Effect of X-Irradiation upon the Enzyme Systems of the Mouse Spleen. (19639)

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Barron, employing tissue slices, reported that the respiration of most tissues and the oxidation of substrates requiring sulfhydryl enzymes was diminished immediately after irradiation, with the thymus as the only exception(1,2). LeMay, using the homogenate technic, found the succinoxidase system of the rat kidney (a sulfhydryl enzyme system) to be unaffected by 400-800 r(3). Dubois *et al.* have demonstrated that cytochrome oxidase and succinic, malic and triosephosphate dehydrogenase are all unaffected by lethal whole body irradiation, and that certain alkaline phosphatases are increased so that liver, kidney and thymus homogenates have a decreased ability to esterify phosphorus or, at least, to maintain it in the form of its high energy esters(4). They were, however, unable to show any effect upon the adenosinetriphosphatase (ATPase) content of the tissues studied.

Recent reports from Jacobson's laboratory (5,6) have centered interest on the role of the spleen in radiation trauma and prompted further study of the biochemical properties of that organ. The present work was undertaken to evaluate the effect of whole body irradiation upon the succinoxidase, alkaline phosphatase and aerobic phosphorylation systems of the mouse spleen.

Methods. The animals used in these experiments were 6-7-week-old white male mice of the NIH brother-sister strain. The radiation factors were 200 KV, 20 ma, 0.51 mm aluminum and 0.25 copper added filtration, 50 cm target distance and a dose rate of 62-66 r per minute. Ten mice were irradiated together in a perforated plastic container 2.5 cm deep, 15 cm in diameter with walls 1.5 mm thick. The animals were given a total exposure of 640 r which has been shown to be an LD_{90} dose in this laboratory(7). The analyses for the *succinoxidase*(8) and *aerobic phosphorylation*(9) systems were carried out in the conventional Warburg apparatus at 37°C with air as the gas phase. The Na β glycerophosphatase(10) 5-nucleotidase(11) and ATPase (8) systems were incubated in centrifuge tubes for 15-30 minutes at 37°C, precipitated with trichloracetic acid and the phosphorus analysis carried out on the supernatant according to the method of Fiske and Subbarow. The hexokinase, prepared from bakers yeast according to Berger, Colowick, Slein and Cori (12), was brought to step 3A and then lyophilized in a 1% glucose solution.

Respiratory enzymes. It was necessary in this, as in the following experiments, to pool the spleens of several animals in order to have sufficient tissue for analysis, more spleens of irradiated animals being required because of the weight loss from a normal average of 110 mg to 33 mg. The homogenates were prepared in a Potter-Elvehjem type homogenizer as a 10% suspension in cold distilled water and were used immediately. The assays were carried out at two or more levels of tissue concentration and the activity was found to be strictly proportional. For the succinic dehydrogenase system 0.2 and 0.4 cc of the 10% suspension were used and for the cytochrome oxidase 0.10, 0.15 and 0.20 cc of a 5% suspension. Assays were run at intervals from 4 hours to 17 days after irradiation simultaneously with the non-irradiated controls. As shown in Table I, there was no effect of irradiation upon the activity of either the succinic dehydrogenase or the cytochrome oxidase in the mouse spleen on the basis of equal dry tissue weight, which agrees with the results of others on liver, kidney and thymus. Because of the loss in weight of the spleens from the irradiated animals, however, it is clear that the absolute quantities of both enzymes are reduced in exact proportion to the weight loss—an average of about 70%.

TABLE I. Effect of X-Irradiation on Succinoxidase System of Mouse Spleen Homogenates. Values expressed as mm^3 O_2/mg dry wt/hour.

No. of assays*	Succinic dehydrogenase		Cytochrome oxidase		Avg spleen wet wt (mg)	
	Control	Irradiated	Control	Irradiated	Control	Irradiated†
21	21.6 ± .4	20.6 ± 2.26	110 ± 6.7	116 ± 3.1	110 ± 6.1	33 ± 2.1

* Assays from 4 hr through 17 days after irradiation with controls run simultaneously.
† Average of 100 spleens from 24 hr after irradiation through 14 days.

TABLE II. Effect of X-Irradiation on Alkaline Phosphatases of Mouse Spleen Homogenates. Values expressed as μg phosphorus liberated/mg nitrogen/hr.

Time after irradiation	ATPase		5-Nucleotidase		β-glycero-phosphatase		Avg spleen wet wt (mg)	
	Control	Irrad.	Control	Irrad.	Control	Irrad.	Control	Irrad.
4 hr	2180	2430	115	135	33.6	30.7	115	112
1–11 days*	2068±89	7120±455	106±3.6	201±30	26.3±1.4	54.1±3	139±3	40±3.4

* Figures are averages from 8 assays beginning 1 day after irradiation and continuing through 11th day.

II. *Alkaline phosphatases.* Since the activity of the mouse spleen was markedly different towards all 3 of the various substrates chosen, slight modifications of the described procedures had to be used. Thus, with Na β glycerophosphatase the test system contained 0.8 cc of a 0.025 M barbital buffer (pH = 8.9); 0.2 cc of 0.015 M Na β glycerophosphate, 0.1-0.2 cc of 3% aqueous homogenate and water to make 1.6 cc. The incubation period was 30 minutes at 37°C. The *5-nucleotidase* system contained 0.35 cc of a 0.025 M barbital buffer (pH = 8.9); 0.05 cc of 0.04 M $MgCl_2$; 0.15 cc of 0.015 M adenylic acid (adjusted to pH = 8.9); 0.1-0.2 cc of a 3% aqueous homogenate and water to make 0.8 cc. The incubation period was 30 minutes at 37°C. The *ATPase system** contained 0.4 cc of 0.05 M barbital buffer (pH = 7.4); 0.05 cc of 0.04 M $CaCl_2$; 0.15 cc of 0.013 M ATP, 0.1-0.2 cc of a 1% aqueous homogenate and water to make 0.8 cc. The incubation period was 15 minutes at 37°C.

Controls for substrate and tissue were run with each determination and the appropriate corrections made. The above systems were found to be proportional to the tissue concentrations at the indicated levels. The values were calculated on both a total nitrogen and a total phosphorus basis, the latter showing the same general picture as the former.

As can be seen from Table II, there was after irradiation a sharp increase in activity of all the phosphatases. This was particularly noticeable in the case of ATP which is in marked variance with that found in other tissues and represents an interesting and perhaps significant tissue specificity. It is important to realize that, although the activity of these phosphatases increased sharply, the total content per spleen decreased due to the marked involution of this organ. Thus, the glycerophosphatase and 5-nucleotidase lost approximately 1/3 to 1/2 of their absolute value; the ATPase remained almost constant.

Aerobic phosphorylation. For these determinations the inorganic phosphorus content per flask was dropped to 0.3 cc of 0.011 M phosphate (103 γ P per flask) and the amount of tissue used was 0.6 cc of a 10% isotonic KCl homogenate. It was found that the spleen is deficient in creatine transphosphorylase and consequently it was impossible to tap off the high energy phosphate as the creatine ester. However, there was a significant uptake of inorganic phosphorus which was unaffected by the presence of creatine at a concentration of 0-30 mg per flask.

It could be shown (Table III) that the disappearance of inorganic phosphorus was dependent upon the oxidative process, for when succinate was omitted there was a marked increase in the inorganic phosphorus, presum-

* Newer developments in this system as reported by V. R. Potter and H. C. Simonson, *Fed. Proc.*, 1952, v11, 270, indicate serious difficulties in interpretation of the "Standard" ATPase Assay. Further work in progress to clarify this situation.

TABLE III. Effect of X-Irradiation upon Aerobic Phosphorylation in Mouse Spleen Homogenates.

Time after irradiation	O_2 uptake, mm^3/mg N/10 min		Phosphorus uptake, μg P/mg N/20 min; control spleen			Phosphorus uptake, μg P/mg N/20 min; irradiated spleen			% loss in overall esterification
	Control spleen	Irrad. spleen	With succinate	Without succinate	Total μg P esterified	With succinate	Without succinate	Total μg P esterified	
4 hr	11.6	10.1	+31	−20	51	+10	−22	32	37
1 day	11	12.1	+30	−26	56	−12	−46	36	36
2–11 days*	11.9±.5	12±.5	+25±1.5	−26.5±1.6	51.7±1.5	−37±4.1	−58±3.9	21.4±2.9	56.4±7.3

* Figures are averages from 8 assays beginning 2 days after irradiation and continuing through 11th day.

ably due to the splitting of the ATP even though the system contained NaF. Thus, the algebraic sum of the changes in the flask with and without substrate represents a net phosphorus uptake against a constant dephosphorylating gradient.

After irradiation, the ability of the spleen homogenate to esterify P, or to maintain it in a high energy state, is strongly impaired.† Whether this is due to the post irradiation rise in ATPase as shown in the preceding section or whether there is a more direct effect of radiation upon the transphosphorylation process cannot be answered by this experiment. That the oxidation of the substrate in this system was essentially unaffected after irradiation would be expected on the basis of the earlier results with the succinoxidase system. Therefore, it is clear that any decrease in phosphorylation cannot be assigned to deficiencies in the oxidation of succinic acid but must be linked with one of the subsequent steps.

Several experiments were run in which hexokinase and glucose were added to the experimental flasks. This procedure increased considerably the net esterification and it was possible to show that all the inorganic phosphorus which disappeared was present as glucose-6-phosphate. This system shows exactly the same phenomenon as the above in which the intermediate phosphate esters were not identified.

† While this work was in progress, a paper by R. L. Potter and F. H. Bethell appeared, *Fed. Proc.*, 1952, v11, 270, in which it was shown that irradiation produced a marked inhibitory effect upon oxidative phosphorylation in a mitochondrial preparation of rat spleen.

Discussion. It is clear that spleen homogenates reveal the same general metabolic response pattern to irradiation as do other tissues, insofar as the respiratory enzymes and aerobic phosphorylation are concerned. The one major point of difference revealed so far is the marked increase in the ability of the irradiated spleen homogenate to break down ATP. Whether this be the cause for the decreased ability of the irradiated spleen to perform coupled phosphorylation or not cannot be answered as yet. Further work is in progress in an attempt to evaluate the significance of this phenomenon.

Conclusions. Total whole body irradiation of white mice with 640 r (L_{90} dose) revealed the following changes in the metabolism of spleen homogenates: 1) no effect upon the activity of the succinoxidase system, 2) marked increase in activity of alkaline phosphatase which was especially marked with ATP as substrate and 3) decreased ability to synthesize (or maintain) the high energy phosphorus compounds coupled with the oxidation of succinate.

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Studies on Chick Embryo Adapted Rabies Virus. III. Duration of Immunity in Vaccinated Dogs. (19640)

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Although attempts have been made to immunize dogs against rabies as far back as 1826(1) and Pasteur succeeded in doing so in 1885(2), only few extensive studies have been conducted on duration of immunity following vaccination of dogs against this disease. A summary of these studies is shown in Table I. Pasteur reported(2) that resistance to intracerebral infection still appeared to be manifest 2 years after the "immunizing" injection. Data obtained by other investigators and summarized by Schnürer(3) seemed to indicate that immunity can be observed even 363 days after vaccination with living virus, although lack of adequate controls makes interpretation of their data more difficult. In the experiments of Miessner and Baars(4), dogs were found to be solidly immune to challenge inoculation with street virus 8 to 14 months after vaccination with fixed virus vaccine. More recently, a series of experimental studies designed to determine the duration of immunity following vaccination of dogs has been published by Johnson(5), and these are also summarized in Table I. It may be observed that preparations of phenolized rabies vaccine conferred an effective immunity upon dogs challenged with street virus one year after vaccination. Of those animals immunized with a single injection of vaccine, 11.5% died after challenge inoculation, as compared with 79% of the controls. Conversely, if the vaccination process consisted of 3 injections given at weekly intervals, none of the vaccinated dogs came down with rabies after challenge one month later, in contrast with 68% which died in the control group.

In view of the fact that an effective single injection method of canine vaccination against rabies was found in the recently developed chick embryo vaccine containing the living Flury strain of rabies(6), it seemed to be of interest to determine the duration of immunity conferred upon dogs by inoculation with this vaccine.

Materials and methods. Virus strains. The Flury egg-adapted strain of rabies virus used in this study has been described(7). An infected chick embryo suspension desiccated from the frozen state was used for vaccination of dogs. It represented the 40th egg passage of the virus, and was prepared 8 months prior to vaccination. The PM (Pitman-Moore 980) rabbit-fixed strain of virus(8) was used for the preparation of phenolized vaccine of horse brain origin. This particular lot of vaccine, bearing Lab. No. 6273-1580, was prepared on April 13, 1948, and the Habel mouse potency test was performed on May 4, 1948, with results indicating that the vaccine protected against 8166 LD_{50} of fixed virus. For challenge purposes the NYC strain of rabies street virus representing either the 6th or 7th passage canine salivary glands in this laboratory was used on the vaccinated animals. The preparation of the challenge inoculum followed that described in detail elsewhere(6).

Animals. Mongrel dogs over 6 months old