5. Exocellular fungal esterase appears to play a large part in bringing about the fungistatic activity of phenolic esters.

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Changes in Lens During the Formation of X-Ray Cataract in Rabbits

3. PHOSPHATES*

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The first biochemical change caused by irradiation of tissues is unknown. There is some evidence, reviewed by Ord & Stocken (1953), that changes take place in the high-energy phosphate ($\sim P$) compounds but assessment of the results is complicated by the fact that in most situations it is difficult to separate primary from secondary effects. Gross histological change may occur in an organ within a few hours of whole-body irradiation, with lysis of some cells and influx of others from the blood stream. No valid biochemical comparison is possible between the tissue in this state and in the normal state before irradiation. Where cell population has changed one can expect large changes in chemical and enzymic make-up.

From this point of view a study of the effect of irradiation on the lens may be peculiarly valuable. It is an isolated non-vascular organ, derived from a single type of embryonic ectodermal cell, and any observed early effect of radiation is more likely to be a primary effect than it is in other tissues where the cell population is complex to start with and may change after irradiation. We have already shown that certain changes in the composition and enzymes of lens occur during the early stages of the formation of X-ray cataract (Pirie, van

Heyningen & Boag, 1953; van Heyningen, Pirie & Boag, 1954). The changes may not be primary effects, however, since their onset occurred some weeks after irradiation. Since changes in $\sim P$ may be an early result of irradiation, we have examined the effect of X-rays on the phosphate compounds of the lens.

We have used rabbits of 14–28 days in this study for two reasons. First, it is known that the concentration of high-energy phosphate compounds is greater in the lens of the young animal than it is in the old (Nordmann & Mandel, 1952; Müller & Kleifeld, 1953) and, secondly, the development of cataract for a given dose of X-rays is far quicker in the young (Cogan & Donaldson, 1951). Both these characteristics are probably related to the higher proportion of dividing cells and nucleated fibres in the young lens. The irradiated lens was compared directly with its normal fellow, as it has been shown that the composition and weight of the two lenses of a single rabbit are very similar, although values may differ widely from animal to animal.

A known early change in the rabbit lens after X-ray treatment is a fall in glutathione (Pirie *et al.* 1953; van Heyningen *et al.* 1954). We have therefore estimated glutathione in some of the lenses, considering that any change in it was a yard-stick by which to assess other changes.

* Part 2: van Heyningen, Pirie & Boag (1954).

METHODS

Irradiation. Dutch rabbits were used. They were anaesthetized by an intraperitoneal injection of sodium pentobarbitone (Nembutal) and placed in a sitting position. The X-ray beam of diameter 1.4 cm. was directed vertically from above on the right eye of the rabbit, which was placed so that the judged position of the lens was in the centre of the path of the beam. With this position of the rabbit and this diameter and direction of the beam, little or no radiation reached the left lens. The rabbits were used when 13–17 days old, the right eye of each of the young in a litter being irradiated with a dose of 1000 r. Otherwise the technique used was the same as before (Pirie *et al.* 1953).

Preparation of lens extracts: (1) *Liquid air.* The rabbit was deeply anaesthetized with Nembutal, and then dropped head first into liquid air. The eyes were chiselled out of the head and the lens dissected out from each eye, dropped into a tared tube containing 1.0 ml. ice-cold 5% (w/v) trichloroacetic acid (TCA) and ground up. The different parts of the eye separated as the temperature rose slightly, and it was possible to get the lens into the acid before it melted. (2) *Ice-cold.* The rabbit was killed by Nembutal injection and the eyes were immediately removed and placed in ice-cold water for a few minutes. Each lens was then dissected out as quickly as possible and ground up in 1.0 ml. ice-cold 5% TCA.

The lens was weighed by difference in the TCA after it had been ground. The suspension was then centrifuged at 7000 *g* for 5 min. The supernatant fluid was removed, the largest possible sample was added to 0.3 ml. aminotrihydroxymethylmethane buffer at pH 7.4, 0.1M, and the extract neutralized with 2% (w/v) KOH from a burette. The extract was kept chilled throughout, and after neutralization was stored at -5° . All determinations were completed within a few hours of extraction of the lens.

Phosphorylated sugars and the energy-rich phosphate groups ($\sim P$) of adenosine triphosphate (ATP) and adenosine diphosphate (ADP). These were determined by the enzymic method of Slater (1953). As little as $0.02 \mu\text{mole}$ $\sim P$ can be estimated with considerable accuracy by this method. It is therefore very suitable when, as in this case, only a small amount of material is available. The method depends on the enzymic formation of dihydroxyacetone phosphate, which oxidizes reduced diphosphopyridine nucleotide (DPNH) in the presence of glycerophosphate dehydrogenase. The amount of DPNH oxidized was measured spectrophotometrically, the reaction being almost complete in 3–5 min. The following reactions occur:

(1) Glucose 1-phosphate $\rightarrow$ glucose 6-phosphate $\rightarrow$ fructose 6-phosphate (phosphoglucomutase and hexose monophosphate isomerase).

(2) Fructose 6-phosphate + ATP $\rightarrow$ hexose diphosphate (HDP) + ADP (phosphohexokinase).

(3) 2ADP $\rightarrow$ ATP + adenylic acid (myokinase).

(4) HDP $\rightarrow$ glyceraldehyde phosphate + dihydroxyacetone phosphate (aldolase).

(5) Glyceraldehyde phosphate $\rightarrow$ dihydroxyacetone phosphate (triose phosphate isomerase).

(6) Dihydroxyacetone phosphate + DPNH $\rightarrow$ glycerophosphate + DPN $^+$ (glycerophosphate dehydrogenase).

The overall reaction is as follows:

where HMP is the sum of the three hexose monophosphates, and $\sim P$ represents the high-energy phosphate groups of ATP and ADP (and creatine phosphate; see below).

All the enzymes necessary for the reactions are contained in two preparations from rabbit muscle (Racker, 1947; Slater, 1953).

The method can be used in two ways: (a) in the presence of excess of $\sim P$ the disappearance of DPNH is a measure of the total concentration of HMP, HDP, pyruvic acid, phosphopyruvic acid and phosphoglyceric acid (PGA). These two last are converted by the enzymes present into pyruvic acid, which oxidizes DPNH in the presence of lactic dehydrogenase; (b) in the presence of excess of HMP

Fig. 1. Oxidation of DPNH by Slater's procedure (b). The complete reaction mixture contained DPNH ($0.22 \mu\text{mole}$ approx.), glucose 6-phosphate ($0.5 \mu\text{mole}$), ATP ($0.01 \mu\text{mole}$), CP ($0.03 \mu\text{mole}$ approx.), MgCl_2 ($7.5 \mu\text{moles}$), aminotrihydroxymethylmethane buffer pH 7.6 ($30 \mu\text{moles}$), suitable dilutions of creatine phosphokinase and Slater's two muscle enzyme extracts; final volume 3.0 ml. Curve 1, complete mixture; curve 2, complete mixture + 10^{-5}M iodosobenzoate; curve 3, complete mixture + 10^{-4}M iodosobenzoate; curve 4, complete mixture + 10^{-3}M iodosobenzoate; curve 5, complete mixture + 10^{-2}M iodosobenzoate; curve 6, CP omitted; curve 7, ATP and CP omitted (i.e. blank). $d_4 - d_1$ gives decrease in optical density due to ATP, $d_3 - d_1$ that due to ATP + CP and $d_2 - d_1$ that due to CP.

the disappearance of DPNH is a measure of $\sim P$ contained in ATP and ADP and of HDP, PGA, phosphopyruvic and pyruvic acids. We have shown by method (a) that only small amounts of these last four compounds are present in lens extracts (see Results) and hence the estimation gives a measure of the $\sim P$ of ATP and ADP in lens.

Creatine phosphate (CP). This was included in the substances determined in procedure (b) by the addition of creatine phosphokinase prepared from rabbit muscle (Kuby, Noda & Lardy, 1954). Insufficient creatine phosphokinase is present in the original enzyme preparations.

There was sufficient material from a single lens for most estimations to be done in duplicate. The lens weights ranged from about 90 mg. at 14 days to about 140 mg. at 28 days old. The results are calculated in terms of 100 g. wet weight of lens.

RESULTS

Estimation of $\sim P$ of ATP, ADP, and CP

Total high-energy phosphate was determined by procedure (b) and the results are given in Table 1.

Table 1. *High-energy phosphate in normal and irradiated rabbit lens*

The right eye of the irradiated rabbits received 1000 r.

Normal rabbits	Days from irradiation	Age at death (days)	$\sim P$ (μ m-moles/lens)		Difference (%) $\left(\frac{R-L}{L} \times 100\right)$
			R	L	
A	—	13	626	614	+2.0
B	—	14	642	616	+4.2
C	—	14	614	642	-4.4
D	—	15	693	705	-1.7
E	—	17	662	677	-2.2
F	—	21	618	675	+7.5
G*	—	28	608	596	+2.0
Irradiated rabbits					
46	0.25	13	620	654	-5.2
47	1	18	677	669	+1.2
48*	1	16	417	475	-12.2
49	1	16	648	680	-4.7
50	2	19	862	891	-3.3
51*	2	17	536	579	-7.4
52	2	17	632	657	-3.8
53	3	18	710	735	-3.4
54	4	17	640	600	+6.7
55*	4	19	640	663	-3.5
56	4	21	628	825	-23.9
57	6	23	925	963	-3.9
58	7	24	796	784	+1.5
59*	7	22	677	755	-10.3
60*	9	26	590	783	-24.6

* Killed with liquid air.

Creatine phosphate, in the presence of the kinase, reacts with ADP to form ATP, and the enzymic analysis of $\sim P$ will therefore include creatine phosphate. It was found that potassium *o*-iodosobenzoate inhibited creatine phosphokinase completely when at a concentration of $10^{-3}M$, without affecting the activity of the muscle enzymes; therefore by addition of iodosobenzoate to the enzyme preparations a value for $\sim P$ of ATP and ADP alone could be obtained. Thus to get a value for creatine phosphate alone two estimations are necessary, one of total $\sim P$ and one of $\sim P$ without CP. Fig. 1 shows the results of such an experiment.

Creatine and creatine phosphate. These were also determined chemically by the method of Ennor & Rosenberg (1952).

Inorganic phosphate. This was determined by the method of Fiske & Subbarow (1925).

Glutathione. This was determined in the TCA extracts before neutralization, by the method of Grunert & Phillips (1951).

The numbers of the irradiated rabbits are consecutive with those in the two previous papers in the series (Pirie *et al.* 1953; van Heyningen *et al.* 1954). The table shows that, with four exceptions, the differences in high-energy phosphate between 15 pairs of irradiated and normal lenses were no greater than the differences found between pairs of normal lenses. A significant fall of $\sim P$ occurred in the irradiated lens of only one rabbit during the first 3 days after irradiation (rabbit 48; decrease 12.2%). From 4 to 9 days after irradiation decreases of 23.9, 10.3 and 24.6% were found in three rabbits; in the remaining four rabbits there was no significant change. One can say therefore that no systematic fall in $\sim P$ can be detected immediately after or within a few days of irradiation. We have found that there is a great fall in $\sim P$ in complete X-ray cataract (unpublished results); this confirms

the work of Nordmann & Mandel (1953) on diabetic cataract in rats, and it is probable that the falls observed at 4-9 days after irradiation are the beginning of this decline. Rabbits 21 days old irradiated with 1500 r show the first signs of

creatine in bovine and rabbit lens (Table 3) showed that part is in a combined form, presumably creatine phosphate, but the amount is too small for determination enzymically in the presence of large amounts of other reactive phosphorus compounds.

Table 2. *Creatine phosphate in rabbit lens and brain, determined enzymically*

The right eye of the irradiated rabbits received 1000 r.

	Days from irradiation to death	Age at death (days)	Creatine phosphate in lens				Creatine phosphate in brain (μ moles/100 g.)
			$(\mu\text{m-moles/lens})$		$(\mu\text{moles/100 g.})$		
			R	L	R	L	
			Normal rabbits				
<i>F</i>	—	17	38	3	37	2	—
<i>G*</i>	—	28	15	40	11	30	140
<i>H</i>	—	<i>ca.</i> 440	108	98	18	18	—
Irradiated rabbits							
47	1	18	33	24	28	18	—
48*	1	16	30	13	33	13	—
50	2	19	44	44	33	34	—
51*	2	17	72	111	71	99	—
56	4	21	0	25	0	20	—
55*	4	19	32	0	29	1	—
59*	7	22	51	21	41	17	—
60*	9	26	51	0	29	0	282

* Killed with liquid air.

Table 3. *Creatine and creatine phosphate in normal rabbit and bovine lens*

Animal	Free creatine (μ moles/100 g.)	Creatine phosphate (μ moles/100 g.)	
		Chemical determination	Enzymic determination
Calf	58	18	—
Cow	6	9	—
Rabbit			
12 days old* (4 lenses)	34	5	9
15 weeks old (2 lenses)	12	3	—

* Killed with liquid air.

cataract 15-20 days later (Cogan & Donaldson, 1951); therefore a nine-day period is a considerable part of the time needed for clinical cataract production.

Estimation of creatine phosphate

An attempt was made to determine creatine phosphate in lens by enzymic methods using *o*-iodosobenzoate as an inhibitor of creatine phosphokinase. Creatine phosphate could be satisfactorily determined in brain in this way, but the results of the determinations in lens were quite erratic, the apparent creatine phosphate being as great in a lens that had been extracted at room temperature as in one that had been frozen (Table 2). Chemical determination of free and combined

Determination of HMP, HDP, PGA, phosphopyruvic and pyruvic acids

Procedure (a) was used to determine these substances in lens extracts. Table 4 shows that there is no consistent difference between irradiated and normal lens. The total amount of these substances in the lens is small, and the accuracy of the determination is therefore lower than that of $\sim$ P.

Determination of inorganic phosphate

The inorganic phosphate in normal lens is a small fraction of the total and we considered that it was worth while to determine whether any change took place in this fraction after irradiation. One complete lens had to be used for each estima-

tion, and Table 5 shows that it was impossible to detect any consistent change in inorganic phosphate after irradiation.

Determination of glutathione (GSH)

Table 6 sets out the values obtained in normal and irradiated lens. In these young rabbits, there

does seem to be a very early fall in GSH. In older rabbits the fall in GSH is a later change but opacity formation is also a far more prolonged process. The rise in total GSH with lens growth, which is very rapid at this stage, can be readily seen from Table 6, and may be contrasted with the much more constant content of ~P (Table 1).

Table 4. *Hexose monophosphates and hexose diphosphate, phosphoglyceric, phosphopyruvic and pyruvic acids in normal and irradiated lenses of rabbits*

The right eye of the irradiated rabbit received 1000 r.

Irradiated rabbits	Days from irradiation to death	Age at death (days)	HMP, HDP, etc. ($\mu\text{m-moles/lens}$)		Difference (%) $\left(\frac{R-L}{L} \times 100\right)$
			R	L	
46	0.25	13	41	43	-4.7
54	4	17	56	56	0
57	6	23	36	34	+5.9
58	7	24	38	49	-22.4
60	9	26	35	40	-12.5

Table 5. *The inorganic phosphate of normal and irradiated lenses of rabbits*

The right eye of the irradiated rabbit received 1000 r.

	Time from irradiation to death	Age at death (days)	Inorganic P ($\mu\text{m-moles/lens}$)		Difference (%) $\left(\frac{R-L}{L} \times 100\right)$
			R	L	
Normal rabbits					
<i>J</i>	—	14	303	295	+2.7
<i>K*</i>	—	15	333	344	-3.2
<i>L*</i>	—	15	349	363	-3.9
<i>M</i>	—	19	324	325	-0.3
Irradiated rabbits					
61*	5 hr.	16	331	308	+7.5
62*	6 hr.	16	338	358	-5.6
63*	1 day	17	312	323	-3.4
64*	2 days	18	383	351	+9.1

* Killed with liquid air.

Table 6. *Glutathione in normal and irradiated lenses of rabbits*

The right eye of the irradiated rabbits received 1000 r.

	Days from irradiation to death	Age at death (days)	Glutathione ($\mu\text{m-moles/lens}$)		Difference (%) $\left(\frac{R-L}{L} \times 100\right)$
			R	L	
Normal rabbits					
<i>A</i>	—	13	730	710	+2.8
<i>B</i>	—	14	772	755	+2.2
<i>C</i>	—	14	710	693	+2.5
<i>D</i>	—	15	837	856	-2.2
<i>E</i>	—	17	997	964	+3.4
Irradiated rabbits					
49	1	16	644	768	-9.6
51*	2	17	823	935	-12.0
52	2	17	752	827	-9.1
53	3	18	1000	1082	-7.6
55*	4	19	1144	1240	-7.8
58	7	24	1637	1726	-5.2
59*	7	25	1387	1700	-18.4

* Killed with liquid air.

Relation between loss of GSH and loss of $\sim P$

In Fig. 2 the percentage difference in glutathione between the right and left lenses of normal and irradiated rabbits is plotted against the difference in $\sim P$.

Fig. 2. Relation between percentage difference of GSH and that of $\sim P$ in the right and left lenses of normal and irradiated rabbits. $\circ$, normal; $\times$, irradiated.

DISCUSSION

Several points of interest arise from these results. The enzymic method makes it possible to determine the high-energy phosphate groups of ATP and ADP, the phosphate esters and glutathione in a single lens of weight about 100 mg. Unfortunately the variation between the $\sim P$ of the two lenses of a normal rabbit appears to be sufficiently large to make it impossible to detect small changes in $\sim P$ after irradiation (Table 1). There is less variation in glutathione content of the two lenses of a normal rabbit and this makes it possible to detect small changes. The results seem to show that there is a fall in glutathione 24 hr. after irradiation (Table 5). If we plot percentage difference in glutathione between normal and irradiated lenses against percentage difference in $\sim P$ (Fig. 2) the points lie on a straight line, loss in one being proportional to loss in the other; no such proportionality exists between the differences in GSH and in $\sim P$ of pairs of normal lenses. Perhaps this is a suggestion that $\sim P$ is in fact diminishing within the first few days after irradiation, although the enzymic method cannot detect this. The number of results is small but it did not seem useful to do more experiments. The point of interest is the early change and it is not possible to establish small decreases of $\sim P$ owing to the normal variation.

The results reported here, with the enzymic method of phosphate estimation, differ consider-

ably from those obtained by the chemical fractionation described by Umbreit, Burris & Stauffer (1949), which has been so generally used. Nordmann & Mandel (1952), using Umbreit's method, have found a total of about 400 μ moles P/100 g. rabbit lens of hexose monophosphate, hexose diphosphate and phosphoglycerate, whereas the enzymic method, which we consider to be more specific, gives a value of only 25–50 μ moles P/100 g. of these phosphate esters. The enzymic method of estimation includes in this fraction phosphopyruvic and pyruvic acids, so that our values do, if anything, overestimate the amount of phosphate esters present. Kratzing & Narayanaswami (1953) noticed the same difference between the phosphate esters of brain estimated enzymically and the values reported by chemical fractionation.

Table 7. Comparison of values obtained, by different methods of estimation, for certain phosphate compounds in tissues

The enzymic method of Slater (1953), procedure (a) (see Methods), was used. The values obtained by chemical fractionation are taken from published results. Animals were killed by immersion in liquid air unless otherwise stated. Results expressed as μ moles P/100 g. wet wt.

Tissue	Enzymic analysis (HMP, HDP, PGA, phospho- pyruvic and pyruvic acid)	Chemical analysis (HMP, HDP, PGA)
Skeletal muscle (rat)	572	—
	547	—
		517*
Kidney (rat)	11	—
	16	—
	22	—
Liver (rat)		433*
	115	—
	75	—
Heart (rat)	92	—
		706*
	244	—
Brain (guinea pig)	135	—
	76	—
		700*
Brain (rat)	26–33†	—
	—	386*
Lens (rabbit)	25-day	—
	7-month	—
	2-year	—
	3-month	402‡
	Old	> 274‡

* LePage (1948).

† Kratzing & Narayanaswami (1953).

‡ Nordmann & Mandel (1952). Eyes rapidly removed and refrigerated after death of animals; 28 lenses pooled for analysis.

In an attempt to determine the significance of these differences we have estimated the phosphate-ester fraction in various tissues of the rat and compared our results with the published figures given by Umbreit's method. Table 7 shows that with skeletal muscle similar results are given by both methods, but in liver, heart and kidney there appears to be very much less of this fraction when it is estimated by the enzymic method than when it is estimated in the standard chemical way. These results show that lens is not unique in this respect. The discrepancy between chemical and enzymic determinations is a large one; in the lens, for example, about 350 μ moles P/100 g., which was estimated as phosphate esters by the chemical method, now appear to be unaccounted for. We are investigating the problem further.

Creatine phosphate measured by the chemical method of Umbreit *et al.* (1949) gave the not insignificant value of 86 μ moles P/100 g. of young rabbit lens (Nordmann & Mandel, 1952), although the animals were not killed in liquid air and the lenses were refrigerated until 28 lenses had been collected for a pooled analysis. The enzymic method gave very variable values, between 0 and 99 μ moles CP/100 g. lens (Table 2) and precautions which usually are necessary to preserve this labile compound had no effect. It is therefore questionable whether either the chemical method of Umbreit *et al.* (1949) or the enzymic method of analysis is actually measuring creatine phosphate in the lens. The method of Ennor & Rosenberg (1952), in which the difference between free and bound creatine is measured, probably gives the most reliable value for creatine phosphate, 3–5 μ moles/100 g. rabbit lens (Table 3).

SUMMARY

1. The effect of irradiation on the organic phosphate compounds in the lens of the very young rabbit has been studied by the enzymic method of Slater (1953).

2. No change could be detected in the high-energy phosphate groups of adenosine triphosphate and adenosine diphosphate, or in hexose monophosphates, hexose diphosphate, phosphoglycerate

or inorganic phosphate within a few days of irradiation.

3. A method for the enzymic determination of creatine phosphate has been worked out, but the amount in lens is too small for satisfactory determination.

4. A fall in glutathione was detected 24 hr. after irradiation.

5. The hexose phosphate and triose phosphate fraction determined enzymically is considerably less than the same fraction when determined chemically by the method of Umbreit *et al.* (1949).

6. This discrepancy between results given by enzymic and chemical methods is found in other tissues besides lens.

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