CCLXX. METABOLISM OF TISSUES GROWING IN CULTURE.

VI. EFFECT OF RADIUM ON THE LACTIC DEHYDROGENASE AND ARGINASE SYSTEMS OF EMBRYONIC TISSUE.

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IN an earlier paper the effect of γ -radiation on the metabolism of tissue growing in culture was described [Holmes, 1933]. It was found that whereas the breakdown of sugars (glucose, galactose and laevulose) was considerably inhibited in the irradiated tissue, the production of ammonia and urea was not altered. The irradiation given was roughly estimated to be sufficient to cause the death of the tissue about 7 days after the dose of γ -rays had been given. It will be remembered [Spear, 1930] that the dose of γ -rays required to cause instantaneous death is far greater than that required to cause delayed death, and within limits, the length of time the cultures will live after irradiation depends upon the dose given. This curious fact makes the study of the metabolism of the tissues during the intermediate period particularly interesting, as it is possible that it may give some indication of the damage the cells have received. During the intermediate period growth and outwandering of the cells may continue normally for some time. Our cultures were only kept for 48 hours, and during this time the extent of outwandering of cells from the culture was not lessened, and the gross microscopical appearance (chromosomes not examined) was little altered. It therefore seemed that the mechanisms responsible for sugar breakdown were particularly sensitive to γ -rays under our conditions, being affected before the movement of the cells was inhibited and before nitrogenous metabolism was altered. It should be added that cell division was not taking place in any of the cultures (control or irradiated), but merely outwandering of sheets of epithelial cells and some fibroblasts, and that this outwandering had previously been found to be accompanied by ammonia and urea formation in the cultures [Holmes and Watchorn, 1927].

On the other hand, Crabtree [1932] found that aerobic and anaerobic glycolysis of tissues in glucose-bicarbonate Ringer solution in a Warburg respiration apparatus were not easily affected by radium, and that the respiration was much more sensitive. It is not possible to compare the actual doses given by Crabtree with the ones used in the experiments just described, because Crabtree used mixed γ - and hard β -radiations. The exact amount of β -radiation reaching the tissue cannot be calculated without the thickness of the glass vessels, the depth of fluid intervening, and so on, being known, and β -radiation is known to be far more biologically potent than γ -radiation. Nevertheless, some comparisons of my own work and Crabtree's are probably worth making.

It is notable that in Crabtree's experiments a considerable drop in respiration caused by exposure to radium did not lead to an increase in aerobic glycolysis, although (judging by his figures for anaerobic glycolysis) the glycolytic mechanism was not impaired to nearly the same extent as the respiratory mechanism. This might be explained on the idea put forward by Laser [1934] that the small

amount of respiratory activity remaining may be just that part of the cell respiration which is responsible for suppressing aerobic glycolysis.

On the other hand, it is possible that after irradiation, when the respiration is much lowered, there is also a much smaller aerobic breakdown of glucose. This would be disclosed by estimating the amount of glucose disappearing instead of estimating only the amount of acid appearing. If it should prove to be the correct explanation of Crabtree's figures, his results and mine would be mutually confirmatory, since I also find a decreased breakdown of glucose under aerobic conditions after irradiation.

Use of the tissue culture method.

There are definite advantages and also definite disadvantages in using the tissue culture technique instead of the ordinary respiration technique for work of this sort.

For work of the type described in this paper it is obviously useful to have the tissues under conditions which are known to be suitable for prolonged life, to be able to observe the effects of irradiation or other treatment on the growth and outwandering of the cells and to be able to continue the experiment for some days after irradiation if necessary. It was important in these experiments, for instance, to observe the tissues for 34 hours after the 14 hours' irradiation was completed, and to see that the dose given had not been sufficient to curtail the cell movements or cause obvious microscopic changes in their appearance.

Against the use of the tissue culture method it must be said that probably metabolic reactions are not proceeding at their optimum speed, since the atmosphere is air and not oxygen and the cultures are not usually shaken, although it is possible to arrange to shake them at intervals [Laser, 1932], or at the end of the experiment for the purpose of measuring respiration or fermentation. It is well known from respiration experiments [Dixon, 1934] that the rate of respiration may be considerably affected by the rate of shaking, since this determines to some extent the speed at which oxygen diffuses into the tissues and the speed at which fresh substrate becomes available and metabolic products are removed from the near neighbourhood of the tissue.

From this point of view it would be of interest to repeat our former experiments in an atmosphere of oxygen, which would ensure a more rapid diffusion of this gas, but some workers are of the opinion that an atmosphere of oxygen inhibits growth in cultures. The condition of partial anaerobiosis which probably exists in parts of the cultures may not be abnormal for embryonic tissue, since low oxygen tensions are experienced by the foetus *in utero*. It does, however, make comparison of experiments done in tissue cultures with those done in a respiration apparatus very difficult.

The question of the retarding of metabolic reactions in the cultures on account of slow diffusion of oxygen and other necessary substances is mentioned here because it may limit the detectable effects of irradiation. For instance, it is possible that if urea and ammonia formation were proceeding at the greatest possible speed, it might be found that irradiation with γ -rays did produce an inhibition, whereas in our experiments no inhibition was found. The same considerations apply to the experiments on arginine breakdown described in this paper. There is some evidence that these considerations apply less than might be expected in cultures where active outwandering is going on, as in our "growing cultures". No doubt the actual movements of the cells and the fact that they spread themselves over a considerable area and in a very thin layer, make the availability of oxygen and substrates greater than in inert cultures, and this may partly account for the considerable differences in metabolic activity we have

always found between growing and non-growing cultures [Holmes and Watchorn, 1927; Watchorn and Holmes, 1927].

Some attempts were made to avoid these difficulties by measuring the respiration of the cultures transferred to a respiration apparatus after the completion of the 14 hours' irradiation from the 300 mm. plaque and also after irradiation with radon. After 14 hours the respiration of both the control and the irradiated cultures was low, but that of the irradiated cultures was not consistently lower. Sometimes the irradiation reduced the respiration by about 50 % and sometimes it had no effect. These experiments were soon discontinued, but later experience suggests that if the irradiation were carried out at room temperature far larger doses could be given without the oxygen uptake of control tissue falling off seriously, and no doubt more consistent results could then be obtained.

There is a definite suggestion that the cultured tissues are less sensitive to irradiation than the tissues in glucose-bicarbonate Ringer's solution. It is not possible to draw this conclusion with certainty, however, since the amount of β -radiation reaching the tissues in Crabtree's experiments is not known.

From the work of Crabtree [1932], Crabtree and Cramer [1934], Frik and Posener [1926] and from my own experiments it seemed plain that the effect of γ -radiation upon the respiratory systems, and particularly those connected with carbohydrate oxidation, should be studied further. It was suggested to me by Prof. Keilin that the lactic dehydrogenase system might prove to be particularly sensitive.

Experiments with lactic dehydrogenase.

The usual cultures of embryo rat kidney tissue were exposed to the same dose of γ -radiation as that used for the earlier work (14 hours' exposure at a distance of 0.5 cm. to 300 mg. element contained in a platinum capsule 0.3 mm. thick) and at the end of the 14-hour period were put into vacuum tubes and tested for their dehydrogenase activity in the presence of lactate by the usual methylene blue technique.¹ On two occasions this was tried, and the irradiated culture showed no change of reduction time when compared with the control culture. The amount of tissue was small and the reduction very slow in both cases, and it was therefore decided not to use the culture method for these experiments. Instead, minced tissue was placed in small flat-bottomed tubes; the area of the bottom of the tubes was almost exactly the same as that of the top of the capsule containing the radium. The depth of tissue in the tubes was about 0.5 cm. and thus much greater than the depth of tissue in the cultures, and in order to secure a sufficiently large dose of γ -radiation throughout the depth of tissue, the tubes were placed directly upon the capsules and the time of exposure increased to 16 hours. The whole experiment was carried out at 37°. The control tubes were kept in the same incubator, but were screened with lead blocks.

It has been our usual practice to use rat embryos as nearly as possible at term, since the amount of kidney tissue available is otherwise too small. On one occasion the rat was killed about 5 days before term, and it was therefore necessary to use brain tissue instead of kidney for the irradiation experiments. On this occasion it was found that the reduction time was very much prolonged by the irradiation. This suggested either that the lactic dehydrogenase system of brain tissue was more sensitive than that of kidney, or that the younger embryo tissue was more sensitive than that of embryos at term.

¹ Experiments carried out with and without added lactate suggest that a considerable proportion of the dehydrogenase activity of the tissue is, in fact, due to lactic dehydrogenase. Some inaccuracy is no doubt involved in speaking throughout this paper of "lactic dehydrogenase" when other dehydrogenases may also be concerned.

To test this point, the brain tissue of embryos at term was next exposed to the radiation, and it was again found that the reduction time was unaltered. It therefore appeared that the sensitivity of the lactic dehydrogenase system was considerably greater in the embryos 5 days before term than in those at term. This result was repeated six or seven times at each age and was confirmed.

The range of embryonic ages at which it is possible to carry out the experiments is limited by the need for a reasonable amount of tissue, and whilst it so happens that, with the breed of rats in use in this laboratory, there is a sharp change in sensitivity during the last 5 days of gestation, it is not likely that it will occur at just this stage in all breeds. In fact, on the two or three occasions when we have used albino or brown and white rats instead of the black and whites, the tissue of the embryos at term has proved to be still sensitive (in respect of its lactic dehydrogenase system) to the γ -radiation.

Brain tissue from the chick embryo is far more satisfactory from the point of view of affording a wider range of ages. The experiments were repeated once with 8- and once with 18-day eggs and it was found once more that the reduction time of the 18-day tissue was unaltered, whilst that of the 8-day tissue was increased 100%.

The examples given in the first part of Table I are quite typical, and it will be noticed that the reduction time of the young embryo tissue was usually doubled after irradiation. The large difference in reduction times between the various experiments is due to the use of different amounts of tissue.

Table I.

Reduction time in min.

Control	Irradiated	Increase %	Tissue
Experiments at 37°.			
40	50	25	Rat at term
120	150	25	Rat at term
25	23	0	18-day chick embryo
150	>480	>300	Young rat embryo
30	60	100	Young rat embryo
30	60	100	8-day chick embryo
Experiments at room temperature.			
13	17	30	Rat at term
15	17	13	Rat at term
*10	*19	*90	*Rat at term
11½	14	23	18-day chick embryo
23	50	117	8-day chick embryo
23	38	65	Young rat embryo
8	16	100	Young rat embryo
5	7½	50	Adult rat

It was found that when the irradiation was carried out at 37° the reduction times of both the irradiated and the control tissues were enormously larger than that of fresh tissue, being half an hour to an hour instead of about 3 min. Tissues kept overnight at room temperature did not lose their reducing power to any great extent, and it was therefore decided to repeat the irradiation experiments at room temperature in case the condition of the tissue and of the dehydrogenase systems was a determining factor in their sensitivity to γ -rays. The results are given in the lower half of Table I; the amounts of tissue taken were smaller and the amounts of dye larger than in the first experiments, so that the difference in dehydrogenase activities between the tissues kept at 37° and those kept at room temperature is greater than appears from the Table.

The number of experiments done at room temperature was not large enough (they are all included in the Table) to allow of any certainty, but it did not appear that the conditions greatly altered the sensitivity of the dehydrogenase systems to radium. The lactic dehydrogenase of the young tissue was still more easily affected than that of the tissue from older embryos, although in one case (marked *) the reduction time of the tissue from an embryo at term was doubled by the exposure to γ -radiation. On one occasion only was the tissue from a young chick egg found to be insensitive to γ -rays at room temperature; this example is not included in the Table, as there was a definite possibility that the tissue was damaged by accidental overheating.

It is of course obvious that before this difference in response to γ -radiation between young and old embryo tissues can be regarded as definitely proved the experiments must be repeated a great many times. Unfortunately that is not possible under the present conditions of work. Experiments with radium have always suggested considerable variations in sensitivity between individual tissues and no doubt some cases would be found where the lactic dehydrogenase of young tissue was less sensitive and that of older tissue more sensitive than usual. On the other hand, in view of the fact that some workers have considered that embryo tissue is more easily killed by irradiation than adult tissue, it would be well worth while establishing definite differences of this sort in their metabolic response to irradiation.

Of much greater immediate interest is the question of what part of the lactic dehydrogenase system—the enzyme itself or the co-enzyme—is destroyed by γ -rays. One or two preliminary experiments have suggested that it is the co-enzyme that is destroyed in the sensitive tissues. The addition of co-enzyme to the tissues increased the rate of methylene blue reduction of the irradiated tissue up to the normal level. It is hoped to investigate this point further in the future.

Experiments with arginase.

In view of the observation that urea and ammonia production in cultures was not altered by the dose of γ -rays given it seemed worth determining whether arginase, which is in all probability one of the enzymes concerned in the urea formation, was similarly unaffected. Arginine was added to the cultures at the beginning of the experiment, and the total urea and ammonia estimated at the end of 48 hours. Part of the urea and ammonia found would of course be due to the ordinary metabolic processes of the cell, but the figures obtained were greatly in excess of those found in the absence of added arginine, and a large proportion of the total urea + NH_3 -nitrogen found must be accounted for as urea formed from arginine. This is especially the case in Exps. 161, 162 and 164, where glucose

Table II. *Arginase experiments.*

Total urea- and ammonia-nitrogen in cultures in mg.

Exp. no.	Control at 0°	Control culture	Irradiated culture	Difference between irradiated and control
159	0.097	0.151	0.204	+ 0.053
160	0.073	0.184	0.192	No difference
161	0.068	0.180	0.180	No difference
162	0.071	0.240	0.196	- 0.044
164	0.090	0.190	0.213	+ 0.023
	14 hours' incubation	3 days	3 days	
189	0.110	0.171	0.171	No difference

was present in the medium, and the ordinary formation of urea and ammonia would therefore have been very small [Watchorn and Holmes, 1927].

It can be seen that the irradiation had no effect on the breakdown of arginine. It is worth mentioning that on two occasions the glucose was also estimated and, although the amounts broken down were too small for great accuracy, it appeared that glucose breakdown was as usual inhibited by the irradiation whilst the arginine breakdown remained unaltered (Exps. 161 and 164).

In order to make certain that any impairment of arginase activity would become obvious in the experimental conditions, Exp. 189 was undertaken. On this occasion the concentration of arginine was high (400 mg. arginine hydrochloride per 100 ml. Ringer's solution, *i.e.* 8 mg. in each culture), the medium was stirred at the end of 14 hours, and the estimation on one culture was made at the end of 14 hours, the others being left for 48 hours as usual. It was hoped by this means to secure sufficient access of the substrate to the arginase and also to demonstrate that arginine breakdown was still continuing after the full dose of irradiation had been received, as in fact proved to be the case.

These cultures were made in the ordinary way in 2 ml. of embryo extract in pyrex flasks, the embryo rat kidney tissue being supported on cotton-wool strands.

In connection with the effect of irradiation on nitrogen metabolism, mention should be made of Lawrie's [1935] paper on the effects of γ -rays on the ammonia production of *Bodo caudatus*, as this illustrates very well the fact that completely different effects can be obtained by varying the dose of γ -rays.

SUMMARY.

1. The advantages and disadvantages of the tissue culture method for studying the effect of irradiation on tissue metabolism are discussed.

2. Experiments are described showing that the lactic dehydrogenase system in the tissue of young embryos is more easily damaged by γ -rays than that in the tissue of older embryos.

3. Reference is made to preliminary experiments which suggest that the part of the dehydrogenase system injured by the γ -rays may be the co-enzyme.

4. Experiments are described showing that the arginase activity of a tissue culture is not impaired by a dose of γ -rays which is sufficient to cause considerable inhibition of glucose breakdown.

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