Examination of Diurnal Variation in Lethally Irradiated Rats

Abstract. *In anesthetized white rats there is no significant diurnal variation in lethality of an x-ray dose that killed 100 percent of the rats in 30 days.*

The remarkable diurnal variation in radiation lethality of anesthetized white rats reported by Pizzarello *et al.* (*1*) is of great interest. An effect of this magnitude might complicate interpretation of many radiation lethality studies. I have, therefore, attempted to confirm this observation, employing larger numbers of animals. Ninety female Sprague-Dawley rats, weighing 172 ± 3 g, were anesthetized with sodium pentobarbital (30 mg/kg, intraperitoneally) and irradiated two at a time with 900 r. Radiation was delivered with a GE Maximar 250-kv unit; radiation factors: target distance, 40.6 cm, 0.75 mm Cu + 1.0 mm Al, ma 15, half-value layer = 1.98 mm Cu, 87.29 r/min; or a Norelco Phillips 300-kv unit (Muller MH301) was used; radiation factors: target distance, 48.6 cm, 0.5 mm Cu + 1.0 mm Al, 10 ma, half-value = 1.98 mm Cu, 90.11 r/min. The experimental groups were replicated, 23 being irradiated at 9 A.M. and the same number at 9 P.M. on 8 March 1963, and another 22 at both times on 4 April 1963. Both machines were used in each experiment.

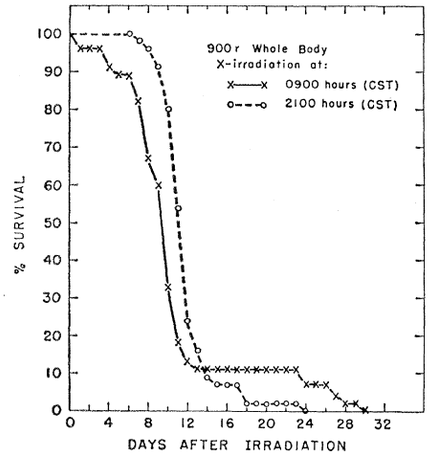

Fig. 1. The mortality response of anesthetized Sprague-Dawley female rats subjected to 900 r of whole-body x-irradiation. $N = 45$ animals for each group.

Table 1. Survival of Sprague-Dawley female rats after 900 r of whole-body x-irradiation.

Days after irradiation	Irradiated at			
	0900 hours		2100 hours	
	S/T*	%	S/T*	%
	Experiment 1			
0	23/23	100	23/23	100
5	20/23	87	23/23	100
10	5/23	22	19/23	83
15	2/23	9	1/23	4
20	2/23	9	1/23	4
25	2/23	9	0/23	0
30	0/23	0		
	Experiment 2			
0	22/22	100	22/22	100
5	20/22	91	22/22	100
10	10/22	45	17/22	77
15	3/22	14	2/22	9
20	3/22	14	0/22	0
25	1/22	4		
30	0/22	0		

* S/T = ratio of survivors to total.

Table 1 shows the mortality responses on the two individual experiments, and from Fig. 1, which summarizes both experiments, there is no significant difference in radiation mortality between the animals irradiated in the morning and those irradiated in the evening. The shapes of the survival curves suggest that the dose used is very close to the LD_{100} for 30 days (*2*).

The discrepancy between these results and those previously reported (*1*) is difficult to explain. The protocol differed from that of the previous investigators in four ways; (i) rats were maintained on a 12-hour on, 12-hour off light cycle (6 A.M. to 6 P.M.), rather than a 9-hour light, 15-hour dark cycle; (ii) prior and subsequent to irradiation the rats were housed five to a cage, rather than individually; (iii) the experiments were performed in March and April rather than June and August; and (iv) Sprague-Dawley rather than Nelson (CFN) strain females were used. Although these factors might conceivably have some influence, it is difficult to see how they could alter a completely lethal to a completely nonlethal response. Mean survival time of the two groups in my study differs by a single day, and this difference is not statistically significant. Thus, no diurnal variation was observed in sensitivity of white rats to x-irradiation in the LD_{100} for 30 days range, in contrast to the work recently reported (*3*).

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References and Notes

1. D. J. Pizzarello, R. L. Witcofski, E. A. Lyons, *Science* **139**, 349 (1963).
2. In order to duplicate the conditions of the experiment of Pizzarello *et al.* (*1*), anesthetized rats were used. In this laboratory unanesthetized rats are used routinely; therefore, LD_{50} values for 30 days for anesthetized rats are not available.
3. Performed under the auspices of the U.S. Atomic Energy Commission.

30 August 1963

Lipid-Protein Particles: Isolation from Seeds of Gossypium hirsutum

Abstract. *Subcellular particles were isolated from cottonseed by tanning of the cell contents followed by differential centrifugation. The particles, high in protein content and containing approximately 28 and 44 percent lipids, are thought to be the site of oil storage and lipid synthesis.*

Numerous seeds have a high lipid content, yet the exact location of the oil within the storage tissue is subject to question; one example is the cottonseed, which contains over one-third lipid in the kernel. Leahy (*1*) was unable to see free oil droplets in cottonseed sections and considered the oil "to occur within the cell as a cytoplasmic emulsion." This concept is in general agreement with the views cited by Tharp (*2*). We confirm these observations. When cottonseeds are sectioned under conditions which prevent spreading of oil, droplets of free oil are not observed.

Free-hand sections of cotyledon parenchyma cells of cottonseed show numerous spherical bodies 1 to 4 μ in diameter (Fig. 1*A*). These are similar to protein bodies, described for many seeds (*3*), which have been isolated from peanut (*4*) and pea cotyledons (*5*) and from wheat endosperm (*6*). In no instance, however, have such particles been reported to contain lipids. It was of interest, therefore, to determine whether the bodies observed in the cottonseed might also be the site of oil storage.

The classical methods of isolating subcellular particles were inadequate for isolation of the spherical bodies in cottonseed. Grinding media which ranged from hypotonic to hypertonic were tried, but most of the bodies were destroyed when the seeds were ground. However, pure glycerol as a grinding medium maintained the integrity of the bodies for extended periods. The bodies stained positively with Kiton pure blue V, indicating that

they were proteinaceous. Water added to the glycerol to change the density caused a derangement of the particles. Similarly, an ethanol solution of Sudan 4 destroyed the particles when introduced to glycerol mounts under the coverslide.

Figure 1*B* shows a small group of bodies in glycerol after the introduction of an ethanol solution of Sudan 4; several of the bodies are swollen and one is about to burst. Figure 1*C* shows the free oil droplet which appeared after the body had burst. The agglutinated proteinaceous material which surrounds the free oil droplet is noteworthy. The appearance of free oil upon disintegration suggested that the intact bodies indeed contained oil.

Since the conventional methods of isolating and handling these subcellular bodies were inadequate, an attempt was made to reduce their fragility by treating them first with tannic acid. Particles isolated by grinding in glycerol containing 5 percent tannic acid were amenable to subsequent manipulation in aqueous solvents. By differential centrifugation of the macerated material, two fractions, both of which contained lipid, were isolated. A fraction sedimenting at 1000*g* in 10 minutes consisted of a homogeneous population of spherical bodies which seem to have an internal structure (Fig. 1*D*). The bodies could be stained with an alcoholic solution of Sudan 4, without being disrupted. They contained from 25 to 29 percent lipids extractable with ethyl ether and in the lipid-free residue there was 55 percent protein. It is estimated that they comprise from 50 to 60 percent of the volume of the cells. A second fraction was obtained from the supernatant by centrifuging for ½ hour at 51,000*g*. Analysis of the pellets so obtained showed 44 percent lipids on a dry-weight basis and 50 percent protein in the lipid-free material. It is possible to account for the lipid content of cottonseed, which is about 35 percent, by the composition of these two particles.

The particulate material obtained at the higher centrifugal forces could originate from the "bodies" by rupture during their isolation procedure, or perhaps they might be specific subcellular particles located in the interbody areas of the cell. For the moment, this question is rather a moot point.

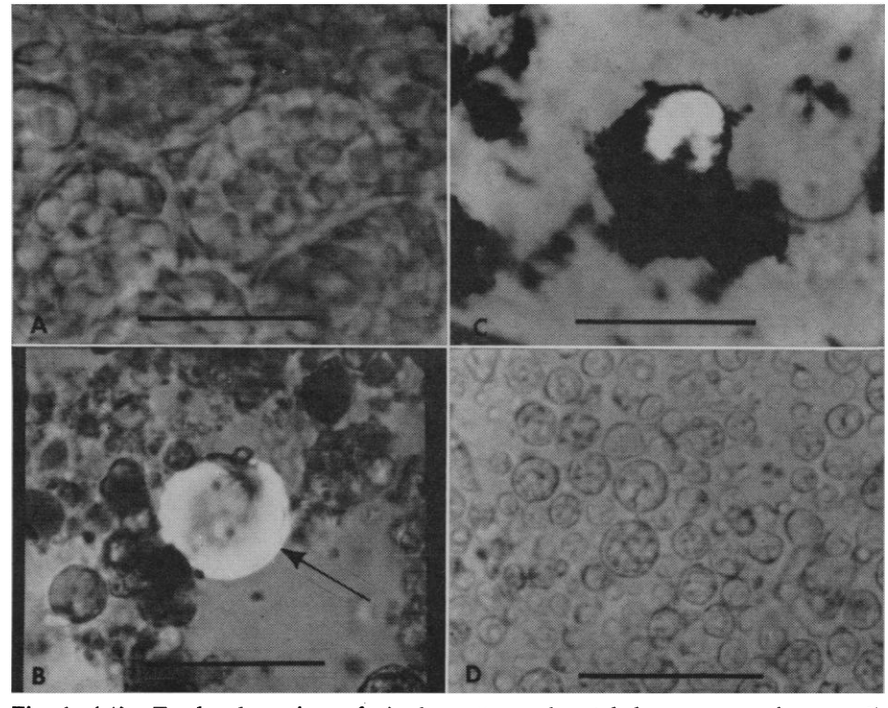

Fig. 1. (*A*) Freehand section of Acala cottonseed cotyledonary parenchyma cell. The section was immersed in glycerol, stained with Kiton blue, and viewed under oil immersion on the light microscope. Note the many spherical subcellular bodies within the cell; these stained blue. Bar represents 10 μ. (*B*) A view of the spherical subcellular bodies obtained by homogenizing the cottonseed in glycerol. A drop of glycerol homogenate was placed under a coverslide, stained with Kiton blue, and counterstained with an ethanol solution of Sudan 4. Note the body which was swollen and is about to burst (see arrow). (*C*) The same general view as in 1*B* several moments later. The discrete spheres have disintegrated into agglutinated masses and free oil droplets have appeared. (*D*) A sample of homogeneous spherical bodies which were obtained by differential centrifugation after tanning treatment. These were stained with Kiton blue and counterstained with Sudan 4. Note the internal structure.

Possibly, the treatment with tannic acid might produce lipid-protein combinations which are artifacts. When cottonseed kernels were homogenized in glycerol-tannic acid, diluted with water and centrifuged, no fatty layer was observed; but when tannic acid was omitted, there was a layer. If the entire contents of the centrifuge tube containing the fatty layer were rehomogenized thoroughly with the correct proportion of tannic acid and recentrifuged, the fatty layer reappeared. The mere treatment of protein by tannic acid in the presence of lipids does not bind the lipids. We are forced to assume that treatment with tannic acid is preserving a lipid-protein relationship existing in the seed.

That tannic acid treatment preserves the integrity of the lipid-protein relationship in cottonseed raises several points. First, it broadens our concept of protein particles. These are now shown in the cottonseed to contain lipid; it might be that a similar situation will hold for other oil-bearing seeds. Secondly, it raises the question of whether some of the proteins in these lipid-rich particles might participate in the biosynthesis of the lipids. Lynen (*7*) has visualized the fatty acid synthetase as a single unit composed of many enzymes whose geometry and proximity are so arranged to facilitate the synthesis of fatty acids. The possibility occurred to us that the particles which we have isolated might contain functional units such as Lynen envisions (*8*).

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8. One of us (L.Y.) is a postdoctoral research associate, funds provided by the National Cottonseed Products Association.

25 September 1963

Teratogenic Significance of Ionic and Fluid Imbalances

Abstract. *Some agents teratogenic to the chick embryo cause serum electrolyte and fluid imbalances which initiate an edema syndrome leading to malformations. Differences in ionic composition of serum and yolk of normal chicks help explain how these imbalances can be produced.*

A study of the effects of moderate hypoxia on young chick embryos has shown that many of the induced malformations can be attributed to a disturbance in fluid balance (*1*). This disturbance, which characterizes the edema syndrome, includes a marked increase in circulating and extracellular fluids, followed by the formation of clear blisters and hematomas which cause malformations mechanically. The edema syndrome can also be induced by Trypan blue (*2*) and injections of 0.01 to 0.02 ml of fresh egg albumen into the allantois of 4- to 5-day chick embryos. The latter can induce malformations in 20 percent (30 out of 149) of embryos treated at 4 days and 9 percent (9 out of 91) at 5 days (*3*). Less than 2 percent malformations were found in 225 controls that were injected with saline. All three agents can induce identical anomalies, indirectly caused by blisters, when applied to embryos of the same age. Because changes in the fluid volume of the embryo initiate the edema syndrome, we decided to study the composition of embryonic fluids in normal and experimental chicks.

We made assays of Na^+ and K^+ in normal extracellular and extra-embryonic fluids of the 5-day chick embryo (Table 1). These fluid compartments can be divided into two groups on the basis of the concentration of these electrolytes. Albumen and yolk, which are separated from the embryo by cellular membranes, are very high in K^+, and low in Na^+ and in total Na^+ and K^+. Blood serum, cerebrospinal, chorionic, and amniotic fluids are low in K^+, and high in Na^+ and in total Na^+ and K^+. The fluid of the allantois, which has an opening into the yolk sac, is intermediate. As might be expected from the data of Table 1, but contrary to the popular assumption that these fluids have equivalent osmotic pressures (*4*), we found that the depression of freezing point of albumen and subgerminal yolk were markedly lower than that of normal serum (*5*). These observations show that the osmotic relationships of the chick embryo to its surrounding fluids are more complex than generally believed.

Because of these differences between the internal and external fluids of the chick embryo we felt that fluid and ionic imbalances could be induced by a variety of physiological stimuli, so we exposed embryos to several teratogenic agents and tested the serum (Table 2). Saline-injected controls showed an increase in the concentration of potassium ions but no changes in the concentration of sodium ions. This change is probably the result of mild trauma from opening the egg. Occasional malformations are also found in these controls. Embryos subjected to hypoxia (*6*) or treated with albumen or Trypan blue, consistently showed a twofold increase in serum potassium and a significant decrease in serum sodium. The decrease in the concentration of sodium ions compared with that in normal sera, when the embryos were subjected to hypoxia or to treatment with albumen, was significant at the 0.1 percent level (*t*-test); when treated with Trypan blue, the decrease was significant at the 3 percent level. The lower total concentration of these two ions in experimental sera helps to interpret the edema which results from these treatments. The depression of freezing point of the serum of embryos exposed to hypoxia is substantially lower than that of normal serum (*5*).

The data clearly demonstrate that fluid and electrolyte imbalances are produced in the embryo by hypoxia, albumen, and Trypan blue. The edema syndrome is a common factor to all of them and leads to the development of similar malformations.

Although these data cannot be assumed to apply directly to the mammal, we feel a similar study would be a profitable approach to problems of mammalian teratology, because effects comparable to the edema syndrome have been described many times in mice and rats, both after the administration of Trypan blue (*2*) and vasopressin (*7*), and in deficiencies of linoleic and pantothenic acids (*8*), as well as in genetic mutants (*9; 10*).

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Table 1. Sodium and potassium in fluids of normal 5-day chick embryos. The results are expressed as milliequivalents per liter. Determinations were made on a Beckman DU spectrophotomter with a flam attachment, subsequent to perchloric acid digestion.

Fluid	No. of assays*	Na^+		K^+		Total Na^+ and K^+
		Av.	S.D.	Av.	S.D.	
Albumen	**7**	65	13.0	61.3	7.5	126
Subgerminal yolk†	**6**	98	1.6	19.4	3.72	117
Allantois	**7**	115	10.6	9.0	1.14	124
Blood serum	**15**	136	9.0	3.2	0.55	139
Cerebrospinal fluid	**7**	131	11.1	4.0	1.38	135
Chorion	**6**	133	5.5	4.1	0.79	137
Amnion	4	131	6.2	2.2	0.25	133

* Each sample obtained from different chicks except for serum and cerebrospinal fluid which were samples pooled from 2 to 3 embryos. † Clear yolk immediately underneath embryo.

Table 2. Serum sodium and potassium in 5-day chick embryos. The results are expressed as milliequivalents per liter. Experimental treatments: Hypoxia, 10.5 percent O_2 for 6 hours (*6*); fresh egg albumen, 0.02 ml injected into allantois; Trypan blue, 0.06 ml of a 0.1 percent solution injected into yolk sac; and NaCl, 0.02 ml of 0.85 percent saline injected into allantois. The serum samples from the embryos subjected to hypoxia were obtained within 1 hour after treatment was terminated. Other samples were obtained 5 to 6 hours after injection.

Treatment	No. of assays	Na^+		K^+		Total Na^+ and K^+
		Av.	S.D.	Av.	S.D.	
None	**15**	136	9.0	3.2	0.55	139
Hypoxia	**9**	117	10.7	6.5	1.06	124
Albumen	**7**	121	6.6	5.7	1.34	127
Trypan blue	**5**	122	11.6	6.6	1.14	129
NaCl	**5**	137	10.7	5.1	0.60	142