the incubation time and is remarkably constant in normal rats. Under the conditions used, a ten-minute incubation period results in the appearance of a thin black line round the luminal surface of the proximal convoluted tubules, whilst after thirty-minutes incubation the black deposit has been extended into about one-third of the depth of the cells concerned. Alkaline phosphatase activity has been mentioned in connection with the nucleus and nuclear membrane, but in an incubation period of thirty minutes or less this is not apparent in the rat.

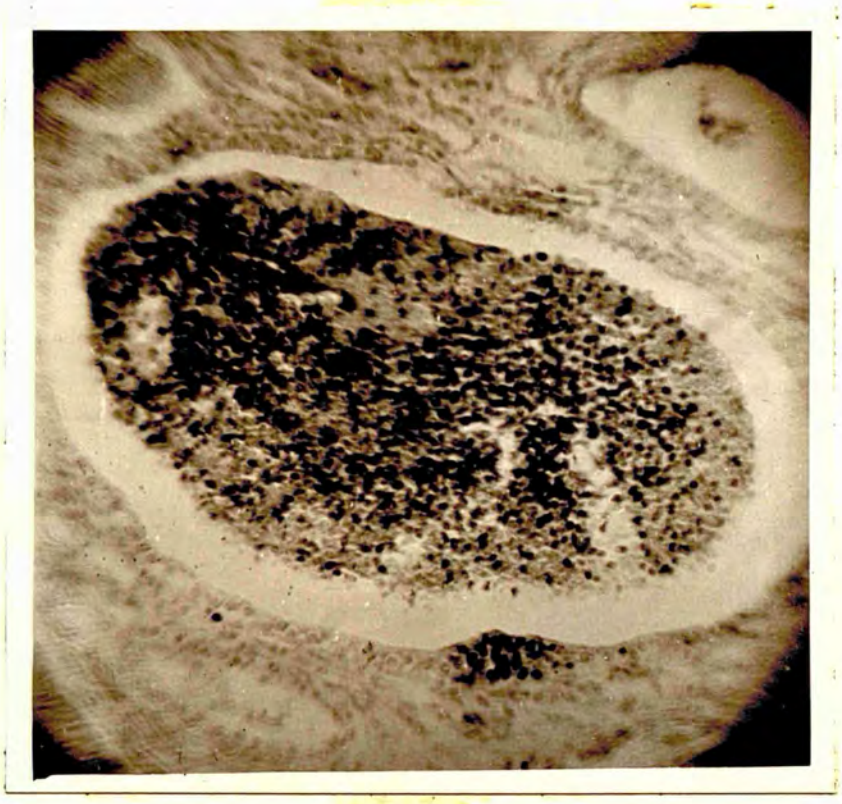


PLATE 4. Photomicrograph x 215 of kidney of rat 173 (given NaF in diet for 10 months). Demonstration of alkaline phosphatase activity: shows activity in cell debris in medullary tubule.

One feature of the kidney of the rat given a fluoride diet for a long time is the appearance of alkaline phosphatase activity in the medulla. A section of such a kidney shows that in and between the tubules of the medulla there is a diffuse, patchy distribution of the enzyme (PLATE 3). The activity of the enzyme in the medulla was found to be less than that in the convoluted tubules. The presence of casts of cellular debris in cortical and medullary tubules has already been commented upon. Suitably incubated sections show that alkaline phosphatase activity can be demonstrated in this material in the form of small discrete round areas, in which the enzyme shows the same degree of activity as in the proximal tubules (PLATE 4).

Activity of the enzyme in casts of cellular debris within the tubules has been noted also in the kidneys of rats to which KF was given in the drinking water. It is likely that the phenomenon is linked with the virtual disappearance of the enzyme from the proximal tubules of the more severely-affected of these animals. It cannot be claimed that fluoride inhibits renal alkaline phosphatase in the rat under these conditions, for it seems more probable that the luminal portion of the tubule cells becomes detached under the influence of fluoride and the tissue fragments having alkaline phosphatase activity pass into the lumina of the tubules.

Histology and Histochemistry of the Dog Kidney.

General Structure. Haematoxylin-eosin and the alcian blue-chlorantine red stains revealed no differences in kidney structure between six dogs which were given 20 mg. F/day in their food for twenty months and their litter-mate controls. The renal epithelium was nowhere abnormally low. There was no evidence of destruction of glomeruli, loss of collagen, dilated tubules or the presence of casts in any of the sections.

Succinic Dehydrogenase. At the end of an experiment in which four young dogs had been given 5-40 mg. F/day in their food according to size for three months, the animals were killed and their kidneys examined for succinic dehydrogenase activity. A comparison with sections from the kidneys of four controls showed no differences that could be ascribed to fluoride, for although there were perceptible differences in the amount of diformazan deposited, the sections of lower activity were distributed evenly between test and control dogs.

Alkaline Phosphatase. No difference in renal alkaline phosphatase activity was found between young dogs given fluoride for three months and their litter-mate controls. Five puppies were given 5-20 mg. F/day and four were given 5-40 mg. F/day according to size.

In another experiment six dogs were given 20 mg. F/day in their food for twenty months. The kidneys of three of these animals appeared to have normal alkaline phosphatase activity, but in the remaining three the enzyme was absent from by far the

greater part of the renal cortex. Small, widely-separated areas of the section showed the presence of the enzyme, but its activity there was much more feeble than in the kidneys of the control dogs of similar age.

In contrast to the results obtained with rats, no evidence was seen in any of the dog kidneys examined of alkaline phosphatase activity in the medulla or the tunica adventitia of blood vessels, or of supra-normal alkaline phosphatase activity in the cortex.

Histology and Histochemistry of the Rabbit Kidney.

General Structure. Three rabbits given 0·05% NaF as drinking-water for one to four years showed no signs of polydipsia, polyuria, impaired appetite or loss of either condition or weight. One rabbit killed after a year and another killed after two and a half years appeared normal post-mortem, and their kidneys showed no irregularity. The remaining rabbit received fluoride for four years. In this animal slight cystic dilatation of convoluted and medullary ray tubules was observed and a little "amyloid" material was present as casts, but the degenerative changes were less pronounced than those in a sibling control rabbit of similar age.

Alkaline Phosphatase. Kidney sections of five rabbits given 6 mg. NaF/100 g/day by mouth for three months were examined for alkaline phosphatase activity. In only one case were there indications of reduced activity of the enzyme. Sections of the kidney of this animal showed that the tubules where alkaline phosphatase activity was feeble or absent altogether had a low epithelium. None of the kidney sections from the test rabbits showed greater activity of alkaline phosphatase than those from the five control rabbits, and activity in the medulla could not be demonstrated.

DISCUSSION.

The Effect of Fluoride on the Kidney.

The experiments presented show clearly that wide differences exist in the susceptibilities of some common laboratory animals to fluoride. Rabbits resist the effects of fluoride. The appearance under the microscope of kidney sections of rabbits given 0·05% NaF to drink for up to four years was in all respects normal. Dogs given NaF in the diet for twenty-two months showed no changes in the minute structure of the kidney, although specimens from half their number had only a feeble alkaline phosphatase activity. This finding was in marked contrast to the rat kidney in which alkaline phosphatase activity was not inhibited by fluoride.

Most of the experiments were carried out on rats. Although individual rats were found to vary in degree of response to fluoride, the manner in which the kidneys were affected showed no variation. Different tests applied to the same animal showed a close correlation, e.g. the kidneys of a rat for which very small clearance values indicated severe renal dysfunction showed negligible enzyme activity and histological evidence of profound disturbance.

Fluoride was administered in two ways. In one set of experiments the dry diet was mixed with an equal weight of 0·1% NaF and fed for periods of six months to two years. In the other, fluoride was given in the drinking-water as KF or NaF for periods of one to three weeks.

Solutions of the same concentration of fluoride are far more toxic if given in the drinking-water than if mixed with the food. This fact has been pointed out by Lawrenz, Mitchell and Ruth (1939) and is easily understood. Fluoride, probably by interfering with gastric acid secretion, depresses appetite, so that after the first meal containing fluoride the food intake, and therefore the fluoride intake itself, is low for several weeks. An animal cannot, however, reduce its liquid intake to a comparable extent, and if fluoride is present in the drinking-water some is bound to be ingested. Moreover, the diet supplied to the rats contained $CaCO_3$, with which fluoride reacts to form the insoluble CaF_2, so that a proportion of the fluoride in the solution mixed with the diet was not available for absorption.

It was found that the effect on the rat of putting fluoride into the drinking-water was in inverse proportion to the animal's appetite. Of five rats given KF either by injection or in the drinking-water, two refused food completely, and when killed were in poor condition with symptoms resembling those of chronic fluorosis. They had kidney clearance values of less than one-fiftieth of the normal figure, negligible renal activity of the enzymes succinic dehydrogenase and alkaline phosphatase, and low epithelium in almost all the surviving renal tubules. Two other rats, drinking the KF solution for eleven and fifteen days respectively, suffered no loss of appetite or condition. Their kidney clearance values and the activities of succinic dehydro-

genase and alkaline phosphatase in the kidneys were normal. Only collagen-loss and the appearance of one or two small "wedges" of tissue replacing functional renal epithelium indicated that they had received fluoride. (The kidney lesions in the rats drinking KF solution were essentially the same as those described earlier (Bond and Murray, 1952) for rats with chronic fluorosis.) The fifth rat, which responded to KF in the drinking-water after a five-day period of normal appetite, was intermediate between the severely-affected and the slightly-affected rats in all respects noted.

Potassium fluoride, rather than sodium fluoride, was put into the drinking-water in the first few experiments in order that Na^+ excretion might be studied in rats maintained on a low-sodium diet. The toxic effects of the KF, however, were far-reaching, and other rats were given either NaF solution or KCl solution to drink to establish that the effects were indeed due to the fluoride ion and not to K^+. This was shown to be the case, since none of the effects shown by the rats receiving KF, i.e. albuminuria, glycosuria, high rate of urea excretion, low kidney clearance values, loss of activity of renal enzymes and alterations in the minute structure of the kidney, appeared in the rats given KCl. The effects were shown, however, and to a similar extent, by the rats to which NaF was given in the drinking-water.

It has been shown (Bond and Hunt, 1956) that the stomach wall of the cat responds to bathing with NaF solution by becoming permeable in both directions to sodium ions. The experiments described here (page 49), carried out on rats in precarious sodium balance, show that the renal epithelium, when under the influence of fluoride, allows Na^+ to escape from the body. The two kinds of experiments, involving as they do two different species and two different periods of exposure to fluoride, do not permit a comparison between the degree of susceptibility to fluoride of stomach and kidney to be made. It is apparent, however, that the rat kidney cell, at any rate, can recover from the increased permeability induced by fluoride. A urine specimen, containing ten to twenty times the amount of sodium regularly excreted by the rat concerned, was collected soon after the drinking-water was replaced with KF solution. The high level of excretion was not maintained, but reappeared intermittently for no obviously discernible reason.

Intermittent also was the presence of glucose in the urine of rats given fluoride in the drinking-water. It appeared when fluoride was first given, and thereafter at intervals of two to five days. Rats given the NaF-containing diet for long periods did not show glycosuria but did have a reduced renal tolerance to glucose (Bond and Murray, 1952).

Evidence of thirst in rats when put on to the NaF-containing diet has been commented upon previously. The rats given KF

solution to drink, however, took no more liquid than they had been accustomed to take during the control period when water was supplied. The rats which developed the symptoms of fluorosis probably were thirsty but found the KF solution unpalatable, since if distilled water was offered they drank eagerly and the output of urine rose. It is of interest that the urine volume was high only after excessive water drinking had taken place. It cannot be argued that fluoride under these conditions of administration hinders reabsorption of water by the kidney tubules and gives rise to a state comparable with diabetes, for in that case a compulsory polyuria would require a compensatory polydipsia and the rat would be forced to drink large volumes of the KF solution provided.

Albuminuria was found always to follow immediately upon the introduction of fluoride into the drinking-water whether or not the rat ceased eating and the signs of fluoride poisoning appeared. Albuminuria was not intermittent, but decreased in severity after about ten days of fluoride consumption, and in some rats had disappeared by three weeks. The urine of rats with chronic fluorosis was found not to contain albumin (Bond and Murray, 1952).

A high rate of urea excretion was noted in rats maintained for several months on the NaF-containing diet (Bond and Murray, 1952). The current experiments show that a raised level of urea is manifested in the urine within a few days of the

administration of fluoride in the drinking-water. There was no tendency for the high urea excretion-rate to fall again within the three weeks during which fluoride was given. On the contrary, it rose steadily within this period. Further experiments are required to determine whether there is a steadily-increasing rate of urea excretion in rats given fluoride for long periods. Only one of eighteen rats given KF or NaF in the drinking-water failed to show a rise within ten days in the amount of urea excreted: the output of urea in this animal continued at the level of its control period. It was killed on the eleventh day of fluoride administration, and an analysis of its blood showed the presence of twenty times the normal concentration of urea. The rise noted in urea output is particularly significant if it is remembered that fluoride-consumption is accompanied, nearly always, by a <u>decreased</u> intake of food. It is possible that in the rise of urea output in rats given fluoride lies a possible test for the diagnosis of a dangerous level of fluoride absorption in human beings. Such a test could be applied long before skeletal changes or other gross disorders are detected.

Rats with chronic fluorosis are thin, with little or no body fat and poor musculature. In particular, the muscle wall of the alimentary canal is so thin as to appear translucent. It may be surmised that the high urea output is derived from the steady breakdown of more of the tissue proteins than are replaced. Loss of tissue from the wall of the stomach is seen also in rats

that have been given fluoride in the drinking-water for a short time, and in these animals a striking feature is a total disappearance of collagen from the kidney. (Collagen is not plentiful in the kidneys of control rats, but collagenous fibres always surround the small arteries and veins.) A study of the kidney of the rat in chronic fluorosis reveals that perivascular collagen fibres are sometimes sparse, but that strands of collagen run in the "wedges" of replacement tissue. There is, however, a more marked increase in the acid mucopolysaccharide-containing type of connective tissue that is distinguishable by its staining reaction with alcian blue. This type of connective tissue (in the normal rat kidney it supports the tubules of the medulla and covers the capillaries of the glomeruli) increases, in chronic fluorosis, in the glomeruli and medulla. It extends into the juxta-medullary region and is seen occasionally in patches in the cortex.

The experiments presented give no support to the suggestion of Pindborg (1957) that the site of the primary lesion in the fluorotic kidney is the loop of Henle. On the contrary, all the evidence indicates that the Malpighian corpuscle is the first part of the nephron to be attacked by fluoride.

The albuminuria, which appears immediately fluoride consumption begins, indicates an effect upon the glomerulus. In clearance studies, which were carried out on a series of rats given the fluoride-containing diet for between six months

and two years, the glomerular filtration rate (measured by inulin clearance) was reduced earlier and to a greater extent than the renal plasma flow as measured by the PAH clearance.

The "wedges" of non-functional renal tissue include all parts of the nephron, and it is not possible to say with certainty at which point of the nephron the lesion first occurs. There are accounts, however, in text-books of pathology of a retrograde change in the human kidney, called amyloid formation, which results in a condition strikingly similar to that seen in fluorosis in the rat. Mallory (1914) describes a state in which amyloid is secreted in the glomeruli (showing at first as a thickening of the connective tissue covering the capillaries) and in the walls of the smaller arteries. The sites of amyloid formation are always "in the closest relation to the cells and fibrils of the connective tissue". At the same time the tubules undergo certain changes. Pressure is raised in the nephrons affected, and the cells in the convoluted tubules become first swollen with a lace-like cytoplasm from imbibition of liquid, and then hyaline droplets appear in the cells. Cells sometimes break off from the basement membrane or become ruptured, so that masses of cell fragments and hyaline droplets fused together appear in the lumina of the tubules. Alternatively, the high pressure inside the tubules may cause the lumina to dilate and the tubular epithelium to become stretched and low. Concurrently with the glomerular changes, the walls of the small

arteries of the kidney show amyloid infiltration. At a later date the veins are similarly affected.

It is possible that the sequence of events described by Mallory for the pathological human kidney apply to the rat kidney under the influence of fluoride. The rats killed after short-term administration of high doses of fluoride had pale yellow kidneys, grossly distended with fluid. Sections of these kidneys showed amyloid formation in some capsules, with disintegration of glomeruli (as in PLATE 1 opposite page 57), and the presence of amyloid and hyaline casts in the tubules. The tubular epithelium was generally low and some lumina were dilated. In places the tubules appeared compressed with the lumina obliterated. The kidneys of rats given the NaF-containing diet for long periods were essentially similar except that the necrotic "wedges" were interspersed with areas of apparently normal renal tissue. The kidneys of these animals were not enlarged. Amyloid formation was seen occasionally in the arteries and veins, but only in rats in which chronic fluorosis was advanced.

From a consideration of the results obtained from rats given fluorides for short or long periods a sequence of events may be postulated to explain the action of the fluoride ion on the kidney. It is suggested that when fluoride in considerable amount enters the blood stream of a rat, the glomerular capillaries react by an immediate increase of permeability, resulting in the appearance of albumin in the

urine. Perhaps all the glomeruli are affected and some of them subsequently return to the normal state, or possibly only a proportion of them respond to fluoride and these glomeruli never assume normal function again. It is evident that the kidney as a whole becomes able to tolerate fluoride, for the rat recovers from albuminuria even though fluoride continues to be consumed with the drinking-water, and in chronic fluorosis albuminuria is not seen. (No investigations have yet been made to find whether there are phases in the development of chronic fluorosis when albuminuria is present.)

Some glomeruli are destroyed and this process is brought about by proliferation of the acid-mucopolysaccharide-containing connective tissue covering the capillaries and lining the capsule. A mass of "amyloid" substance is formed, replacing the capillaries. The amyloid substance eventually passes down the tubule, losing its retiform character on the way and becoming structureless. The destruction of a glomerulus must necessarily arrest the blood supply to the nephron concerned. This fact could account for the disintegration of the epithelial cells which occurs; hyaline casts and fragments of shed cells distend the lumina of affected tubules.

A change in metabolism of connective tissue cells in response to fluoride is reflected not only in the proliferation of the connective tissue covering the glomerular capillaries (and, in chronic fluorosis, in the increase of connective tissue of the

same type in the medulla) but in the rapid disappearance of collagen from the perivascular layers of the kidney. It appears likely that the removal of collagen (probably also from organs other than the kidney) is linked with the dramatic rise in urinary urea that begins a few days after giving a rat fluoride in the drinking-water. The urea excretion is high in chronic fluorosis, in which state collagen is not absent from the kidney but wasting of muscles is pronounced. Brittleness of the bones is a well-marked characteristic of rats with chronic fluorosis, and this observation would be explained if the organic matrix of bone were depleted and the periosteal layers deficient in collagen in this condition.

The extremely low kidney clearances recorded for rats severely affected by KF given in the drinking-water probably reflects the loss of a high proportion of functional nephrons. Nevertheless, that the surviving tubular epithelium was affected to a continually-varying degree was shown by the intermittent appearance in the urine of glucose and sodium in excess of the control period excretory level. Succinic dehydrogenase activity was severely inhibited in these rats. Alkaline phosphatase activity, too, was almost completely absent, but more probably because the parts of the cells containing the enzyme were torn away than because of its inhibition.

During slow development of chronic fluorosis rats show themselves able to tolerate fluoride to a considerable extent. In the early stages of chronic fluorosis in rats there are well-

marked individual variations, but these disappear after one to two years of the fluoride diet. Kidney sections of rats which have consumed the fluoride diet for about twelve months present a regular appearance of "wedges" of non-functional renal tissue alternating with groups of apparently normal nephrons. The areas of replacement tissue are devoid of enzyme activity and contain casts of amyloid substance, hyaline substance and cell fragments. The surviving cortical epithelium is normal with regard to its general appearance and succinic dehydrogenase activity, and usually has an alkaline phosphatase activity higher than that of control rats. Alkaline phosphatase activity is present, moreover, in patches in and between the tubules of the medulla.

With increasing time of consumption of the fluoride diet more nephrons are destroyed. It is rare for rats to die from fluorosis, however, and they continue to survive even when hardly any recognisable kidney tissue remains.

Administration of fluoride to dogs for nearly two years failed to produce kidney abnormalities in any way comparable with those of the rat. Three of six dogs, however, had areas of reduced alkaline activity in the renal cortical epithelium, although in other respects this tissue appeared to be normal.

The resistance of the rabbit to fluoride is so great as to make it an unsuitable animal for the study of fluorosis. No kidney lesions could be demonstrated even after four years of continuous NaF consumption.

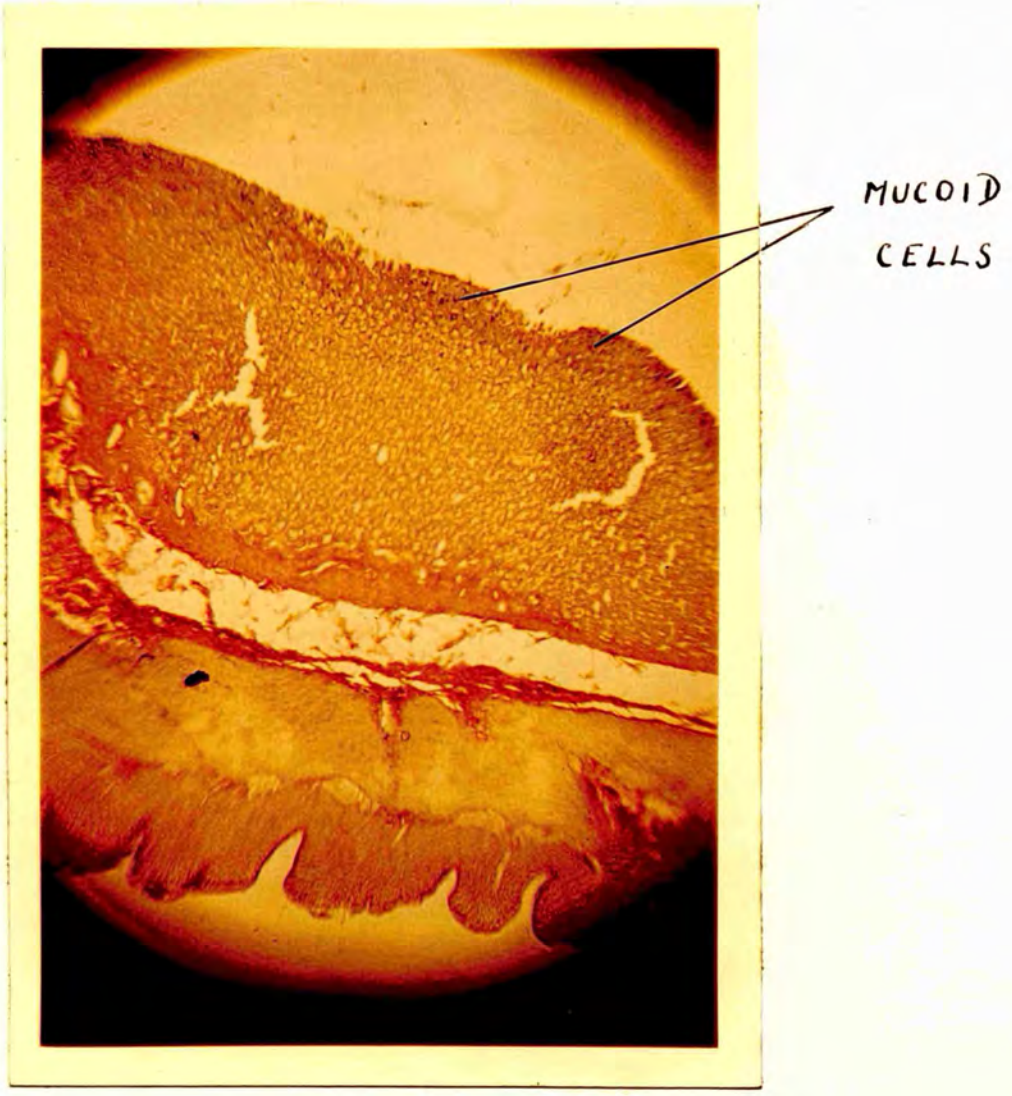


PLATE 5. Photomicrograph x 55 of fundus of stomach of control rat 37. Alcian blue and chlorantine red: shows distribution of active mucoid cells (blue-green) near luminal surface only.

RESULTS

Histology and Histochemistry of the Fundus of the Stomach of the Rat.

General Structure. The part selected for histological study, that is, the most anterior region of the fundus of the stomach, presents a definite and reproducible picture in control rats. The tissue of the mucosa is tightly packed with cells and individual fundic glands cannot be distinguished. A layer of columnar epithelial cells with some mucus-secreting cells covers the surface on which a little mucus sometimes lies. The activity of the mucus-secreting cells, whether at the surface or in the "neck" region of the glands, was never found to be high in control rats. Secretory activity of the "neck" mucoid cells was absent in four of the twelve control specimens examined, barely detectable in six (see PLATE 5) and easily seen in only two. Mucoid cells were never found below the neck region except where the periphery of the squamous part of the stomach had been included in the section.

A dense layer of parietal cells lies below the epithelium and makes up between one-half and one-third of the depth of the mucosa (PLATE 6). The remainder of the mucous coat consists of groups of peptic cells interspersed with parietal cells. The specific pepsinogen stain shows that a few groups of peptic cells appear in the middle of the mucosa, but for the most part they occupy the lowest one-third of it.

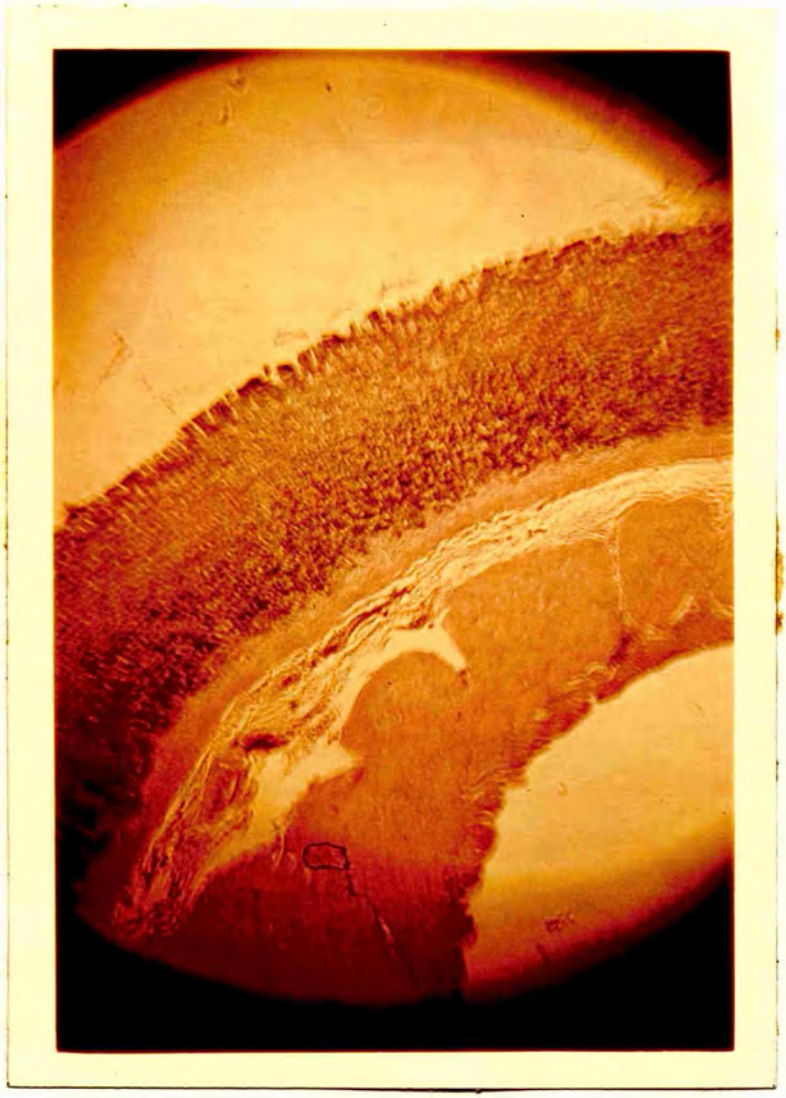


PLATE 6. Photomicrograph x 55 of fundus of stomach of control rat 45. Acid eosin and toluidine blue: shows wide band of parietal cells (pink).

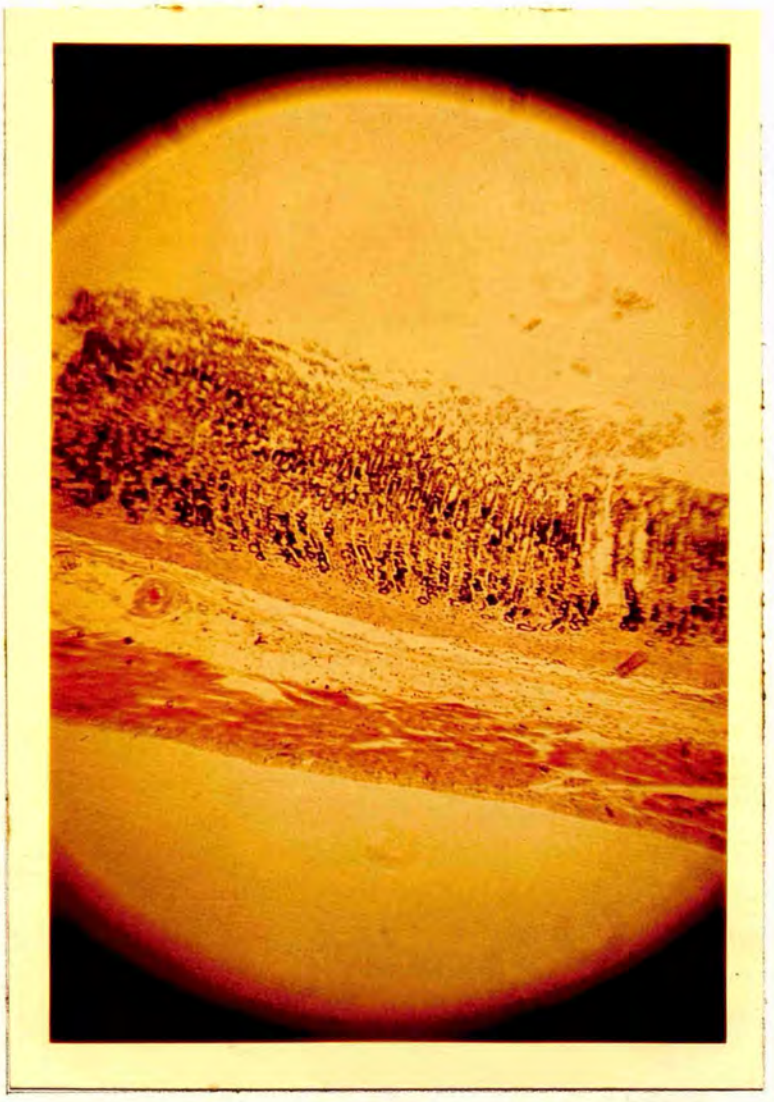


PLATE 7. Photomicrograph x 55 of fundus of stomach of rat 130 (given NaF in diet for 12 months). Acid eosin and toluidine blue: shows irregular luminal surface, narrow band of parietal cells and "erosion" in layer of circular muscle.

(COMPARE WITH PLATE 6)

A number of conspicuous changes were seen in the stomachs of rats to which sodium or potassium fluoride had been administered. Gastric haemorrhage, however, which has been mentioned by many workers as a sequel to acute fluoride poisoning in a variety of animals, was absent. There was no sign of bleeding in the stomachs of rats to which KF or NaF was given for short periods in the drinking-water. Blood was only rarely found in the stomachs of rats subjected to long-term sodium fluoride feeding, and, in these animals, although the surface of the mucosa was torn and roughened, there was no sign of extravasation of blood in the tissues of the fundus.

The thin-walled aspect of the alimentary canal that is observed at a post-mortem examination of a rat with chronic fluorosis is seen microscopically to be due to a reduction in thickness of both mucous membrane and muscle wall. An eroded appearance of the muscle layers of the stomach is characteristic of rats given NaF for long periods in the diet (PLATE 7) as well as rats given KF or NaF for short periods in the drinking-water (PLATE 9). The amount of collagen, also, in the connective tissue between the muscle layers was found to be reduced in both groups of rats.

The most striking change consequent upon giving NaF for long periods is in the number and appearance of the parietal cells. The reduction in depth of the fundic mucosa is due to the disappearance of a high proportion of the parietal cells.

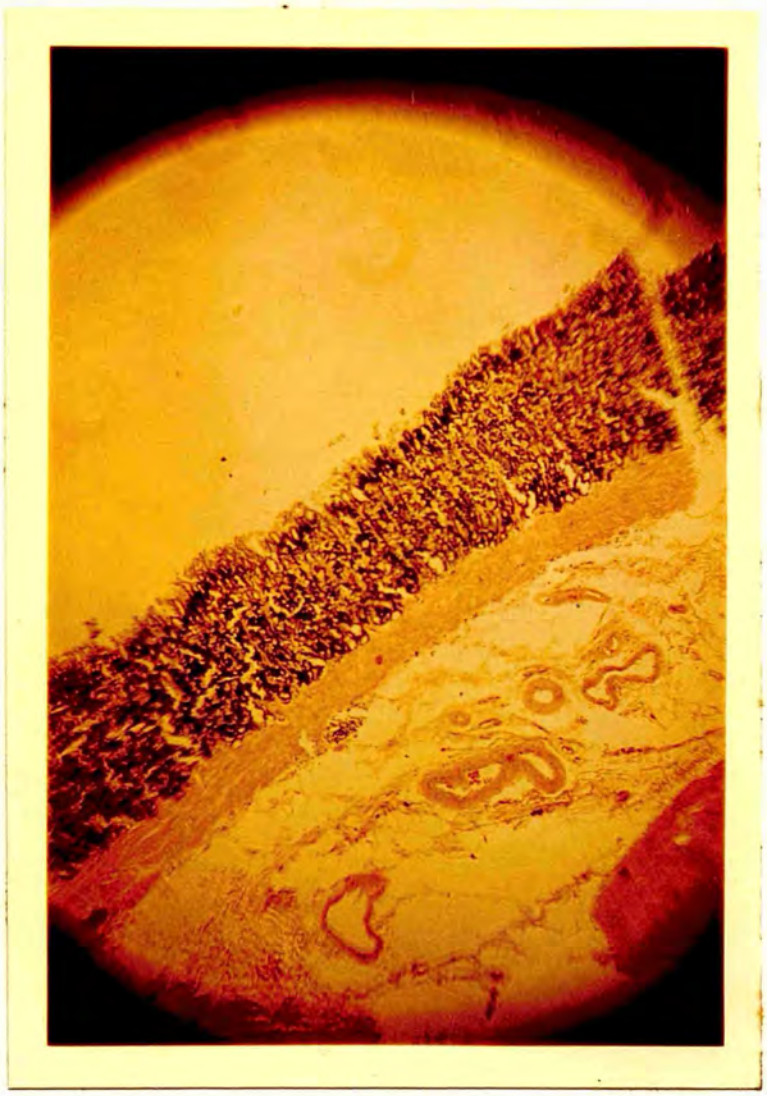


PLATE 8. Photomicrograph x 55 of fundus of stomach of rat 133 (given NaF in diet for 12 months). Acid eosin and toluidine blue: shows absence of band of parietal cells.

(COMPARE WITH PLATE 6)

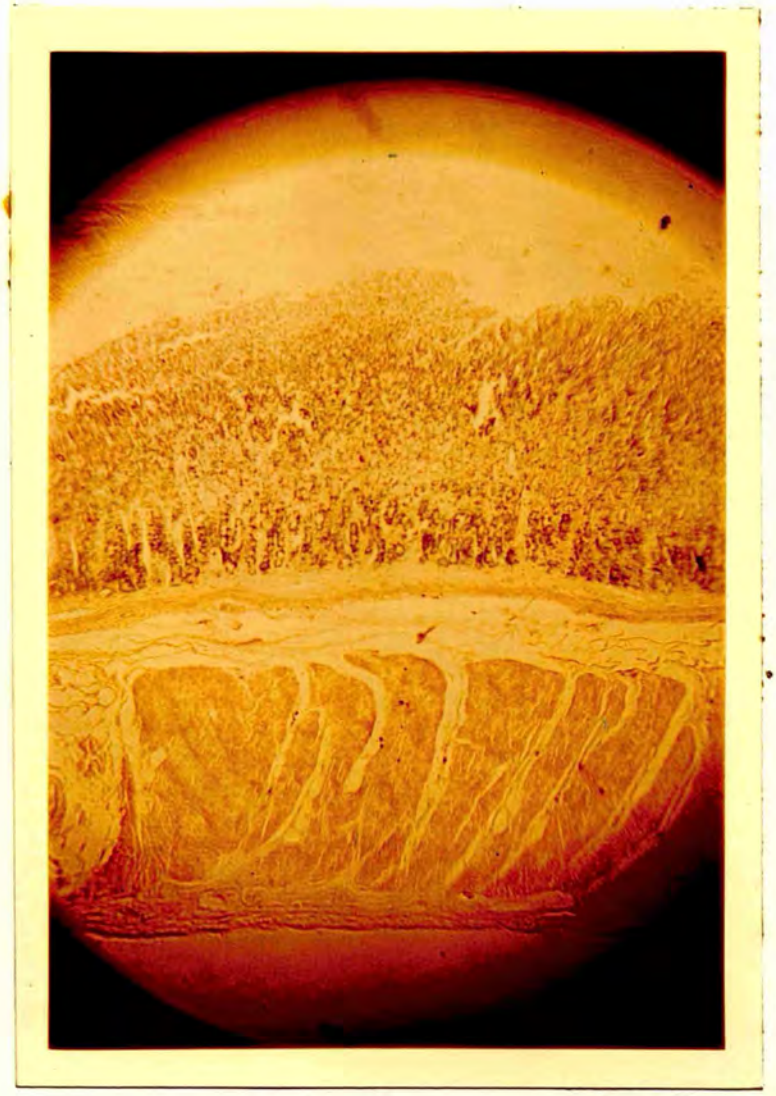


PLATE 9. Photomicrograph x 55 of fundus of stomach of rat 206 (given KF in the drinking-water for 6 days). Acid eosin and toluidine blue: shows feeble staining reactions, particularly of parietal cells, and "erosion" in layer of circular muscle.

(COMPARE WITH PLATE 6)

In the stomachs of fourteen rats (given the NaF-containing diet for six to twenty-four months) examined the parietal cell layer never exceeded one-quarter of the depth of the mucosa (PLATE 7) and often the layer did not exist at all (PLATE 8). High-power examination of the sections showed that sometimes the position of a parietal cell was marked by an isolated nucleus or a few fragments of cytoplasm. No specimen examined was devoid of parietal cells, for there were always some, in apparently good condition, in the lowest part of the mucosa between the peptic cells. Prolonged exposure to fluoride is evidently not necessary for damage to the parietal cells to take place. The rats given KF or NaF in the drinking-water showed disrupted and feebly-staining parietal cells (PLATE 9).

The response of the peptic cells to fluoride is in sharp contrast to that of the parietal cells, for they appear to be unaffected. Cell-counts were not done, but visual comparison with control sections indicated that the depth of mucosa occupied by peptic cells was no less in the fluoride-fed animals, whilst its proportion was, of course, greater. Pepsinogen granules tended to be more numerous in fluoride-fed than in control rats.

The fluoride ion has a stimulating effect on mucoid cells. Mucus was not found to any marked extent at the surface of the mucosa, but the secretory activity of the neck mucoid cells was tremendously increased (PLATE 10). This photograph shows also that mucoid cells, singly and in groups, were found below the

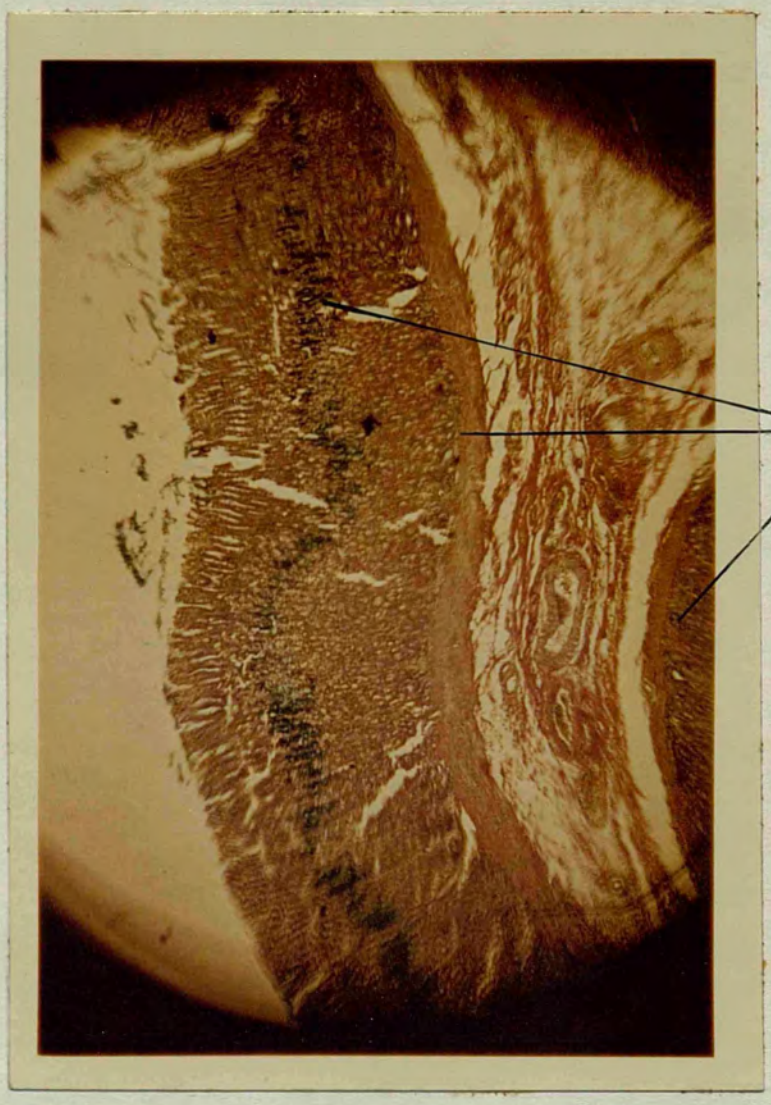


PLATE 10. Photomicrograph x 55 of fundus of stomach of rat 140 (given NaF in diet for 10 months). Alcian blue and chlorantine red: shows intense activity of neck mucoid cells (green) and appearance of mucoid cells close to muscularis mucosae.

(COMPARE WITH PLATE 5)

level of the neck region, between the peptic cells and adjacent to the muscularis mucosae.

A number of sections of the stomachs of fluoride-fed rats showed shedding of epithelium and sub-epithelial tissues into the lumen. PLATE 7 shows a typical specimen. The detached tissue includes epithelial cells, mucoid cells and a few parietal cells. All the cells lining the lumen are, however, of epithelial type, and it may be that when cells of any kind are torn away from the mucosa a layer of epithelial and mucoid cells grows over the surface, so that this process assists in the gradual attrition of the parietal cells.

Succinic Dehydrogenase. Within the short incubation periods specified, succinic dehydrogenase activity in the rat stomach is largely restricted to the parietal cells. The peptic cells show an inferior degree of activity, and the epithelium and muscle layers have none. No difference in enzyme activity could be detected between the parietal cells of the control rats and the remaining parietal cells of the rats to which NaF had been given in the diet for long periods. In rats 205 and 206, however, which received KF in the drinking-water, succinic dehydrogenase showed negligible activity in the fundus of the stomach even after thirty minutes incubation.

Histology and Histochemistry of the Fundus of the Stomach of the Dog.

General Structure. The organisation of the fundus of the dog's stomach differs, in several respects, from that of the rat. The individual fundic glands are readily distinguished. The epithelium consists of a single layer of columnar cells, each of which secretes mucus, and there are no other mucoid cells.

No differences in the number or staining reaction of parietal cells, in the distribution of mucoid cells or in the integrity of the muscle could be detected in six control dogs and six dogs given a fluoride-containing diet for twenty-two months.

Succinic Dehydrogenase. As in the rat, succinic dehydrogenase activity in the dog stomach is high in the parietal cells, low in the peptic cells and absent, within the incubation period used, from the other cells of the glands and muscle layers. Preparations from stomachs of dogs given fluoride for three months could not be distinguished from control sections.

Histology of the Fundus of the Stomach of the Rabbit.

General Structure. The fundus of the stomach of the rabbit is, like that of the rat, closely packed with cells. Mucoid cells are scattered between the luminal surface and the region of the neck of the glands. No evidence was seen of any changes in the gastric tissues of rabbits given fluoride: the stomach of even the animal given 0·05% NaF to drink for four years presented a normal appearance.

DISCUSSION

The Effect of Fluoride on the Stomach.

It is well-known that NaF depresses the functions of the gastric mucosa (Crane, Davies and Longmuir, 1946) and, in particular, inhibits the secretion of HCl. In acute experiments on cats Bowie, Darlow and Murray (1953) showed that bathing the mucosa of the stomach with 0·005 M NaF caused a 50-60% inhibition of the HCl secreted in response to histamine injection. These authors commented upon an apparent secretion of alkali in response to the NaF treatment, for, in some experiments, the acid content of the solution recovered from the stomach was less than that of the 0·005 M HCl solution instilled into it. The experiments of Bond and Hunt (1956) indicate that loss of hydrogen ions from HCl instilled into the cat stomach may come about by exchange of H^+ across the gastric mucosa for Na^+ in the blood. Fluoride was found to alter the permeability of the mucosa to sodium: the passage of radioactive Na^+ through the mucosa being increased forty-fold by the instillation of an NaF solution into the stomach.

Bowie et al. observed that the inhibition of HCl secretion by NaF was not accompanied by an inhibition of pepsin secretion. In one experiment reported, the peptic activity of the fluid recovered from the stomach was substantially greater after an instillation of fluoride solution. A secretion of mucus, too, was seen to follow the fluoride treatment in some cats.

It might be expected from a consideration of all these experiments that long periods of fluoride ingestion would result in parietal cells becoming damaged or destroyed, peptic cells being unharmed and the protective mucoid cells being stimulated to an increased activity. These assumptions have been borne out in the histological studies described above (pp. 78-81).

Sensitivity of the parietal cell to fluoride is confirmed in long-term and short-term experiments on rats. The stomachs of several rats given KF in the drinking-water for one to two weeks showed that all the cells were swollen and feebly-staining and that many parietal cells were ruptured. Succinic dehydrogenase activity was virtually absent from the parietal cells in these rats. After a year's consumption of a diet containing 0.05% NaF the depth of the fundic mucosa of the rat's stomach is considerably reduced. This observation reflects a severe diminution in the number of parietal cells. Sections show that a band representing one-half to one-third of the depth of the mucosa, which is occupied exclusively by parietal cells in control rats, is reduced to a narrow strip or is absent altogether in fluorotic rats. No sections from the stomachs of rats with chronic fluorosis examined were without some parietal cells, and, as previously noted, their succinic dehydrogenase activity compared closely with that of control specimens.

A decrease in the effective concentration of succinic dehydrogenase present in the gastric mucosa, either because of

inhibition of the enzyme or by destruction of some parietal cells (as in the rat with chronic fluorosis) would be a factor in the inhibition of HCl secretion by fluoride. The enzyme is, no doubt, required for the sequence of aerobic chemical changes necessary for the "active transport" of hydrogen ions which ultimately appear in acid gastric secretion (Conway and Brady, 1948).

Even after two years of the fluoride-containing diet peptic cells appear normal in the rat and their numbers show no reduction. In many specimens examined, the number of pepsinogen granules was in excess of that in control rats.

Both the activity and the number of mucoid cells are increased in rats subjected to prolonged fluoride feeding. The control rat shows only slight activity of the superficial mucoid cells and less, or no activity at all, of the mucoid cells in the region of the necks of the fundic glands. With prolonged fluoride feeding the activity of the superficial mucoid cells does not alter, but that of the "neck" cells is greatly increased. In addition, mucoid cells appear in positions where they are not seen in control rats, i.e. low down in the mucosa and even in the crypts adjacent to the muscularis mucosae.

Gastric haemorrhage, observed by many workers to follow fluoride feeding in several species, has not been found in these experiments. The rat stomach tissues examined were free from extravasated blood. The luminal surface of the mucosa, however,

was frequently torn and irregular, and showed evidence of groups of detached cells embedded in the surface mucus.

In the rat stomach, as in the rat kidney, fluoride lowers the amount of stainable collagen. Another response to fluoride ingestion which, also, may be a factor in the high excretion rate of urea that accompanies fluoride feeding is erosion and hence loss of tissue in the muscle layers. In a section, the effect produced is of patchy staining showing up small areas where the muscle cytoplasm is absent. This effect has been seen in both long-term and short-term feeding with fluoride.

Prolonged administration of fluoride to dogs and rabbits caused no detectable change in the constitution or staining reactions of the fundic mucosa in either species. This particular species difference corresponds with other general observations on fluoride toxicity in the animal types studied.

THE ACCUMULATION OF CITRIC ACID IN THE KIDNEY FOLLOWING THE ADMINISTRATION OF FLUORIDE

Citric acid has been shown to accumulate in certain tissues following the administration in vivo of fluoroacetate, which becomes converted to monofluorocitrate and inhibits the enzyme aconitase. Figures given by Buffa and Peters (1949) show that, in the rat, sodium fluoroacetate exerts its greatest effect on the kidney: the citrate content of this organ rising from an average value of 14 μg/g. wet tissue in the control animal to an average value of 1036 μg/g. wet tissue in the rat poisoned with sodium fluoroacetate injected at a dose-level of 5 mg/kg. body weight.

Although there is no direct evidence for the fluoride ion taking part in a lethal synthesis resembling that of fluoroacetate, the possibility is worthy of consideration since there are several properties common to both poisons. Fluoride, for instance, exerts a toxic effect on the living animal at much lower concentrations than are required to inhibit an in vitro preparation.

With the intention of testing this supposition, a few preliminary experiments were carried out which involved the administration of sodium fluoride solution by one or two intravenous injections, followed by estimation of citric acid in the kidney. The animal selected for the experiments was the rabbit. The concentration of citric acid in its kidney under

control conditions has been reported by Dickens (1941) to be 60 μg/g. In the light of long-term feeding experiments carried out subsequently, the choice of animal proved to be unfortunate, for the rabbit was shown to tolerate fluoride to an extraordinary degree.

A suitable dose-level for injection was obtained from the work of Leake (1928). This author stated that the lethal dose of NaF injected intravenously into rabbits was 87·5 mg/Kg., and that death was apparently due to cardiac and respiratory failure, preceded, usually, by clonic convulsions.

Procedure. Adult rabbits were used. NaF was injected as a 3·3% solution into the lateral vein of the ear. One rabbit was kept until it died, 55 minutes after the injection, and another died after 10 minutes. The remainder were killed by a blow when, about 30 minutes from the time of the injection, symptoms of distress developed. Muscular weakness, retraction of the head and salivation were noted in every case, and were usually accompanied by defaecation and urination. In two cases the righting reflexes were severely affected. Convulsions, however, were not general, being seen in one animal only.

Three rabbits were selected as controls, and these received no injections.

The kidneys were taken from the body immediately upon death, and their capsules removed. For the estimation one kidney was cut into two parts of approximately equal size and composition, and one part was weighed and ground in a mortar.

Method for Estimation of Citric Acid. The procedure followed was that described by Buffa and Peters (1949), who used the method of Pucher, Sherman and Vickery (1936) with modifications of their own. The estimation, which is highly specific, depends on the oxidation of citric acid in the presence of bromine to form pentabromacetone. The latter is then extracted with petroleum ether and made to react with sulphide, thus producing a yellow colour.

The standard used by Buffa and Peters (1949) for comparison was sodium citrate equivalent to 196 µg. anhydrous citric acid, and they stated that the colour developed from this gave a reading of 0·60 ± 0·02 on the Hilger Spekker photometer with a 1 cm. micro-cell and an Ilford 601 (violet) filter. In the experiments to be reported, a similar standard was used, and a reading in close agreement with that of Buffa and Peters was obtained.

TABLE IV

The citric acid content of the kidneys in control rabbits and in rabbits injected with sodium fluoride solution.

Rabbit No.		Weight kg.	Citric acid concentration µg/g. kidney	Remarks
1041 ♀	control	0·90	78	
1043 ♂	control	0·80	70	
1046 ♀	control	1·90	52	
1042 ♀	test	1·66	208	died after 55 min.
1044 ♂	test	1·70	103	
1045 ♂	test	1·90	104	
1047 ♂	test	2·20	110	
1048 ♀	test	2·94	138	
1049 ♀	test	1·90	83	died in mild convulsions after 10 minutes.

Discussion. Although the results given above are in no wise comparable with the results of Buffa and Peters (1949), using fluoroacetate as the toxic agent and the rat as an experimental animal, a rise in citric acid content of kidney following fluoride injection has nevertheless been indicated. It is intended to pursue this investigation using rats. A generally-preferred method for the estimation of citric acid, which employs vanadate instead of permanganate for oxidation, is now available (Taylor, 1953).

Several observations made on the effects of fluoroacetate poisoning are equally true of the effects of fluoride poisoning. Sodium fluoroacetate, when administered to the dog, has been shown (Farah, Graham and Koda, 1953; Farah, Koda and Frazer, 1955) to reduce renal blood flow, the clearances of PAH, inulin and creatinine, and the transport maxima for PAH and glucose. Fluoride poisoning reduces PAH and inulin clearances in the rat, as has been shown in the foregoing account of experiments.

In his review, Peters (1957) discusses the relationship between ketonaemia produced by the administration of fluoroacetate and disturbance of sugar metabolism. Elliott and Phillips (1954) and also Cole, Engel and Hewson (1954) showed that among the effects of fluoroacetate administration was a temporary increase in the blood sugar level. Blood sugar determinations were not made on the rabbits in the experiments reported here. It may be, however, that the transient glycosuria exhibited by the rats given fluorides in their drinking-water may reflect a hyperglycaemia instead of, or in addition to, a reduced Tm glucose. Further experiments are required to clarify this point.

A characteristic of fluoroacetate, shared by fluoride, is the great difference in the degree of tolerance shown to the poison by different species. Peters (1957) attributes the lack of effect seen in some animals, e.g. the toad, to the absence of some component of the activating system for forming

fluorocitric acid. The rabbit responding to 0·2 - 0·5 mg. fluoroacetate/kg., is some ten to twenty times more sensitive than the rat.

There is evidence that the CF bond, long thought to be completely stable in the animal body, can be split. Miller and Phillips (1955) found extra fluorine in the bones of growing rats after feeding for some weeks on a diet including 20 p.p.m. fluoroacetate, and Ott, Piller and Schmidt (1956) found that fluorovalerate increased the fluoride content of teeth. If the CF bond can indeed be broken, the possibility of its synthesis should be considered.

Protection against fluoroacetate or fluorocitrate in vivo is given only rarely by such small molecules as acetate or alcohol. Of the substances tested, the most effective have been glycerol monoacetate (monoacetin), reported by Chenoweth, Kandel, Johnson and Bennett (1951), and acetamide, used by Gitter, Blank and Bergmann (1953). It is presumed that these substances, being both lipid- and water-soluble, can penetrate into the cell and liberate acetyl groups at active centres, e.g. enzyme sites in the mitochondria. The use of monoacetin or acetamide provides a possible method for comparing poisoning by fluoroacetate and by fluoride in future experiments. The results of the experiments described here, i.e. the dramatic effects of fluoride on the parietal cells of the stomach and the renal epithelium, are consistent with mitochondria in both tissues suffering severe damage from the fluoride ion.

SUMMARY.

The purpose of the investigation was to study the effects on the stomachs and kidneys of rats, dogs and rabbits of long-term and short-term administration of fluoride.

One series of experiments was designed to determine whether the epithelium of the kidney tubules can be made "permeable" to Na^+ by exposure to fluoride, as has been shown to occur in the gastric mucosa (Bond & Hunt, 1956). Rats which had been maintained on a sodium-free diet were given fluoride (as KF, in a 0·15% solution) in the drinking-water. Some rats then refused to take food, and these animals responded to fluoride ingestion by loss of sodium from the body (the Na^+ excretion varied and sometimes reached 10-20 times the value of the level recorded during the control period for the rat), severe albuminuria and intermittent glycosuria. Renal clearances of inulin and PAH were reduced to a fraction of the values for control rats. Succinic dehydrogenase activity in kidney and gastric mucosa was negligible. In the stomach the muscle layers and the secretory cells (especially the parietal cells) showed histological evidence of damage. Lesions resembling those seen in chronic fluorosis appeared in the kidney, and alkaline phosphatase activity could not be demonstrated, probably because the enzyme sites had been torn away. Disappearance of collagen from the tissues studied was characteristic.

A close correlation was observed between the degree of severity of the response to fluoride ingestion and the extent to which the rat's appetite had been depressed. In order to establish that the effects recorded were really due to F^- and not to toxicity of the accompanying K^+, NaF solution (0·1%) or KCl solution (0·2%) was put into the drinking-water of other rats. Those receiving NaF responded as had the rats ingesting KF: the rats to which KCl was given remained indistinguishable from the control rats.

A striking feature of short-term administration of fluorides was a rise in urea output, evident within a week of the beginning of fluoride administration and increasing during the 3-4 weeks of the experiment.

The nature of the kidney lesions appearing in rats after prolonged fluoride feeding (i.e. the inclusion of 0·05% NaF in the food supplied) has been investigated more fully than in 1952 (Bond & Murray). Renal function tests indicated that for the first 6-8 months of fluoride feeding there is an appreciable individual variation in the extent to which rats are able to tolerate fluoride. Rats that had been consuming the fluoride-containing diet for a year or more, however, consistently showed very low clearances of inulin and PAH.

Histochemical and histological studies of the kidneys of rats with chronic fluorosis showed the presence of areas of compressed and degenerating tubules, wedge-shaped in a radial

section, alternating with areas of normal renal parenchyma. The "wedges" were found to be devoid of succinic dehydrogenase and alkaline phosphatase activities and to contain casts of three kinds, viz: a substance with the "amyloid" staining reaction, a substance with the "hyaline" staining reaction and particles of cell debris.

Observations made on rats in various stages of fluorosis are consistent with the suggestion that the glomerulus is the primary point of attack on the kidney by fluoride. The connective tissue lining the capsule and covering the glomerular capillaries proliferates (in a proportion of Malpighian corpuscles increasing with increasing time of fluoride consumption) until the capillaries are occluded. A mass of "amyloid" material replaces the capillaries, and it later passes into the lumen of the tubule. This process must interfere with the blood supply to the tubules, and could cause breakdown of the epithelial cells. The presence of "amyloid" material, "hyaline" material from ruptured cells and other cell fragments in lumina of affected nephrons can be explained on this basis.

Succinic dehydrogenase activity in the functional renal tissue remaining was found to be fully as high as in control rats' kidneys. Alkaline phosphatase activity was even higher than in control rat kidneys and was not confined to the proximal convoluted tubules but had invaded the medulla.

The fundus of the rat stomach was found to be characteristically thin in chronic fluorosis, due largely to the disappearance of a high proportion of the parietal cells. Peptic cells seemed to be unaffected. This finding complements an observation of Bowie, Darlow & Murray (1953) who noticed that instillation of NaF solution into the cat's stomach abolished HCl secretion in response to histamine injection but did not depress pepsin secretion. Mucous cells, never very obvious in the stomachs of control rats, became very active and much more numerous during chronic fluorosis. The mucous cells in the necks of the glands were particularly evident, and mucous cells appeared low down in the mucosa. The luminal surface of the mucosa was usually torn and irregular, and in severe fluorosis the circular muscle layer presented an "eroded" appearance.

Examination of the stomachs and kidneys of dogs given an NaF-containing diet for up to two years revealed no lesions. Except for patches of low alkaline phosphatase activity in some kidneys, there was no abnormal distribution or unusual degree of activity of the enzymes alkaline phosphatase and succinic dehydrogenase.

Rabbits displayed a remarkable ability to tolerate fluoride. Inclusion of 0.05% NaF in the drinking-water for periods of up to four years produced no changes in the appearance of kidneys or stomach.

The results of some acute experiments included show that injection of NaF solution into rabbits raised the citric acid content of the kidneys. The possibility that this finding is due to the synthesis of fluoro-fatty acid and represents a biochemical lesion has been considered.

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KIDNEY FUNCTION AND STRUCTURE IN CHRONIC FLUOROSIS.

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CHRONIC fluorosis is known to occur in man as an endemic disease and also as an occupational disease in persons engaged in industrial undertakings where fluorine compounds, such as fluorspar (CaF_2) or cryolite ($NaF.AlF_3$) are used as fluxes, or where materials are used which incidentally contain fluorine, as, for example, rock phosphates, certain phosphatic iron ores and even some coals. Persons residing in the vicinity of industrial undertakings which emit fluorine-containing gases, fumes or dusts are subjected to a fluorine hazard (Murray and Wilson, 1946).

Fluorine intoxication of a severe degree after prolonged exposure is readily diagnosed in man and animals by reason of the skeletal changes which result, but by the time these changes are demonstrable in man by X-ray studies, the functional disability may be considerable. It is proposed here to consider only the effects on the kidneys. In a survey of cryolite workers, Roholm (1937) concluded that it was doubtful whether renal lesions were typical of fluorine intoxication. He made post-mortem examinations of the kidneys in two workers ; in one case he found proliferation of interstitial tissue, in the other case he found some stasis.

In studies on endemic fluorosis in man due to high fluorine content of the drinking water, attention has always been centred on the teeth and bone changes, but Linsman and McMurray (1943) reported a case in which they considered that renal impairment with retention of fluorine had been partly responsible for the osteosclerosis. Shortt, McRobert, Barnard and Nayar (1937) studied ten cases of endemic fluorosis and found in the majority impaired kidney function, but this might well have been due in part to other causes. The studies of McClure, Mitchell, Hamilton and Kinser (1945) have shown that in man a fluorine intake of 6 mg. per day can be eliminated completely by the kidneys, but that above this level there is retention ; from then on the effects are cumulative. The use of fluorine in industrial processes has rocketed in recent years, and several outbreaks of animal fluorosis have been reported in Great Britain. Such outbreaks might also involve a hazard to the human population (Murray and Wilson, 1946).

Experimental animals, when fed small amounts of NaF, also exhibit bone changes, digestive disturbances, excessive water intake and polyuria (Roholm, 1937 ; Kick *et al.*, 1935). Post-mortem examination shows that the soft tissues also suffer changes.

An investigation has been carried out on the effects of the intake of small amounts of sodium fluoride on kidney structure and function in rats, with a

view to the possibility of establishing some means of testing for the toxic effects of fluorine in human beings, and of demonstrating the existence of a fluorine hazard before such severe intoxication has resulted as to cause disability and obvious skeletal lesions. The effects on kidney function are immediate.

METHODS.

Rats of an inbred hooded strain, kept at a controlled temperature of 68–70° F., were fed on a diet (Coward, 1938) consisting of maize meal 2600 g., wholemeal wheat 800 g., casein 360 g., dried yeast 200 g., NaCl 20 g., $CaCO_3$ 20 g., KI 1 g. *Addenda :* wheat germ 2 g., milk powder 5 g., cod liver oil 2 ml., green vegetables 2 g., liver or meat 2 g. The animals were put on the fluoride-containing diet at different times after weaning and received, according to age, 8 to 15 g. of the diet per day. The items of the addenda were each given once a week. In the control group the diet was mixed with tap-water and in the experimental group with 0·05 or 0·1 per cent sodium fluoride solution, using 1 ml. of either liquid to each gramme of diet. The amount of fluoride ingested, therefore, was from 4 to 15 mg./day. Different species of animals vary in their susceptibility to the effects of fluorine, and rats appear to be less readily affected than man, as judged by the effects on teeth. The animals were weighed weekly. Except where otherwise stated, all animals received tap-water *ad lib.* The volume drunk was measured daily, and at certain times the animals were transferred to metabolism cages so that the urine could be collected, measured and analysed. Blood samples were taken from the tail vein. Blood-sugar determinations were made on 0·05 ml., using a method described by King (1951). An endeavour was made to carry out renal clearances with inulin and *p*-amino hippuric acid, but the results on control rats were not consistent ; hence these results were inconclusive.

RESULTS.

The results reported below have been gathered over some years, and are representative of observations made on a large number of control and experimental rats.

1. *General observations.*

The intake of fluorine was sufficient to produce a state of chronic fluorosis. The animals developed definite symptoms, including the characteristic bleaching of the dental enamel with gross hypoplasia of the dental tissues and also bone changes, all of which have been fully described by previous authors (Roholm, 1937).

Investigations on fluorosis have been carried out in this laboratory for many years, and only very exceptionally have rats died during the fluoride administration at the relatively low level used, and then only after a year or more of exposure. The experimental rats showed retarded and diminished growth (see Fig. 1), which could in part have been due to the fact that at times, owing to the abnormal tooth development, the fluoride rats consumed less food than the controls. Most observers have attributed the diminished growth and loss of weight in adult animals to low food intake and to poor digestion and absorption, but our recent experiments have shown that in adult rats there is a much greater urinary excre-

tion of nitrogen. After several months on the diet there was a general loss of condition, and in some animals a staring coat. This could not have been due to deficiencies in the diet, but might have been due to a failure to absorb a sufficiency of essential nutrients (Spira, 1950). The fluoride rats ultimately showed greatly diminished body fat.

It was particularly noticeable that the fluoride-fed rats exhibited a much greater water intake as seen in Fig. 2(*a*) and (*b*) ; the effect came on immediately (see Fig. 2(*b*)), but was greater the longer the exposure to fluoride. Fig. 2(*c*) depicts the effect of administration of a natural water of high fluorine content (3 to 5 parts per million) which the animals had drunk, and which after con-

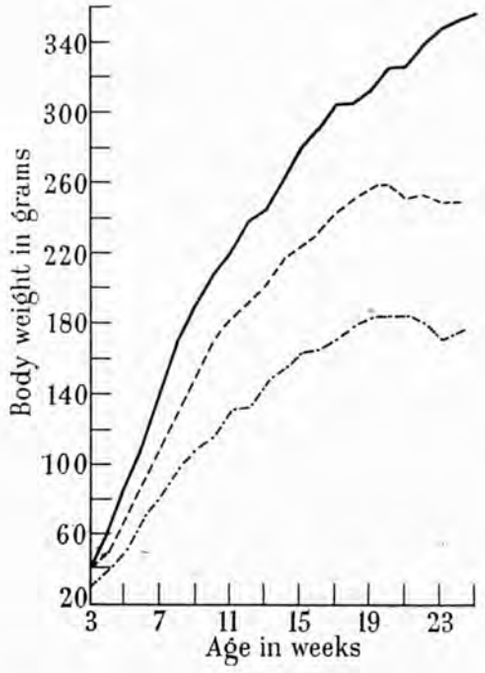


FIG. 1.—Growth rates of control and fluoride-fed male rats.

Control rats : ————
Diet mixed with 0·05 per cent NaF : – – – – – –
Diet mixed with 0·1 per cent NaF : ·—·—·

centration to ten times its original strength and then equivalent to 0·008 per cent NaF was used for mixing the diet. In none of these experiments could the increased water intake and polyuria have been due merely to an increased salt intake, because a replacement of the NaF added to the diet by NaCl had no effect on the water intake. The effect was therefore judged to be due to the specific action of the fluoride ion.

The fluoride ion is well known to inhibit several enzyme systems (Borei, 1945), and noticeably those which are activated by magnesium. In some respects NaF acts, in the body as a whole, like KCN ; animals injected with sub-lethal or lethal doses of NaF show marked cyanosis. This fact taken in conjunction with the increased water intake and polyuria led to a study of kidney function in the experimental rats.

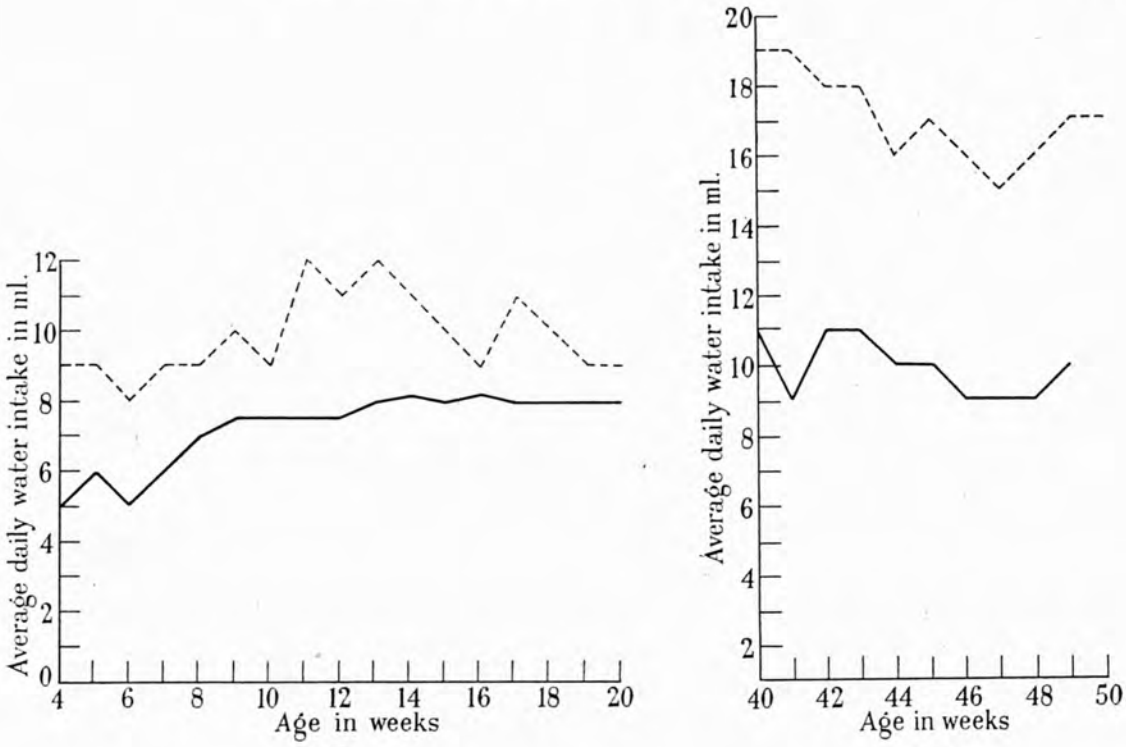
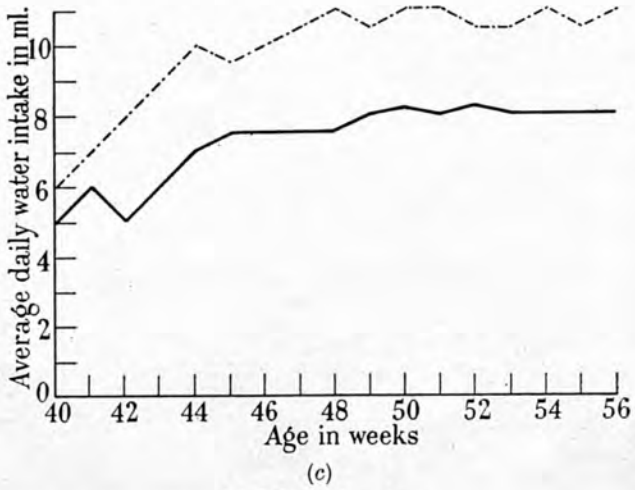


FIG. 2.—(*a*) Water intakes.

Water intake of control rats : ————
Water intake of fluoride-fed rats : – – – – – –

(*b*) Water intakes.

Water intake of control rats : ————
Water intake of fluoride-fed rats after 40 weeks on diet : – – – – – –



(*c*) Water intakes.

Water intake of control rats : ————
Water intake of rats drinking a natural water high in fluorine (3 to 5 p.p.m.) and receiving a diet mixed with this water concentrated 10 times and equivalent to 0·008 per cent NaF : ·— ·— ·

Observations recorded when animals had been on diet 40 weeks.

At autopsy the kidneys of rats fed sodium fluoride for 10 months or more showed definite macroscopic changes; they were dark, shrunken and nodulated. A description of the histological findings in the kidneys is given below.

2. *Kidney function.*

Two groups of control and fluoride-fed rats kept in special metabolism cages were investigated. The rats of one group were 20 months old and had received the NaF for 10 months; the others were 6 months old and had been on the fluoride diet for 5 weeks.

The volumes of urine excreted by comparable groups of control and fluoride-fed rats can be compared from the figures given in Table II, which shows that there was a polyuria in the fluoride-fed rats. The average hourly volume of urine was 1 ml. for the fluorine rats and 0·25 ml. for the controls. The urine of the fluoride-fed rats was very pale and dilute and had a low specific gravity; it was free of albumin, blood pigments and casts. When food was withheld overnight, the urines of the fluoride-fed rats gave a positive test (Cole, 1944) for sugar at blood-sugar levels of between 70 and 80 mg./100 ml.

Both control and experimental rats were given a glucose tolerance test. Each rat was given 0·2 g. glucose/100 g. body weight injected intraperitoneally as a 5 per cent w/v solution in physiological saline. The controls showed a definite glycosuria, although the blood-sugar levels were never greater than 125 mg./100 ml. These results are given in Table I.

TABLE I.—*Sugar in Blood and Urine of Control and Treated Rats.*

	Control rats. Age 10 months.			*NaF diet rats.* Age 20 months. (NaF given for 10 months.)		
Fasting blood sugar (mg./100 ml.)	70	80	67	80	66	70
Sugar in "fasting urine"	0	0	0	+	+ ?	+
Blood sugar (mg./100 ml.) 1 hour after intraperitoneal injection of glucose	80	105	76	125	103	85
Sugar in urine for 3-hour period after glucose injection	0	0	0	+	++	+++

It would therefore appear that the mild glycosuria of the fluoride rats was due to a lowered threshold rather than to diminished tolerance.

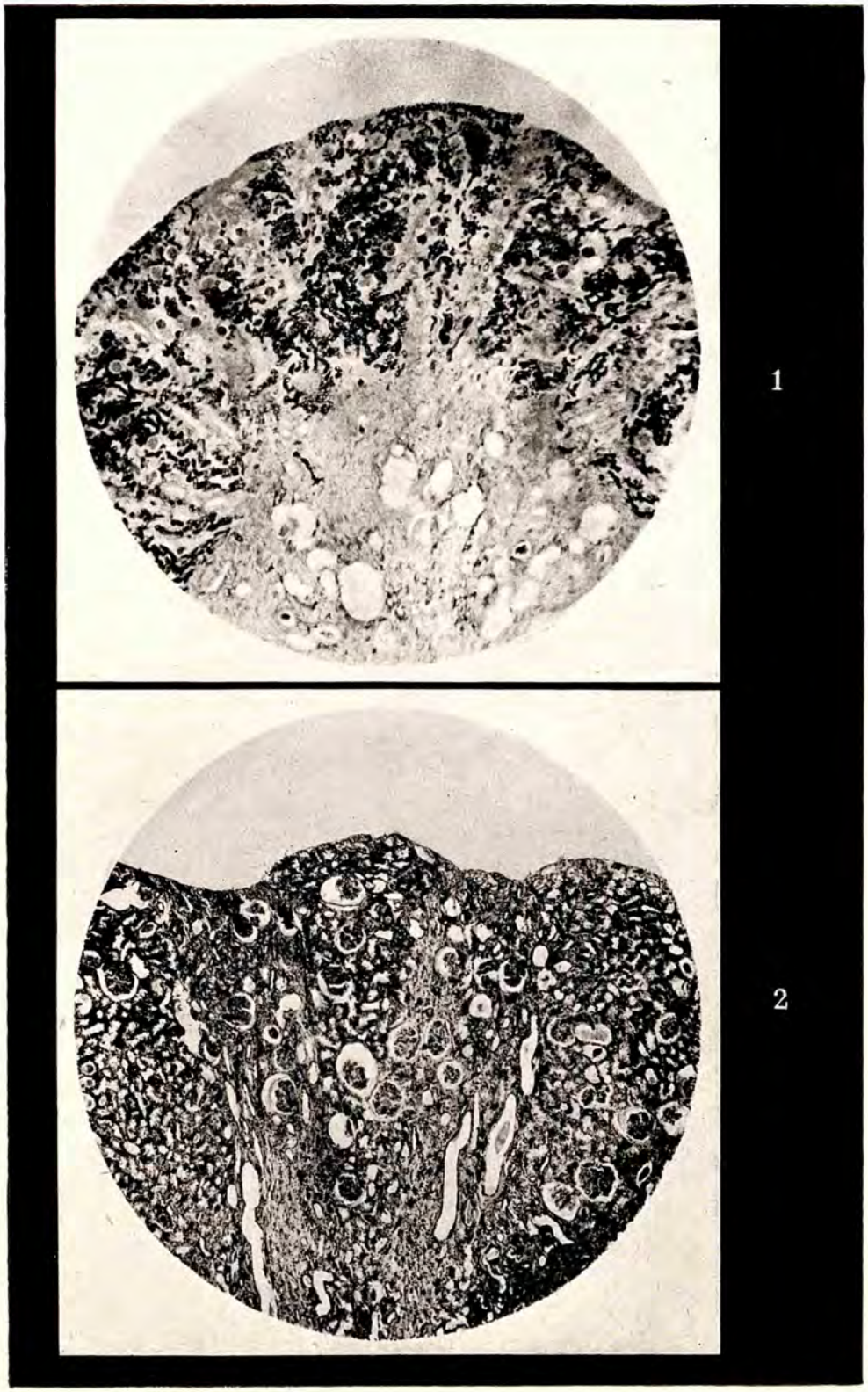
DESCRIPTION OF PLATES.

PLATE 1.—Photomicrograph × 15 of section of kidney of NaF-fed rat F23. Gomori's method for alkaline phosphatase; shows bands where the normal kidney tissue has been replaced by fibrotic tissue in which the phosphatase is absent.

PLATE 2.—Photomicrograph × 38 of section of kidney of NaF-fed rat F20. Haematoxylin and eosin; shows the fibrotic lesions giving rise to the mulberry-like appearance of the surface.

PLATE 3.—Photomicrograph × 190 of section of kidney of NaF-fed rat F20. Haematoxylin and eosin: shows at A arteriole with thickened walls. Changes in Malpighian corpuscles and convoluted tubules can also be seen.

PLATE 4.—Photomicrograph × 315 of section of kidney of NaF-fed rat F22. Silver impregnation for reticulin fibres; shows thickening and increase of these fibres; see B.



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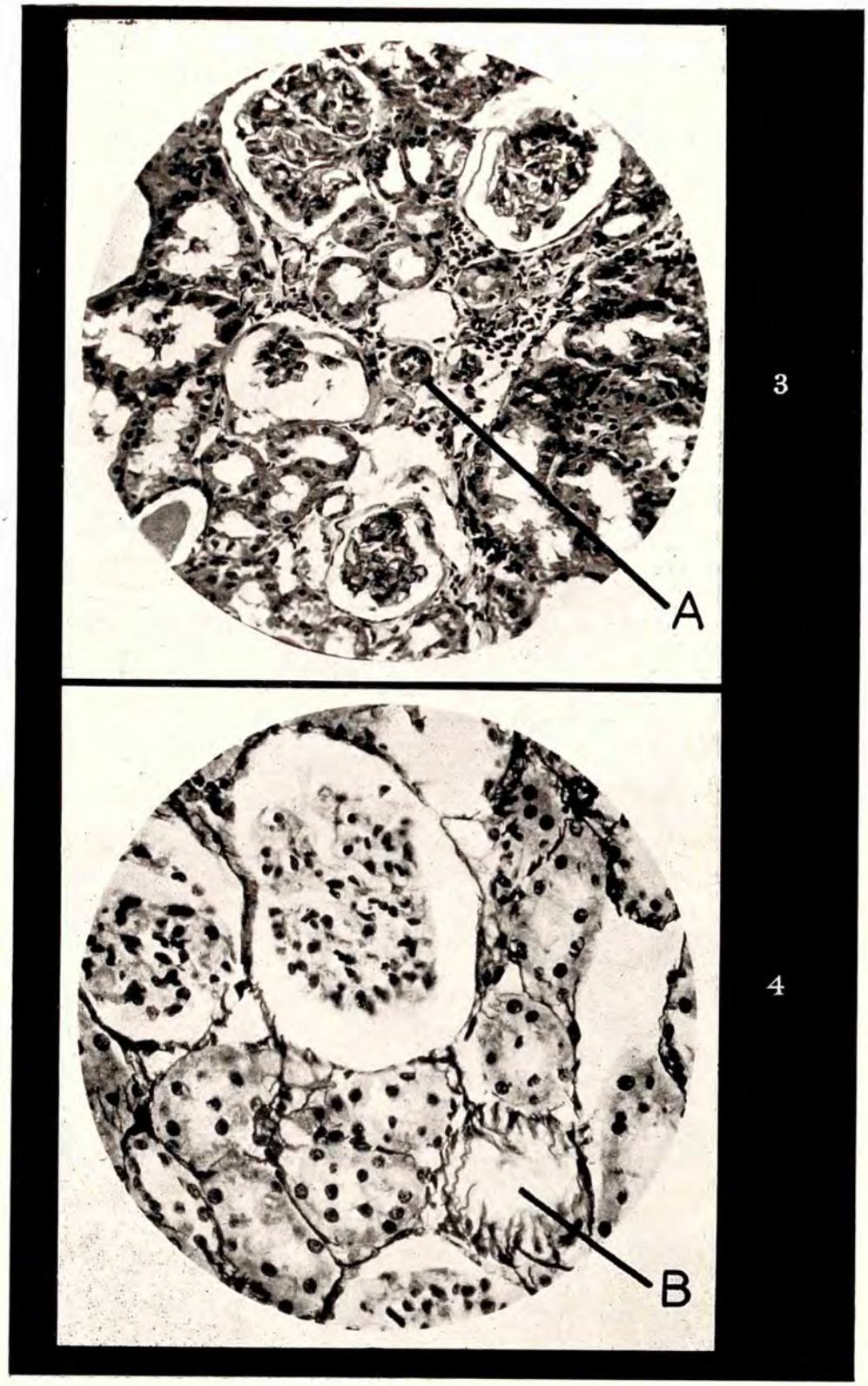
Bond and Murray.



Bond and Murray.

Nitrogen metabolism.—Emaciation is a marked feature of all farm animals suffering from chronic fluorosis, but this has usually been regarded as due to the difficulty in grazing, in eating or in rumination resulting from lameness in cattle or jaw deformity or abnormality of the dentition in sheep. The values we have obtained for urinary nitrogen excretion show that the emaciation could in part be due to an increased metabolism. Table II gives the results of urine analyses on rats kept in metabolism cages without food for 18 hours.

TABLE II.—*Characters of Urine in Control and Treated Rats (18-hour collections).*

	Control rats. Age 10 months.			*NaF diet rats.* Age 20 months. (NaF given for 10 months.)		
Volume in ml.	5	5	3	18	18·5	17·5
Mean hourly volume in ml.		(0·25)			(1·0)	
pH	7·5	7·5	7·5	8	8	8
Specific gravity	1·026	1·026	1·026	1·011	1·010	1·014
Total nitrogen (mg.)	56	65	44	134	120	132
Ammonia nitrogen (mg.)	4	4	2	7	7	5
Urea nitrogen (mg.)	47	51	33	116	102	111
Undetermined nitrogen (mg.)	5	10	9	11	11	16

3. *Alkaline phosphatase activity.*

Since it appeared that tubular function rather than that of the glomeruli was affected and because tubular function depends largely on enzymic activity, histological demonstration and chemical estimations of the level of alkaline phosphatase were made on both control and fluoride kidneys. In the latter the distribution of the alkaline phosphatase was very irregular (Plate 1); it was practically absent from the fibrotic pyramidal areas described in the histological report given below. In the other regions the phosphatase reaction was more intense than in sections of normal kidneys which had been similarly treated. Estimations of alkaline phosphatase in homogenates of the cortex of control and fluoride kidneys showed no consistent significant difference. This was at first surprising, but could probably be accounted for by the greater concentration in those regions of the fluoride kidneys where it was still present. This might be a response to the considerable loss of functional tubular tissue in the fluoride kidney. It may be mentioned here that in sections of the intestinal mucosa of the fluoride-fed rats we also found a patchy distribution, but in some places greater alkaline phosphatase activity, incubation for 5 min. giving as intense a test as 20 min. incubation of sections of normal intestine.

4. *Histological examination of kidneys.*

As a routine procedure whenever a fluoride rat was killed a control rat of the same age was sacrificed, and after post-mortem examination certain tissues and organs, including the kidneys, were taken for histological preparations. At least twelve fluoride and twenty control rats' kidneys have been given a general histological examination.

The sections described below were representative; the fibrotic changes seen in the fluoride kidneys varied in degree with length of exposure and from animal to animal, but in none of the control rats were these typical changes encountered.

It is our experience that in rats maintained on a good and varied diet as used in these experiments nephritis is not commonly seen, as it is in stock laboratory rabbits.

The following is a report on the histological examination of kidneys, by the late Dr. S. Roodhouse Gloyne:

Rats F20, F21 and F22 had been put on the NaF diet together at the age of 6 weeks. Rat F20 died, probably from another cause, aged 24 weeks. Its general condition was then poor, exhibiting a staring coat and marked dental fluorosis. The kidneys were dark, shrunken and nodulated. Rats F21 and F22, with rat A14, a control animal also aged 24 weeks, were sacrificed at the same time. Rat F23 was killed after receiving the NaF diet for 48 weeks. In appearance, including that of the kidneys, it resembled F20.

The kidneys of rat F23 were fixed in acetone for demonstration of alkaline phosphatase (Gomori, 1946) and those of the other rats in formalin saline. All sections were 5μ in thickness. They were stained either with haematoxylin and eosin, or iron-haematoxylin and van Gieson's stain, or Mallory's connective-tissue stain, or by Foot's silver-impregnation technique for reticulin fibres.

Microscopical examination.—In all the kidneys examined the lesions were irregularly distributed. In the affected portions of the kidney tissue which corresponded with the areas devoid of alkaline phosphatase (see Plate 1) the following appearances were noted:

Rat F20.—Malpighian corpuscles: The basement membrane of Bowman's capsule was thickened and the capsular epithelium flattened and inconspicuous. The normal cleft-like or sickle-shaped subcapsular space was dilated. The glomerulus was contracted and markedly lobulated, the capillaries were thickened and in many instances difficult to define, but the nuclei of the visceral glomerular epithelium were generally demonstrable and erythrocytes were still present in considerable number (Plates 2 and 3). No leucocytic reaction was noted, and there were no haemorrhages.

Convoluted tubules: The lumina of the convoluted tubules were often small. The cytoplasm of the cells lining these tubules was granular and here and there contained small vacuoles. The outlines of the cell envelopes were frequently difficult to define, the free borders were ragged and brush borders obliterated. Nuclei had disappeared in places, and there were tubules in which in cross-section all the cells seen were without nuclei (Plates 2 and 3). A few cystic dilatations of tubules occurred. The collecting tubules were also occasionally dilated and contained cellular debris.

Arterioles: In the neighbourhood of the glomeruli and along the collecting tubules arterioles exhibited considerable thickening of their walls as seen in Plate 3.

Interstitial fibrosis: Radiating outwards from the internal medullary zone to the cortex were elongated fibrotic strands of tissue (Plates 1 and 2), sometimes pyramidal in shape with their bases towards the cortex. These corresponded in position to the medullary rays of the normal organ, and they consisted of collecting tubules and arterioles irregularly compressed by collagen and reticulin fibres. Often all that was left of a tubule was an interrupted double row of nuclei of the epithelial lining. In other places the compression of the collagen fibres had resulted in cystic dilatations. Generally speaking the constricted arterioles and venules were found along the margins of the pyramidal strands.

The reticulin fibres were substantially thicker than those in a section of the kidney of the normal animal, and showed proliferation in some parts of the kidney. The region of the collecting tubules was most affected and there were dense ramifications of argyrophil fibres (Plate 4). Adjoining sclerosed glomeruli or atrophied tubules a thickened fibrous network was usually apparent.

Rat F21.—Microscopically the lesions noted resembled those seen in rat F20 but were on the whole rather more advanced. For instance, the cells of the convoluted tubules contained more fat droplets and had become more vacuolated and disorganised until in some areas nothing but a skeleton of cell envelopes lined the tubules. Here and there were collections of darkly-stained rounded or polygonal nuclei without cytoplasm surrounding the glomeruli and convoluted tubules. These appeared to be the remains of the epithelial lining of neighbouring convoluted tubules which had become collapsed, disorganised and obliterated. The collecting tubules tended to become more cystic in this animal. The pyramidal radiations of collagenous tissue were present as in rat F20.

Rat F22.—The kidney sections from this animal presented a picture which was in general similar to that of rat F20, but there was a greater proportion of normal tissue and the areas of the lesions were smaller.

The noteworthy features of the histological findings were :

(1) The exudation of plasma into the subcapsular space of the Malpighian corpuscles and the contraction of the glomeruli.

(2) Degeneration and collagenous thickening of arteriolar walls.

(3) Progressive degeneration and disorganisation of the cell lining of the convoluted tubules.

(4) Cystic dilatation of tubules.

(5) The formation of pyramidal strands of collagen fibres containing constricted blood-vessels and compressed collecting tubules.

(6) Coarsening of reticulin fibres.

These lesions suggest a chronic intoxication leading to a vascular, glomerular and tubular degeneration and finally an interstitial fibrosis. There is no evidence of an infective inflammatory lesion.

SUMMARY.

Rats given small amounts of NaF in the diet exhibited, in addition to the well-known skeletal and dental lesions, marked polydipsia and polyuria.

The urine of these rats was of low specific gravity, but free of albumin and casts.

There was slight glycosuria after 18 hours' fasting at blood-sugar levels of 70–80 mg./100 ml.

Administration of glucose intraperitoneally produced definite glycosuria at blood-sugar levels less than 125 mg./100 ml. Thus there was a low kidney threshold.

At autopsy the kidneys were dark, shrunken and nodulated.

Demonstration of alkaline phosphatase showed that there were fibrotic lesions in the cortex, where the enzyme was practically absent owing to absence of functional tubular tissue.

The histological examination indicated that in the kidneys there was a vascular,

glomerular and more obviously tubular degeneration leading finally to interstitial fibrosis.

The fluoride-fed rats showed diminished growth, and at low body weights there was a marked diminution of fat stores.

Nitrogen metabolism was greater in the fluoride-fed rats than in the controls.

This work has been financed in part by an expenses grant from the Medical Research Council, for which we wish to record thanks.

We should also like to thank Dr. D. C. Wilson for advice throughout the investigations.

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THE EFFECT OF SODIUM FLUORIDE ON THE OUTPUT OF SOME ELECTROLYTES FROM THE GASTRIC MUCOSA OF CATS

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Bathing the gastric mucosa with a solution containing 5 m-equiv of sodium fluoride/l. has been shown to reduce the gastric secretion of hydrochloric acid in response to histamine or gastrin in cats (Bowie, Darlow & Murray, 1953). The experiments to be described in this paper were made to study the influence of intragastric instillations of fluoride on the outputs of chloride, sodium and potassium by the gastric mucosa. Comparative studies of the outputs of electrolytes by the gastric mucosa under the influence of other inhibitors of enzymes were made to give insight into the effect of the fluoride on gastric secretion.

METHODS

The experiments were made on seventeen cats anaesthetized with 4 ml./kg. body weight of a solution containing 1 g chloralose and 5 g urethane/100 ml. given intravenously after induction with ether. Swallowing, which disturbed the experiment in some instances, was abolished by giving an extra 5 or 10 ml. of the anaesthetic solution. Alternatively, a few ml. of 5% procaine run into the pharynx was effective in abolishing swallowing. After tracheotomy, 50 mg of mepyramine maleate were injected intramuscularly to minimize the vascular effects of the large dose of histamine which was used to stimulate gastric secretion. The pylorus was occluded by a ligature and a rubber tube passed into the stomach. After closing the abdomen a continuous intravenous injection of 0·045 mg histamine acid phosphate/kg body weight/min in 1 ml. of warm saline (0·9 g NaCl/100 ml.) was given. This is ten times the dose used by Bowie *et al.* (1953) and, in contrast to their findings, a satisfactory rate of secretion was obtained in all the cats used. The rate of injection was about twice the rate of gastric secretion. Although some experiments lasted as long as 6 hr, no animals died, which shows how effective was the mepyramine maleate in protecting the cats against the vascular effects of histamine. To measure the effect of fluoride and other agents on gastric secretion 20, 25 or 50 ml. of various solutions were run into the stomach at regular intervals, and after 10, 15 or 30 min respectively the fluid was aspirated and a new portion introduced. In the early experiments the blood pressure was recorded, but since gastric secretion continued when the blood pressure fell to 40 mm Hg, in the later experiments the blood pressure was not studied. The early experiments were usually ended because of blood staining of the gastric contents which probably occurred because heparin was given to prevent clotting in the cannula used for recording blood pressure. Other experiments were ended because of fluid leaking up the oesophagus.

Acid in the aspirates was titrated to pH 7·0 or to pH 5·4 which gives a more satisfactory correspondence between total anions and total cations (Gudiksen, 1950). Chloride was titrated electrometrically (Ihre, 1938). Sodium and potassium were determined by means of a flame photometer with an external standard. In the majority of experiments there was close agreement between determined anions and cations: any imbalance can possibly be attributed mainly to the presence of mucus and carbon dioxide in the aspirates.

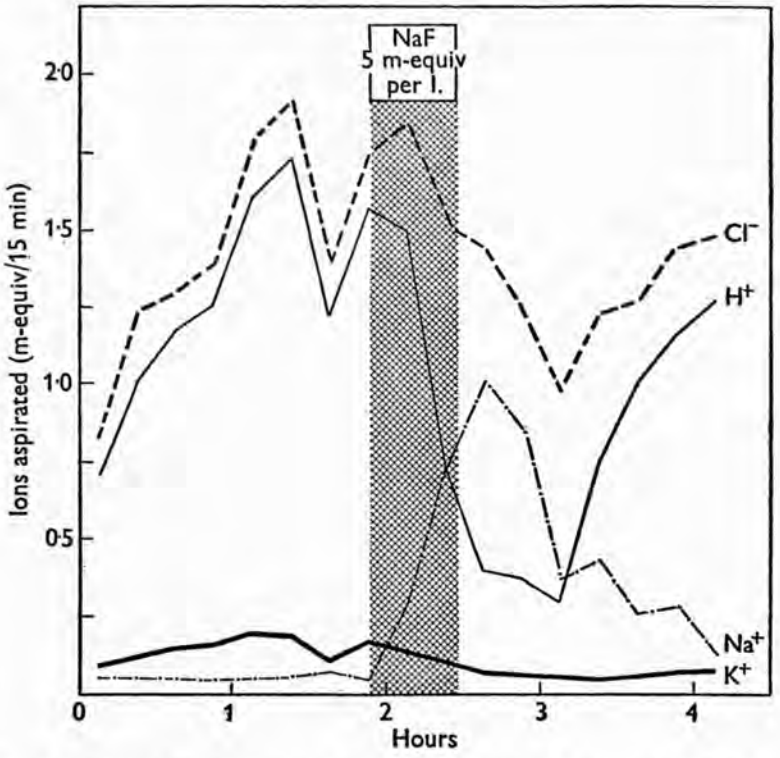

Fig. 1. The influence of sodium fluoride on the amounts of electrolytes aspirated from stomach of a cat stimulated by a continuous intravenous injection of histamine.

RESULTS

The influence of sodium fluoride on the output of electrolytes

Typical experimental results which show the amounts of ions aspirated from the stomach stimulated by histamine before and after treatment with fluoride are given in Fig. 1. Corrections in all the data have been made for any sodium ions instilled into the stomach. It may be seen that after instilling the solution of sodium fluoride (5 m-equiv/l.), the measured output of acid fell to a greater extent than that of chloride. It is clear therefore that some cation must have replaced the hydrogen ion in maintaining electrical equilibrium. Fig. 1 shows that the cation deficit is quantitatively accounted for by an increase in the output of sodium. As the output of acid declined after the instillation of sodium fluoride, so did the output of potassium, which was small relative to the outputs of acid and chloride. During the later part of the experiment the outputs of acid and chloride rose and the output of sodium fell correspondingly.

The data given in Fig. 1 were confirmed by those of seven similar experiments. In order to summarize these results a comparison was made between the amount of each particular ion aspirated per collection period immediately before the instillation of fluoride and the minimum amount aspirated which was reached within an hour of the removal of the fluoride. It showed that the amounts of acid obtained fell by a mean of 70% (S.E. of mean ±8·5); the amounts of potassium fell by a mean of 52% (S.E. of mean ±10) and the amounts of chloride obtained fell by a mean of 48% (S.E. of mean ±4·5). In each experiment the amounts of sodium aspirated increased by several hundred per cent. The effect of the fluoride was reversible in that the amounts of acid and chloride aspirated increased and the amounts of sodium decreased in the four experiments which continued for more than 90 min after the instillation of the fluoride. The amounts of potassium obtained fell and rose again in a manner more or less corresponding to the aspirates of chloride, although the amounts of potassium were very much smaller than the amounts of chloride.

These experiments were not designed to determine the volume of the gastric secretion, which was small relative to the volume instilled. However, it was found that the volume recovered from the stomach tended to fall after the instillation of fluoride. This was best seen when water absorption was minimized by instilling 11% sucrose, which is iso-osmotic with plasma. In these circumstances the water absorption, which is known to occur in cats (Sleeth & van Liere, 1937) and in dogs where it has been shown to be influenced by the osmotic gradient (Bandes, Hollander & Glickstein, 1940), was presumably negligible.

The influence of sodium fluoride on the fate of acid instilled into the unstimulated stomach

It is usual to think of the output of hydrogen ion by the gastric mucosa as being linked to the output of chloride ion, but in the present experiments the output of chloride fell less than the output of acid. This raises three possibilities: the secreted hydrogen ion may have been neutralized; it may have exchanged across the gastric mucosa with some other cation; or some other cation may have been secreted in place of hydrogen ion. The finding of increased outputs of sodium after instillations of fluoride could be explained by any of these possibilities, but the work of Bowie *et al.* (1953), who noted an alkaline mucoid fluid in the stomach after instillations of higher concentrations of fluoride than we were using, shows that neutralization is a possible explanation. If it were correct to regard our results in this light we should expect solutions of acid introduced into the stomach to be similarly neutralized after the instillation of fluoride solutions. Fig. 2 shows the results of an experiment in which 50 ml. portions of hydrochloric acid solution (50 m-equiv/l., a

concentration which is about the maximal reached in the stomach in the experiments reported here), were instilled into the unstimulated stomach every 30 min. It may be seen that after the instillation of the fluoride less acid but more sodium was aspirated from the stomach, whereas the output of potassium was small and almost constant. This experiment was repeated and similar results were obtained. Thus it appeared that instillations of fluoride were followed by a loss of instilled hydrogen ions, which might be accounted for by supposing that they had been neutralized by a secretion of sodium ions and bicarbonate ions. Since the aspirated output of sodium by the gastric mucosa was much greater than the secreted output of chloride the sodium could not have been secreted by the parietal cells in place of hydrogen ion.

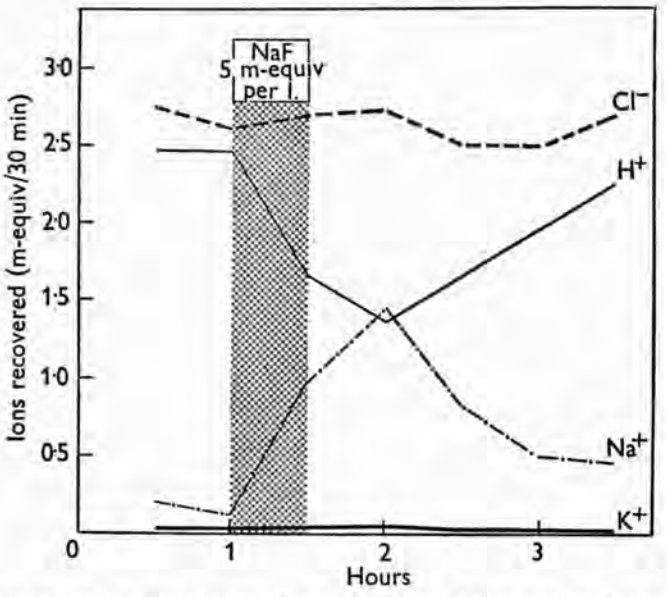

Fig. 2. The influence of sodium fluoride on the movement of ions across the unstimulated gastric mucosa of a cat. 50 ml. HCl (50 m-equiv/l.) instilled every 30 min.

The influence of sodium fluoride on the fate of acid instilled into the stimulated stomach

The relevance of the two experiments in which acid was instilled might be questioned on the grounds that the instillations were not made into a secreting stomach. To test the validity of this objection a similar experiment was made in a cat given a continuous intravenous injection of histamine. The results are given in Fig. 3. In the period $1\frac{1}{2}$–2 hr the rate of aspiration of hydrogen ion was 1·5 m-equiv/$\frac{1}{2}$ hr. Half an hour after the instillation of 2·5 m-equiv HCl in 50 ml. 3·1 m-equiv of hydrogen ion were aspirated, so that apparently the rate of secretion had fallen from 1·5 to 0·6 m-equiv/$\frac{1}{2}$ hr whilst the instilled acid was in the stomach. There was no corresponding increase in the output of sodium. An instillation of fluoride solution had the usual inhibitory effect on the secretion of hydrochloric acid. While this effect was prominent an instillation of

2·5 m-equiv HCl in 50 ml. was again made, but the recovery of hydrogen ion at the end of the period 4–4½ hr was only 1·6 m-equiv instead of the expected 2·9 m-equiv. Thus the loss of acid was greater than could be accounted for on the basis of complete suppression of secretion of acid. The discrepancy between chloride recovered and the sum of chloride instilled plus chloride expected to be secreted was of the same order as the corresponding discrepancy for acid.

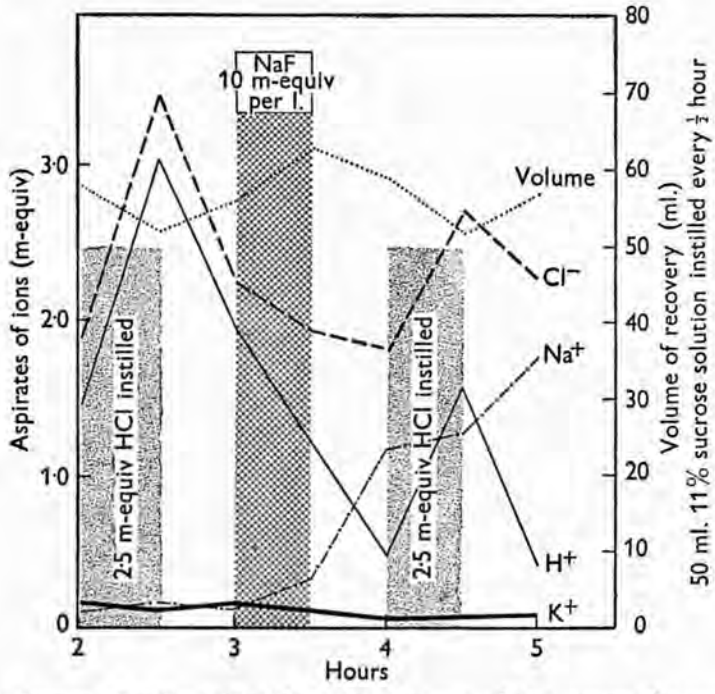

Fig. 3. The influence of sodium fluoride on the passage of ions across the gastric mucosa stimulated by a continuous intravenous injection of histamine.

Since there is no possibility of chloride disappearing by neutralization there must either have been a greater suppression of secretion of chloride than of acid, which seems unlikely (see Fig. 4), or chloride must have passed from the stomach across the mucosa. Whatever is the correct interpretation of these findings, it is clear that events in the secreting stomach are not as simply analysed as they are in the unstimulated stomach (Fig. 2).

The influence of instilled acid on the secretion of acid by the stimulated stomach

It may be seen from Fig. 3 that the instillation of acid into a secreting stomach seems to reduce gastric secretion, since the failure to recover the acid instilled plus that expected to be secreted cannot be explained by the secretion of sodium bicarbonate, as might have occurred in the experiments of Figs. 1 and 2.

In order to interpret the data presented in Fig. 3 it was desirable to know whether instilling acid under our conditions *did* inhibit secretion, in a stomach

untreated with fluoride but stimulated by a continuous intravenous injection of histamine. Fig. 4 shows the results of such an experiment, instillations of 50 ml. of water being alternated with those of 50 ml. of hydrochloric acid solution (50 m-equiv/l.). The rate of secretion of acid and chloride during the instillations of water was stable from period to period; but when the acid was instilled the recovery of instilled acid plus secreted acid was about 1·5 m-equiv less than would be expected had the rate of secretion remained constant. The outputs of potassium and sodium remained low throughout the experiment and were not significantly changed by the instillation of acid. The chloride

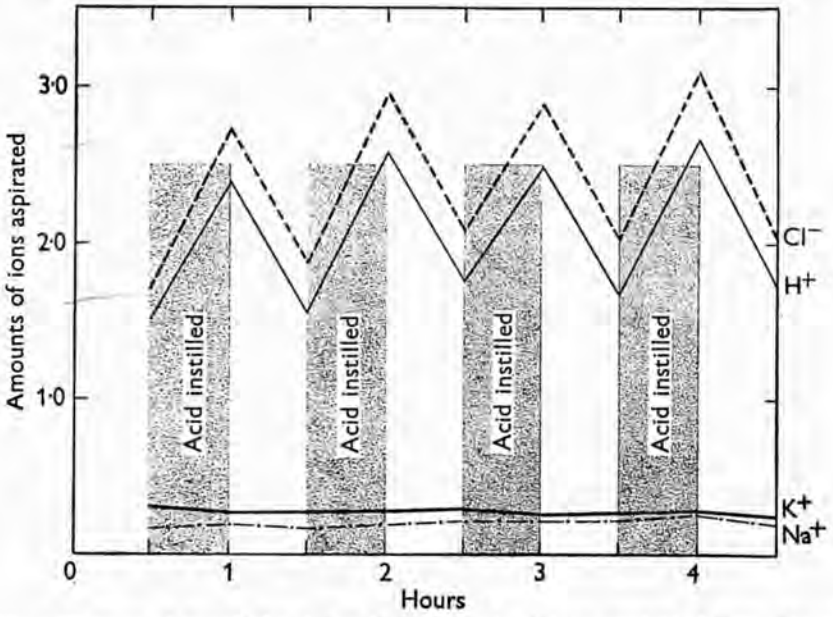

Fig. 4. The influence of instilled acid on the amounts of ions aspirated from the stomach stimulated by a continuous injection of histamine.

aspirated was also less than was to be expected had the rate of secretion remained unchanged. A repetition of this experiment gave similar results. It is interesting that this large reduction in the output of acid, about 1·5 m-equiv/$\frac{1}{2}$ hr, occurred without any increase in the output of sodium. Usually when the secretion of acid and chloride falls there is a corresponding fall in the output of potassium, but in the experiment of Fig. 4 and in another similar experiment the output of potassium was not reduced during the period after instillations of acid. The implications of this point will be considered later.

The influence of sodium fluoride on the efflux of ^{24}Na *from the stomach*

Another possibility suggested by the finding of a fall in the output of acid greater than the fall in the output of chloride (shown in Fig. 1) after instillations of fluoride may be drawn from an idea put forward by Bowie *et al.* (1953). They suggested that the effect of fluoride on the parietal cell was to create an altered permeability of the cell membrane to hydrogen ions. In this event

sodium might be supposed to enter the gastric cavity as a result of an ion-exchange with hydrogen ion. This would be in accord with Teorell's diffusion hypothesis (Teorell, 1933), which one school of thought believes to be responsible for the major portion of the variation in the concentration of ions in the gastric secretion (Heinz & Öbrink, 1954).

It is difficult to design an experiment to decide whether hydrogen ion is neutralized in the stomach by bicarbonate or is lost from the stomach by a process of ion-exchange with sodium. It seems useless to determine the efflux of tritium or deuterium from the stomach after instilling fluoride, because

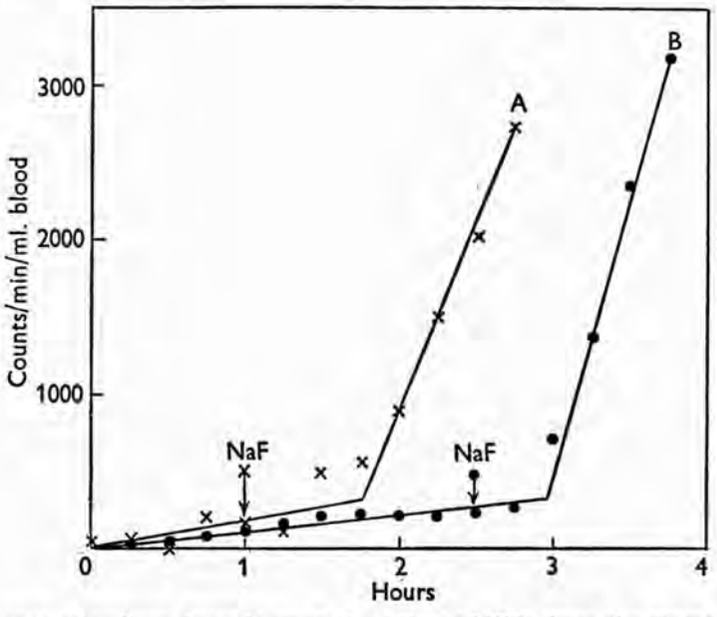

Fig. 5. The influence of sodium fluoride upon the efflux of ^{24}Na from the stomachs of two cats. In both experiments the gastric contents were 25 ml. of a solution containing 50 m-equiv HCl/l. and 100 m-equiv NaCl/l. At the arrow sufficient fluoride was introduced to give 10 m-equiv NaF/l. in Expt. A and 20 m-equiv/l. in Expt. B. The counts for Expt. A have been multiplied by 10 before plotting on the ordinate.

these markers might be lost not only by movement in their ionic state but as water after neutralization by incoming bicarbonate. If the hydrogen ions were lost by a process of ion-exchange through a membrane damaged by fluoride, it might be expected that such a membrane would allow efflux as well as influx of sodium across the gastric mucosa. On the other hand, if the sodium entered the stomach as a result of *secretion*, either as sodium bicarbonate or as sodium chloride, it would seem probable that a corresponding increase in the efflux of sodium from the stomach would not occur. Thus, if there were no increase in the efflux of sodium from the stomach after instillations of fluoride, the hypothesis of exchange of hydrogen ions for sodium ions would appear to be less likely than some hypothesis involving secretion of sodium.

Fig. 5 shows the results of two experiments in which after ^{24}Na had been instilled into the stomach, serial blood samples were taken. The early part of

both experiments confirm the finding of Reitemeier, Code & Orvis (1955) that the gastric mucosa is relatively impermeable to sodium. As judged from counts of the blood, after the fluoride was instilled to give a concentration of 20 m-equiv/l. the rate of efflux of ^{24}Na from the stomach increased about 40-fold. The total amount of sodium leaving the stomach was of the order of 0·2 m-equiv/hr. The maximum rate of loss of instilled hydrogen ions has been about 2 m-equiv/hr. Although this rate is ten times greater than the estimated efflux of sodium from the stomach the gradient for the diffusion of sodium into the stomach is much greater than that used for measuring the efflux of sodium from the stomach. In addition, the efflux of hydrogen ions, if it occurs, must be supposed to increase the influx of sodium into the stomach.

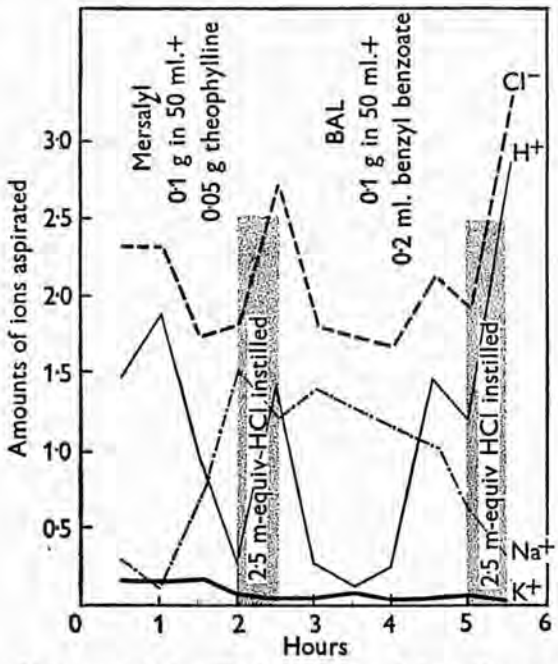

Fig. 6. The influence of intragastric instillation of mersalyl on the movement of electrolytes across the gastric mucosa stimulated with histamine.

This demonstration of the increased efflux of sodium from the stomach after bathing the gastric mucosa with fluoride does not provide positive support for the hypothesis of ion-exchange through the gastric mucosa, but it shows that a change in permeability for effluxing sodium does occur so that the requisite change in permeability for influxing sodium is reasonable.

The influence of instillation of mersalyl on the gastric output of electrolytes

Several substances other than fluoride have been described as inhibiting secretion of acid by the gastric mucosa, and it seemed probable that the reversible effects produced by fluoride could be obtained with other agents which are inhibitors of enzymes. The results of an experiment carried out to test this hypothesis are shown in Fig. 6. Secretion was stimulated with hista-

mine with the usual blocking of side effects with mepyramine maleate. After instilling a solution of mersalyl, the output of hydrogen ion and chloride ion fell, whilst the output of sodium rose. At this point an instillation of 2·5 m-equiv HCl in 50 ml. was made but only 1·5 m-equiv of hydrogen ion were recovered half an hour later. The recovery of chloride was 2·7 m-equiv, which is considerably less than the 4 m-equiv which would have been expected, bearing in mind the rate of secretion of chloride before and after the instillation of acid: but the output of sodium was not increased by the instillation of acid. The gastric mucosa was now bathed with a suspension of dimercaprol, British anti-Lewisite, which is a recognized antidote for mersalyl. The rate of secretion of hydrogen ion and chloride ion rose while the output of sodium fell. At this point a further 2·5 m-equiv HCl in 50 ml. were instilled and 2·9 m-equiv of hydrogen ion were recovered after $\frac{1}{2}$ hr. This experiment clearly shows that under the influence of mersalyl the output of sodium ion by the mucosa rises as the output of hydrogen ion falls, a change which is similar to that produced by gastric instillations of fluoride.

The influence of acetazolamide on the properties of the gastric mucosa

On the other hand, all inhibitors of enzymes which reduce the output of acid by the stomach do not work in the same way as fluoride and mersalyl. Fig. 7 shows that an intravenous injection of 160 mg/kg body weight of acetazolamide did not reduce the recoveries of acid instilled into the unstimulated stomach. Fig. 8 shows that this dose was sufficient in another cat to reduce considerably a secretion of acid stimulated by histamine, with a correspondingly marked fall in the output of chloride and only a small increase in the output of sodium.

DISCUSSION

It has been known for some years that the application of fluoride reduces the amount of acid which may be aspirated from a secreting stomach. The results of the foregoing experiments show that this effect is not due entirely to the inhibition of secretion of hydrochloric acid, because the fall in the output of hydrogen ion is accompanied by an increased output of sodium. When the unstimulated gastric mucosa is exposed to fluoride there is a fall in the recovery of hydrogen ion that has been instilled into the stomach, with a corresponding rise in the recovery of sodium. The result of instilling acid into the secreting stomach treated with fluoride is slightly different in that it does not augment the output of sodium. This might indicate either that the transfer of sodium has a limit, or that the instilled acid does not gain access to the site of exchange or neutralization, or more likely that the instillation of acid inhibits the secretion of acid so reducing the amount available for exchange.

Experiments in which the inhibitor of carbonic anhydrase, acetazolamide, was employed instead of fluoride gave evidence of inhibition of parietal

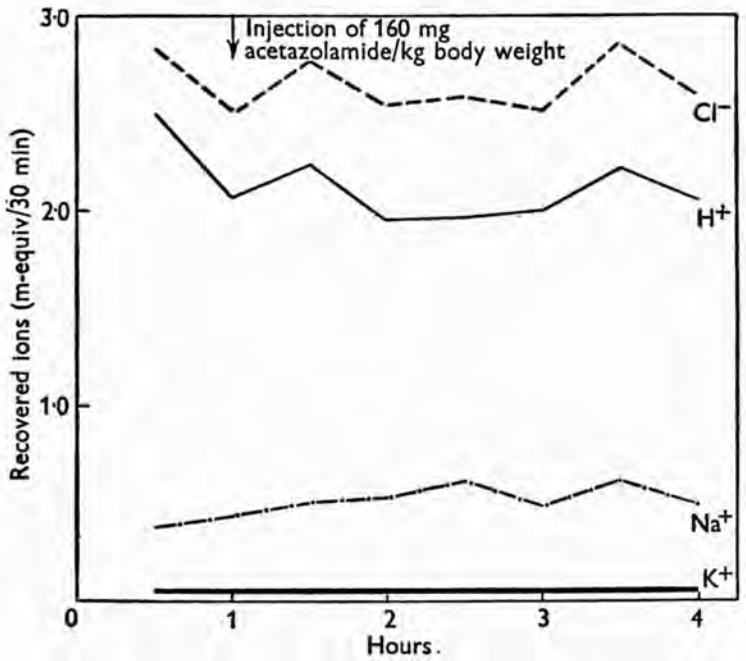

Fig. 7. The influence of an intravenous injection of 160 mg acetazolamide/kg body weight upon the permeability of the unstimulated gastric mucosa to instilled hydrochloric acid. 2·5 m-equiv HCl in 50 ml. 11% sucrose were instilled every ½ hr.

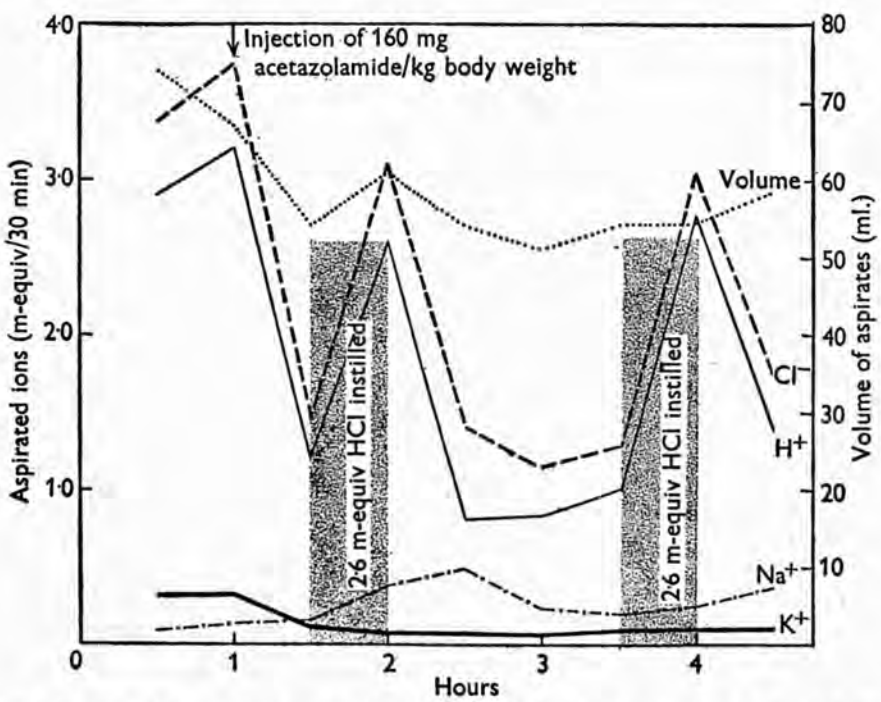

Fig. 8. The influence of an intravenous injection of 160 mg acetazolamide/kg body weight on the output of electrolytes by the stomach in a cat given a continuous intravenous injection of histamine. Instillations were 50 ml. of 11% sucrose solution.

secretion relatively uncomplicated by other factors. The secretion of hydrogen ion and chloride ion from the stimulated stomach was much reduced by acetazolamide with only a small increase in the output of sodium, but hydrochloric acid put into the unstimulated stomach treated with acetazolamide was recovered almost in full.

Mersalyl, an inhibitor of enzymes which presumably acts by combining with sulph-hydryl groups, has similar effects to fluoride on the gastric mucosa. The effects of mersalyl can be reversed by the application of British anti-Lewisite; the effects of an application of fluoride are much reduced within an hour of the fluoride being washed out.

It is inferred that an inhibitor such as fluoride either stimulates secretion of $NaHCO_3$, thus removing hydrogen ions by neutralization, or causes the secretion of sodium instead of hydrogen, or so alters the permeability of the gastric mucosa to certain cations that hydrogen ions leak back into the blood and sodium ions pass into the gastric cavity. We have not designed any critical experiment to decide between these three explanations but the possibility of an increased ion-exchange is enhanced by the evidence provided by the use of ^{24}Na. The increase in the rate of efflux of sodium ion instilled into the stomach after exposure of the gastric mucosa to fluoride shows an altered permeability to this cation, which might explain the entry of sodium ions under the conditions of the previous experiments.

The results of Figs. 3 and 4 taken together weigh against the hypothesis of secretion of sodium instead of acid by the parietal cells.

The effect of the inhibitor of carbonic anhydrase was to reduce the recoveries of hydrogen ion and chloride by approximately the same amount. Fluoride and mersalyl, on the other hand, reduced the recovery of hydrogen ion to a much greater degree than the recovery of chloride. Whether this differential effect may be explained as a leakage of both hydrogen ion and chloride ion or as partial inhibition of secretion of hydrochloric acid, together with partial exchange of hydrogen ion for sodium ion, remains unestablished. Since we have never recovered less chloride than was put into the stomach, whether in the secreting or non-secreting state, we cannot claim to have shown that any leakage of chloride ion that may occur normally (Teorell, 1933) is accentuated by fluoride.

It is well known that a rough parallelism is generally observed between recoveries of hydrochloric acid and of potassium. If part of the reduction in the recovery of hydrogen ion after the application of fluoride, or mersalyl, is ascribed to an inhibition of secretion, the corresponding decrease in the recovery of potassium ion suggests that the secretion of potassium is intimately connected with the secretion of acid. Alternatively, the secretion of potassium may be unaffected, and the fall in the recovery of potassium which occurs may be due to potassium ion leaving the gastric cavity in company with hydrogen

ion in exchange for sodium ion. The exchange of potassium for sodium across the membrane of rabbit's erythrocytes is affected by fluoride (Davson, 1941). Potassium recovery is reduced, however, during the inhibition of parietal secretion by acetazolamide, where there is no reason to suspect increased permeability. Hollander (1952) reported that an injection of acetazolamide reduced the recovery of potassium from dogs' stomachs to a low, steady value during stimulation by histamine.

In this connexion it is interesting that the recoveries of hydrogen ion and chloride from the secreting stomach after acid instillation were not as large as might be expected, judging from the previous and subsequent rate of secretion. The recovery of chloride was reduced by about the same amount as the recovery of hydrogen ion, but there was no fall in the recovery of potassium or rise in that of sodium. It seems more probable that there was a loss of acid through the mucosa rather than inhibition of secretion by instilled acid. It has been stated by Wilhelmj, Neigus & Hill (1933) that the presence of instilled hydrochloric acid in the stomach did not affect secretion of hydrochloric acid induced by histamine, but their results were obtained under substantially different experimental conditions.

The experiments reported here clearly indicate that a fall in the recovery of hydrogen ion from a stimulated gastric mucosa on exposure to an inhibitor of enzymes is not necessarily the result of inhibition of secretion. This finding complicates the interpretation of quantitative studies of the metabolism of the secreting gastric mucosa.

SUMMARY

1. After instillation of 0·005 N-NaF into the anaesthetized cat's stomach secreting in response to histamine, a reduction of the outputs of chloride, potassium and particularly in hydrogen ion was observed. The output of sodium rose. The effects were reversible on removal of the fluoride.

2. Hydrogen ions were lost from a solution of hydrochloric acid instilled after the unstimulated gastric mucosa had been treated with fluoride, with a corresponding rise in the output of sodium equivalent to the amount of hydrogen ion lost. Chloride instilled was recovered in full.

3. The instillation of hydrochloric acid into the stomach stimulated with histamine was followed by equivalent reductions in the recoveries of hydrogen ion and chloride, but the aspirates of sodium and potassium were unaltered.

4. Fluoride altered the permeability of the gastric mucosa. The rate of efflux of ^{24}Na from the gastric cavity was increased 40-fold when the mucosa was exposed to fluoride.

5. An intragastric instillation of mersalyl evoked the same changes in electrolyte output as an application of fluoride. Those effects were reversed by an application of British anti-Lewisite.

6. Different results followed an injection of acetazolamide. The output of hydrogen ion fell by about the same amount as the output of chloride. There was a reduction in the output of potassium. Only a small rise in sodium output occurred.

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