XCI. ESTIMATIONS OF IRON IN THE LYMPH GLANDS OF MICE DURING TREATMENT WITH A CARCINOGENIC COMPOUND

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PARSONS and her fellow-workers [Mayneord & Parsons, 1937; Clarkson *et al.* 1938; Parsons, 1938] have reported the deposition of Fe in the lymph glands of mice undergoing treatment with the water-soluble carcinogenic substance sodium 1:2:5:6-dibenzanthracene-9:10-*endo-αβ*-succinate [Cook, 1931]. Similar results were obtained in animals treated with other carcinogenic substances. The process involved may consist of a slow destruction of red blood cells, followed by storage of the liberated Fe in the lymph glands of the animal. Occasionally the lymph glands have the macroscopic appearance of haemolymph glands, the mesenteric lymph glands being particularly liable to this change. The water-soluble compounds used by Parsons and her co-workers have been shown to have a haemolytic action *in vitro* [Warren, 1939].

The experiments described in the present paper were carried out with a view to placing on a quantitative basis the histological demonstration of the accumulation of Fe in the lymph glands during the latent period before tumour formation. A few measurements of Fe in the lymph glands of mice bearing spontaneous tumours are recorded, and, in view of the interest of the results, this investigation is being extended.

METHODS

The determination of Fe in biological material is notoriously difficult. The most serious error which is likely to occur is loss of Fe during ashing on account of the volatility of ferric chloride. It became clear at an early stage of the present experiments that the variation in the Fe content of the glands of mice which had received the same dose of carcinogenic compound was great enough to make it desirable to employ as many animals as possible to obtain a reliable mean value. The method employed had to be a reasonably rapid one, even if some degree of accuracy had to be sacrificed to achieve this. With this in mind, the method described below was adopted. Control experiments with known quantities of Fe were satisfactory. With the reservation that great accuracy is not claimed for the absolute values, there is no doubt that the results give a satisfactory picture of the relative Fe contents of the lymph glands.

Stock male mice were injected thrice weekly subcutaneously in the right flank with 0.3 ml. of a 0.4% solution of the sodium salt of 1:2:5:6-dibenzanthracene-9:10-*endo-αβ*-succinic acid (hereafter abbreviated as "D.B.A.-mal"). 10 mice were killed after receiving 20 doses. Further groups of 10 mice were killed at various dose intervals. One axillary gland from each side, one mesenteric and one inguinal gland (left) were dissected entire with the surrounding fat. The glands were washed free from extraneous blood with saline and dried

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roughly with filter paper. The four glands from one mouse were ashed together. The glands were transferred to ashing tubes (made by blowing bulbs 25–30 mm. diameter on 16 × 150 mm. pyrex test tubes). 0.5 ml. of a mixture of conc. HNO_3 (3 parts) and conc. H_2SO_4 (1 part) was added to the glands and carefully evaporated. This process was repeated until no carbonaceous material remained. In practically all cases a total of 3 ml. of acid mixture was used. The residue after the final evaporation of the acid was dissolved in 2–3 ml. of water and transferred to a 10 ml. standard flask. The Fe was estimated colorimetrically by measurement of the colour produced by salicylsulphonic acid in the presence of ammonia. To the contents of the standard flask were added in order 2 ml. 2N NH_4Cl , 2 ml. 20 % salicylsulphonic acid and 2 ml. 10 % ammonia, and water to 10 ml. The purple colour at first developed in the acid solution changes to yellow or orange on the addition of the ammonia. Blank solutions were prepared by carrying out the evaporation of the appropriate amount of acid mixture without lymph gland and making up the solution with reagents and water to 10 ml.

The colorimetric measurements were made on a Hilger "Spekker" Absorptiometer. The mode of operation of this instrument has been previously described [e.g. Callow *et al.* 1938]. The instrument was calibrated by means of standard ferric alum solution employing Ilford "Spectrum Violet" filters. From Fig. 1

Fig. 1. Iron estimations. Calibration curve $E(v) = 0.0077 + 0.08496 \times \mu\text{g. Fe}^{+++}/\text{ml.}$

it is seen that there is a straight line relationship between the measured absorption of violet light and the quantity of Fe present in the solution over the range of concentration employed.

RESULTS

(1) Normal mice

Parsons has noted that it is rarely possible to demonstrate the presence of Fe in the lymph glands of normal untreated mice by the use of the prussian blue reaction. In order to have a basis for comparison with the glands of the mice undergoing treatment with the carcinogenic substance, the total Fe in the four glands (see above) of 21 normal male mice, 6 months of age (an age comparable

with that of the experimental mice in the later stages of the injections), was determined. The amounts found were all small. The maximum value in any mouse was 6.0 $\mu\text{g.}$ and the average value approximately 3.0 $\mu\text{g.}$ The individual values are shown in Table I. The figures denote $\mu\text{g. Fe.}$

Table I. *Fe in the lymph glands of 21 untreated male mice*

2.0	2.5	2.0	1.5	5.0	4.0	3.0	1.0	3.0	2.5	1.0
4.0	4.5	6.0	3.5	4.0	3.5	4.0	2.0	2.5	4.0	

(Mean value 3.1)

(2) *Mice treated with the carcinogenic compound*

The results for 70 mice at various dose levels are given in Table II. In the case of those figures marked with an asterisk the animal showed signs of haemolymph gland formation and these values are not included in the averages. In Fig. 2 the average values are plotted against the number of doses injected before

Fig. 2. Total Fe in glands of mice treated with Na-1:2:5:6-dibenzanthracene-9:10-*endo*- $\alpha\beta$ -succinate.

Fig. 3. Fe in glands/log (no. of doses).

Table II. *Fe in the lymph glands of 70 treated male mice*

No. of doses ...	20	25	30	35	40	50	60
Total Fe in glands of mouse, $\mu\text{g.}$	7.00	9.0	8.5	9.0	14.0	18.0	13.5
	7.75	11.5	10.0	10.0	15.0	29.5*	13.5
	7.50	10.5	10.0	13.5	20.0*	12.5	17.0
	3.50	16.0	10.5	10.5	13.5	18.5	15.5
	15.00	13.5	9.5	10.0	16.0	13.5	14.5
	7.75	10.0	12.5	8.5	13.0	16.0	14.5
	8.25	6.5	11.0	11.5	11.5	11.5	20.0
	9.00	11.0	11.0	12.0	12.0	12.5	24.5*
	8.25	10.0	16.5	10.5	16.0	15.0	14.5
	7.50	14.0	15.5	12.5	10.5	15.0	15.0
Mean value	8.15	11.2	11.5	10.8	13.5	14.7	15.3

* Haemolymph gland.

the animal was killed. The vertical lines indicate the range of the individual Fe values at that dose level.

In Fig. 3 the mean values from Table II are plotted against the logarithms of the number of doses injected. The seven points fall approximately on the straight line given by the equation:

$$\mu\text{g. Fe}^{+++} = 14.1 (\log \text{ no. of doses}) - 9.58.$$

The broken curve shown in Fig. 2 has been calculated from this equation. The inflexion of the curve shown between zero and 20 doses has, of course, not been experimentally determined.

During the later stages of the injections 9 mice developed sarcomas at the site of injection. Estimations of Fe in the four lymph glands of these animals gave the following values (not included in Table II): 24.0, 13.5, 15.0, 6.5, 17.5, 36.0, 10.5, 17.5, 16.5 (mean 17.4 $\mu\text{g.}$).

In order to be certain that the increase in total Fe in the glands of the injected mice represented a true increase per unit weight of the gland tissue it was necessary to investigate the possibility of any considerable increase in size of the glands from the injected animals as compared with the glands from normal uninjected animals. With this in view, the glands (2 axillary and 1 inguinal) from 20 normal mice and 10 mice injected with 50 doses of D.B.A.-mal. were removed, together with their fat capsules, and immersed in Bouin's fixative (saturated picric acid 75 ml., formalin 15 ml., glacial acetic acid 10 ml., urea 1 g.) for 24 hr. The three glands from each mouse were then shelled out, roughly dried and weighed. The weights in mg. of the glands from the control mice were: 17.0, 23.0, 21.5, 50.0, 21.0, 13.0, 12.0, 17.0, 21.0, 34.0, 35.0, 18.0, 12.0, 20.5, 29.0, 26.5, 45.0, 33.0, 18.0, 21.0 (mean 24.4 mg.). In the case of the injected mice the corresponding weights were: 28.0, 24.0, 28.5, 25.0, 26.5, 30.0, 24.0, 20.0, 25.5, 32.0 (mean 26.35 mg.). The difference between the two means is approximately 2.0 mg. and is not significant.

In the experiments described above the mice were killed 24 hr. after the last injection. It seemed of interest to determine whether the increased Fe deposit in the glands would be removed on discontinuing the injections. For this purpose two groups of mice which had received 48 and 54 doses respectively of carcinogenic compound were kept untreated for 3 weeks before killing. The Fe values found were: 8.0, 8.5, 6.5, 3.0, 11.0, 9.5, 8.5, 9.0 (mean 8.0 $\mu\text{g.}$) for the first group and 12.5, 15.0, 13.5, 11.0, 14.0, 12.5, 7.5, 12.0 (mean 12.25 $\mu\text{g.}$) for the second. Comparison of these values with Fig. 2 indicates that there is some removal of the accumulated Fe from the glands on discontinuing the injections. In connexion with this question of the lability of the Fe deposits it is perhaps worth mentioning that the glands from the injected mice, which were treated with Bouin fixative in the experiment described above, gave values for the Fe content indistinguishable from those for normal glands. That is, Bouin's fluid dissolves the abnormal deposit of Fe.

(3) *Mice bearing spontaneous tumours*

An opportunity presented itself of estimating the total Fe in the lymph glands of 13 mice bearing spontaneous tumours. The animals were mainly female mice of a high cancer strain (Little Dilute Brown) bearing spontaneous mammary carcinomas. The values for total Fe in the same four lymph glands were as follows: 14.5, 18.0, 8.5, 8.5, 11.0, 13.5, 14.0, 25.0, 8.5, 14.0, 9.0, 19.0, 11.5 (mean 13.5 $\mu\text{g.}$). Thus the average amount of total Fe present in the lymph

glands of these animals is of the same order as the amount present in the glands of stock mice after treatment with 40 doses of the carcinogenic D.B.A.-mal. compound.

DISCUSSION

The results obtained with the lymph glands of mice injected with D.B.A.-mal. are in excellent agreement with the histological findings of Parsons. Seven weeks after the first injection the total Fe in the glands has risen to 3 times the normal amount: at 12 weeks the Fe content is 4 times, and at 20 weeks 5 times the normal value. The Fe content increases most rapidly during the early stages of the injections. The rise during the interval between the 20th and 40th doses is approximately twice as great as the rise between the 40th and 60th doses. Fig. 3 indicates that the relation of dose to Fe deposition is that of a normal drug action.

Evidence for the lability of the additional Fe is afforded by the fact that the glands of mice killed 3 weeks after the last injection show lower Fe contents than the glands of mice at the same dose level killed 24 hr. after the last injection.

No proof can yet be offered that the phenomenon investigated in the present work has any causal relationship with the malignant growths which arise on continued injection of the D.B.A.-mal. compound. It must be borne in mind that the haemolytic and carcinogenic actions of this substance may be two entirely distinct properties. The question is, however, of some importance and the preliminary results on mice bearing spontaneous tumours reported in the present paper appear sufficiently encouraging to warrant an extended application of the method to cancer-susceptible strains before the appearance of malignant growth.

SUMMARY

Estimations of the Fe contents of the lymph glands of mice undergoing injection with a carcinogenic substance have been carried out.

Increase in the total Fe content up to 5-fold the normal value has been demonstrated.

Evidence of the lability of the additional Fe has been obtained.

Preliminary experiments suggest that a similar process may occur in mice bearing spontaneous tumours.

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