LXIII. FURTHER OBSERVATIONS ON THE EFFECTS OF LARGE DOSES OF IRRADIATED ERGOSTEROL.

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RECENT observations on the effects produced in animals by excessive feeding with irradiated ergosterol have provided definite results which are at variance. Pfannensteil [1927], Kreitmair and Moll [1928], and Harris and Moore [1928] have shown that rats and some other animals become ill in a few days and die in a week or two when they are given some milligrams of this substance daily in addition to a normal diet; and Kreitmair and Moll [1928] and Kreitmair and Hintzelmann [1928] described an atrophic spleen, haemorrhagic enteritis, and extensive and severe arteriosclerosis as the prominent pathological features. On the other hand, Dixon and Hoyle [1928] were unable to confirm these results. They found that rats failed to gain in weight when fed with similar amounts of irradiated ergosterol, and when killed, after being fed in this way for about 40 days, showed calcium phosphate calculi in the urinary tract. Dixon and Hoyle used ergosterol which had been irradiated in oily solution, and they suggested that the medium in which irradiation is effected may be a deciding factor in determining the toxicity of the product as shown by feeding experiments with very large doses. In the experiments of Harris and Moore the ergosterol had been irradiated in alcoholic solution. The German observers did not state their method of irradiation.

In the experiments described in the present paper the work of Dixon and Hoyle has been repeated with the addition of certain quantitative estimations on the urine in an attempt to determine the immediate cause of the formation of the calculi found in such experimental animals.

EXPERIMENTAL.

Twenty-four half-grown albino rats each weighing about 180 g. were chosen for the experiment and kept throughout in cages of the Hopkins pattern, two rats in each cage. The urine was collected by Hopkins's method in 3-day periods, and that from three cages mixed and analysed together. In this way four groups, each consisting of six rats, were maintained, and two of these groups received the experimental diet and two were kept as controls. The

volume of urine passed by each group was measured separately. The contents of the three flasks from each group were poured into a graduated cylinder and the volume noted. In this way the average amount of urine passed per rat per day could be calculated. The urine was then poured back into one of the flasks; the graduated cylinder and the two remaining flasks were washed out with 20 cc. of distilled water and these washings were added to the main bulk of urine. The analyses were made on this diluted urine and allowance made for the dilution by calculation. In the earlier estimations the urine was acidified with 5 cc. of *N* HCl in order to dissolve any calcium phosphate which might have crystallised out: in the later estimations, which were made daily, no acid was added. When the estimations were not carried out immediately the urine was preserved with chloroform.

We intended to estimate the urinary calcium excretion, but we found that in using the ordinary Shohl and Pedley technique [1922] for the calcium estimations it was impossible to obtain with certainty a sufficiently clear solution after digesting the urine with ammonium persulphate. In the case of rats' urine this oxidation gives rise to an insoluble substance which has not yet been thoroughly investigated. As it was partly organic in nature, we considered that further investigation of the completeness of the oxidation of urates and allied substances in rats' urine by this method was necessary before it could be relied upon as a step in the estimation of calcium. For this reason we were compelled to abandon calcium estimations in the present experiments.

Urinary phosphate was estimated by Briggs's method [1922]. The acidified urine was shaken and allowed to stand; 10 cc. were withdrawn and the HCl previously added neutralised by the calculated amount of *N*/10 NaOH. The solution was then made up to 100 cc. and 1 cc. used for the estimation. During the last 9 days of the experiment daily estimations of urinary phosphates, chlorides, and p_H were made. Chlorides were determined by Whitehorn's modification of Vollhard's method. The hydrogen electrode was used for the p_H determinations. In all the estimations duplicates were done as far as possible, and gave satisfactory readings.

All the animals received a control synthetic diet for a preliminary period of 14 days. The synthetic diet consisted of caseinogen 20 %, potato starch 35 %, sugar 10 %, cacao butter 20 %, orange juice 5 %, marmite 5 %, salt mixture 5 %. The salt mixture was that given by Hume and Smith [1928], with the addition of 0.1 % potassium iodide and traces of sodium fluoride and manganese sulphate. All the animals received in addition to this diet 2 drops of a standardised cod-liver oil daily. The experimental diet contained 1 % of irradiated ergosterol in the cacao butter so that 10 g. of the completed diet contained 20 mg. of irradiated ergosterol. The irradiated ergosterol, kindly supplied by the British Drug Houses, was irradiated in oil as described by Dixon and Hoyle [1928]. Distilled water for drinking was supplied in ample quantity in glass bulbs. After the preliminary control period, half the animals were given the experimental diet, and the other half continued on the control

diet. The experimental period was continued for 45 days. In all cases the diets were given mixed into a stiff paste with a little distilled water in order to reduce scattering to a minimum. Each rat was allowed 15 g. of the dry diet daily, and the leavings from each cage were weighed each day. In this way the average quantity of food and irradiated ergosterol taken per rat could be accurately estimated.

In the urinary estimations there are two main sources of probable error, contamination of the urine with faeces and with food. It is impossible to prevent faecal particles adhering to the urine collectors occasionally; and it is impossible entirely to prevent scattering of food. In order to decide the extent of the errors involved in these ways, two estimations were made. In the first case four faecal masses were shaken up for 5 minutes with 100 cc. of distilled water. After sedimentation 1 cc. of the fluid was withdrawn for the estimation of phosphate. The amount of soluble phosphate present, however, was so small that no detectable blue colour was produced with the Briggs reagents. In the second place 2 g. of control diet were shaken up with 100 cc. of distilled water. The phosphate in this solution was then estimated and it was found that 5.5 mg. had passed into solution. Supposing that 50 cc. of urine are contaminated with 2 g. of diet, then approximately 11 mg. of phosphate are added; this is equivalent to an error of about 3 % and is negligible when the additional sources of error that are inseparable from such animal experiments are considered. But it is extremely improbable that so large a quantity of food as 2 g. was ever mixed with the urine.

RESULTS.

All the control rats throughout the entire experiment and the experimental rats during the preliminary control period maintained an approximately steady food consumption. When the experimental rats began to take the diet containing the irradiated ergosterol they showed a decrease in appetite during the first week. During this time they lost weight to a corresponding degree and although subsequently they returned to their normal level of food consumption this was not accompanied by a gain in weight. During the remaining period of the experiment the average weight curve for the twelve experimental animals remained practically steady. This is shown in Fig. 1. The average amount of irradiated ergosterol taken per rat per day over the whole experimental period was 26.4 mg.

The daily output of urine and the urinary phosphate excretion for the control and experimental groups of rats are shown in Figs. 2 and 3 respectively. The figures for both control and both experimental groups have been averaged and presented together since there were no important differences between the two sub-groups. It will be seen that the urine output of the control group was constant within definite limits throughout the whole experiment. The daily urinary phosphate excretion varied, however, considerably from day to day and is throughout placed at a very high level relative to that of other animals.

During their control period the experimental animals were strictly comparable with the controls both as regards their urine output and urinary phosphate excretion. Within 3 days of the administration of the irradiated ergosterol these animals developed a considerable diuresis which persisted throughout the subsequent 42 days of the experiment. During this time the extent of the diuresis remained roughly constant and represented an increase of 200 to 300 % on the previous water excretion. The urinary phosphate passed by these animals per rat per day remained almost at its previous level, though still showing considerable daily fluctuations.

Fig. 1. Showing the average weight curves of the control and experimental animals.

Fig. 2. Showing the urine passed in cc., and the total urinary phosphate in mg. P per rat per day. The two curves are based upon average figures from 12 control animals.

During the last 9 days of the experiment urinary chloride and p_H determinations were made. The figures in Table I show that the total chloride and phosphate passed per rat per day during this period by the control and

experimental groups show no significant differences. The average values of the p_H determinations were also similar.

Table I.

The figures in column 1 are the arithmetic means of the individual readings.

Column 2 is calculated from the formula used by Hill, Long and Lupton [1924].

Column 3 expresses as a percentage the largest observed discrepancy between an individual reading and the mean.

Column 4 expresses as a percentage ratio the difference between the mean values for the control and experimental animals.

Phosphate expressed in mg. P; chlorides expressed in mg. Cl.

		1	2	3	4
		Mean per rat per day	Probable percentage error of mean (calculated)	Maximum percentage error of mean (observed)	Percentage deviation of experimental mean from control mean
Urine	{Controls	7.06 cc.	2.8	21	151
	{Experimentals	17.8	3.3	26	
Phosphate	{Controls	32.5 mg.	2.4	17	17
	{Experimentals	38.0	4.8	32	
Chlorides	{Controls	44.0 mg.	3.8	23	17.5
	{Experimentals	52.8	3.6	26	
p_H	{Controls	6.21	3.5	31	4.7
	{Experimentals	6.5	3.2	26

Fig. 3. Showing the urine passed in cc., and the total urinary phosphate in mg. P per rat per day. The two curves are based upon average figures from 12 experimental animals.

Towards the end of the experiment several of both the control and experimental rats were X-rayed; only in one of the experimental animals was a calculus defined with certainty, and in this case a calculus, some 4 mm. in diameter on the plate, was observed in the bladder. At the end of the experiment all the rats were killed and examined *post mortem*. No pathological changes were observed in any of the control animals. In all the experimental rats the kidneys presented a streaky, yellowish white appearance at the cortico-medullary junction, in some cases extending into the papilla, similar to that already reported by Dixon and Hoyle. In nine of these rats calcium phosphate calculi were found in the kidneys, ureters or bladder. They varied in number, size and position, but the frequency of secondary mechanical changes was not so marked as in the former experiments of Dixon and Hoyle. In no case was there evidence of gross infection of the urinary tract. In four animals there was some degree of arteriosclerosis of the thoracic or abdominal aortae. In all these cases the other main arteries showed no lesions.

DISCUSSION.

This experiment affords complete confirmation of the results already published by Dixon and Hoyle. In no case did the irradiated ergosterol administered prove fatal to the experimental animals, and the weight changes and *post mortem* findings confirm their results except in the presence, in four of our experimental animals, of some degree of arteriosclerosis in the aorta. As this was present in a few animals only, and was limited in distribution even in these, and as further the experiment had been continued about four times longer than in the case of those reported by the German workers, it is evident that the arterial changes found in the present case have considerably less significance.

All the experimental animals showed a persistent diuresis without any significant increase in either the total phosphate or chloride output in the urine, so that the concentrations at which these substances were passed varied inversely to the extent of the diuresis. As there was no increase in the total phosphate output this substance was not the cause of the diuresis, since, with a low threshold substance such as this, a diuresis directly due to it causes an increase in total output without any marked fall in the concentration at which it is passed. No further light was thrown on the matter by the p_H determinations. The average urinary p_H of the experimental animals was slightly more alkaline than that of the controls, as would be expected with a diuresis. The figures show that there is no evidence that phosphate is precipitated in the urinary tract as the result of a marked change of p_H towards the alkaline side. Moreover, during the diuresis the total outputs of phosphate and chloride, and the relative concentrations at which they were passed, followed an approximately parallel course. This shows that there is no evidence of any selective effect on the tubular reabsorption of phosphate.

The cause of the diuresis remains at present unexplained. It may well be that a marked increase in the urinary calcium excretion is the determining factor. If this is not so, the occurrence of urinary calculi composed of calcium phosphate in animals that have exhibited a diuresis in which the urinary phosphate concentration is always less than half the normal, without any increase in the total phosphate passed and without any change in the reaction of the urine, must be a very unusual condition.

CONCLUSIONS.

1. Confirmation of the effects described by Dixon and Hoyle as occurring in rats given large doses of irradiated ergosterol have been obtained. Emphasis is laid on the non-lethal effects, the absence of persistent or marked loss in weight, and the presence of urinary calculi *post mortem* as the main features of such experiments when ergosterol that has been irradiated in oily solution is used.

2. Such animals show a persistent diuresis without any increase in the total phosphate and chloride passed per day, and without any significant change in the reaction of the urine.

The work in this paper has been done during the tenure by one of us (J. C. H.) of the Ernest Hart Memorial Scholarship of the British Medical Association.

NOTE (added 11. v. 29, Ed.).

In a paper published since this was sent to press, Harris and Moore [1929] have confirmed their previous findings with ergosterol irradiated in alcohol, and have further found similar effects with ergosterol irradiated in oil. In both cases the *post-mortem* findings were identical with those described by the German workers, with in addition atrophy of the thymus, and in many cases the presence of urinary calculi. Further, they found that ergosterol which had been over-irradiated was completely innocuous, and on the basis of these results they consider that the suggestion made by Dixon and Hoyle, that some additional toxic factor dependent upon the method of irradiation is responsible for poisonous effects, is not valid. If their findings are confirmed this must be so. But in the experiments described in our paper no such results have been found.

Harris and Moore suggest that the reason why Dixon and Hoyle failed to obtain the toxic effects in full measure is because insufficient irradiated ergosterol was given, or that it was not administered for a sufficient length of time. We do not think that this can be the explanation. In the first place, Harris and Moore have obtained the full toxic effects in about 40 days when the equivalent of only 1 to 2 mg. of ergosterol irradiated in alcohol was given daily, as "radiostol," *i.e.* irradiated in oil. Assuming that this ergosterol was of maximal antirachitic activity—that is, about 10,000 units per mg.—the daily dose would be some 20,000 antirachitic units. In Dixon and Hoyle's experiments up to

17 mg. daily were given, and in the experiments in the present paper an average of 24 mg. daily, in both cases for the same period of time as in Harris and Moore's experiments. Although this particular sample was not tested for antirachitic activity, samples irradiated under exactly similar conditions for the same length of time have shown an antirachitic activity of 1600 units per mg. In our experiments, therefore, some 30,000 to 40,000 units at least were given daily. As the samples used by Harris and Moore were irradiated under similar conditions to ours it is improbable that they were of maximal antirachitic activity, and although these writers state that they were assayed they do not give the values found.

The cause of the difference in experimental findings has yet to be determined.

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