dye which does not affect clotting because the dye from the medium in general is concentrated within the zone of mineralization.

Our investigation has not found bone phosphatase to be an important factor in alizarin inhibition of calcification. In conjunction with this finding, Siffert(12), in a publication appearing after the completion of this work, reported finding phosphatase activity associated with preosseous cellular metabolism and not with the process of calcification.

Summary. Alizarin red S has been found to stop calcification without inhibiting the enzyme phosphatase either *in situ* or when added to solutions containing the enzyme extracted from bone. It has been shown that alizarin inhibition of calcification is not due to removal of the calcium ion from the medium in general as evidenced by the facts that there is no interference with the clotting process of plasma or with the action of embryonic hearts. Supporting evidence is presented from a study of bones growing on litmus-containing medium and from bones stained with alizarin and transferred to alizarin-free medium. An explanation of alizarin stoppage of calcification based upon the idea that locally there is a drop in the pH is advanced.

Received May 3, 1951. P.S.E.B.M., 1951, v77.

12. Siffert, R. S., *J. Exp. Med.*, 1951, v93, 415.

Adenosine Triphosphate Levels in Mouse Blood after Whole-Body Irradiation and During Tumor Growth.* (18787)

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Whether the excessive release or depletion of high-energy adenosine polyphosphates is associated with pathogenesis has only recently been amenable to quantitative tests. Two pathological states in mice, both overtly characterized by wasting, were investigated in this regard: (1) that produced by whole-body X-ray irradiation; (2) that produced by a growing sarcoma implant.

Irradiation. Methods and procedure. Rockland Swiss male mice of 20-22 g in groups of 10 were simultaneously irradiated with a dual-tube X-ray machine.† A whole-body dose of 800 r was chosen as best suited to the experiment, as at this dosage a weight loss and general wasting was clearly evident after 48 hours (Fig. 1); deaths began on the

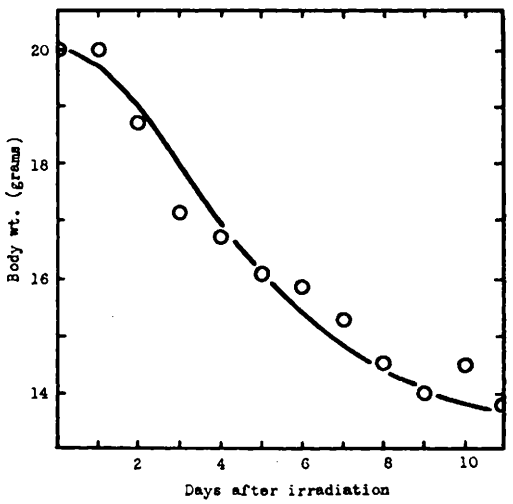

FIG. 1. Mean Body Wt of 20 Male Mice Following a Whole-Body X-ray Dose of 800 r, Plotted Against Time.

* This project was supported in part by the U. S. Atomic Energy Commission, Contract No. AT(30-1)-863.

† Made available for our use through the courtesy of the Physics Dept., Memorial Hospital, New York City. KV—180; MA—25; distance from targets to mice—30 cm; delivery—480 r/min; diameter of aluminum-grill mouse container—16.6 cm; depth of container—3 cm.

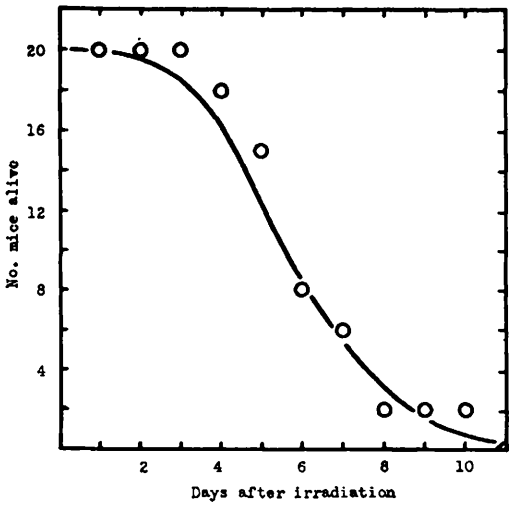

FIG. 2. Typical Survival Curve of 20 Male Mice, Following a Whole-Body X-ray Dose of 800 r.

4th day (Fig. 2), and all the mice were dead by the 11th day following irradiation. In one experiment a dose of 1000 r was used. Animals were sacrificed at various times after irradiation; hematocrit and nucleotide determinations were made on their blood. The technic for blood removal was as follows: the mice were anesthetized with parenterally injected nembutal, and the thoracic cavity was opened to expose the heart. Cardiac puncture was made with a No. 27 hypodermic needle into whose syringe 0.02 ml of an aqueous 5% heparin solution had previously been drawn. Usually about 1 ml of blood was withdrawn from each mouse, although in the advanced stages of irradiation sickness a diminution of total blood meant that less blood could be withdrawn than from non-irradiated mice. In these cases, enough mice were sacrificed from the same group to yield a total blood pool of 5 ml, the heparin content not exceeding 1 mg/ml. Neither nembutal nor the heparin, by test, interfered with the accuracy of the nucleotide assay. The analyses were carried out with 1 ml aliquots of blood, which were immediately deproteinized with 4 volumes of cold 2% perchloric acid. The protein precipitate was washed with an additional volume of cold 2% perchloric acid. The combined supernatant solutions, collected after centrifugation, were adjusted to pH 7 and made to a final volume of 10 ml. In some experiments, the cell sediment, or the serum withdrawn after centrifugation, were deproteinized and a protein-free filtrate prepared in similar fashion. Total nucleotide, and known nucleotide (adenylic acid, AA; adenosine diphosphate, ADP; and adenosine triphosphate, ATP) were assayed spectrophotometrically according to the method of Albaum and Lipshitz(1). In brief, total nucleotide was determined from the density reading at 2600 Å. The known nucleotide (AA, ADP, and ATP) levels were determined with specific enzymes. ADP and ATP were converted to AA and the latter in turn to inosinic acid in accordance with the following reactions:

$$(1)\ \text{AMP} \xrightarrow[\text{adenylic acid deaminase}]{} \text{Inosinic acid}$$

$$(2)\ 2\text{ADP} \xrightarrow[\text{Myokinase}]{} \text{ATP} + \text{AMP}$$

$$(3)\ \text{ATP} + \text{glucose} \xrightarrow[\text{hexokinase}]{} \text{ADP} + \text{glucose-6-phosphate}$$

This procedure has already been used for the measurement of these compounds in human blood(2).

Results. The alterations in mean body weight plotted as a function of days after irradiation is shown in Fig. 1. It is evident that a sharp decrease in weight begins about 24 hours after irradiation. Blood samples were assayed for total and known nucleotides at intervals of 2, 3, 4, 5, 6, and 8 days after irradiation. No consistent changes in either total nucleotide or in individual known nucleotides, as compared to non-irradiated controls, were observed. Nor did hematocrit levels change significantly during this period. The data for all the runs are shown in Table I. As indicated in the lower portion of Table I, such differences as occurred in the blood of irradiated and control mice, regarding total nucleotide and known nucleotides, are without statistical significance. Similar experiments carried out on red cells alone and serum alone revealed no significant differences between irradiated and non-irradiated mice.

1. Albaum, H. G., and Lipshitz, R., *Arch. Biochem*, 1950, v27, 102.

2. Albaum, H. G., Cayle, T., and Shapiro, A., *J. Clin. Invest.*, in press.

TABLE I. Total Nucleotide and Known Nucleotide Content of Whole Mouse Blood from Irradiated and Control Animals. The experimental values for mice sacrificed at varying times (see text) after irradiation showed no significant differences, and are therefore presented below in summated form. The figures in the "Known nucleotide" columns may be considered substantially to represent the ATP levels in the blood under test, for most of the known nucleotide was present as ATP. In only a few cases were AA and ADP detected, and their trace appearance showed no significant relation to one animal group or the other.

	Control group		Irradiated group	
	Total nucleotide	Known nucleotide (combined AA, ADP, ATP)	Total nucleotide	Known nucleotide (combined AA, ADP, ATP)
No. of mice	39	39	34	32
Mean value: mg %	13.3	7	12.8	6.1
Range	10.6–17.2	4.7–10.5	8.6–17.9	2.9–9.4
Stand. dev.	1.68	1.27	2.36	1.89
S. E. mean	.27	.20	.43	1.06
Coeff. variab.	12.7%	18.1%	19.2%	30.9%

Total nucleotide: S. E. difference .55
d–control group, exper. group .5
$$d/S.E. = \frac{.5}{.55} = .91 \text{ (no significance)}$$

Known nucleotide: S. E. difference 1.07
d–control group, exper. group .9
$$d/S.E. = \frac{.9}{1.07} = .84 \text{ (no significance)}$$

% packed cells in blood. Mean*	42.5	39.4

* Range of individual values approximately the same for both groups.

Sarcoma growth. Methods and procedure. During the course of the experiments described above, exploratory observations were made also on the nucleotide levels in the blood of mice bearing implanted tumors. Preliminary results appeared to justify more extended experiments in this direction. Accordingly, large groups of Rockland Swiss male mice, 20-22 g, were implanted with sarcoma 180, using a trocar technic(3) for subcutaneous deposition of the tumor tissue fragment in the axillary position. Groups of such tumor-bearing mice were sacrificed at daily intervals from the time of implantation to 17 days. Blood was taken under the same conditions as for the irradiated mice described above; and the same assay procedure for total and known nucleotides was followed. Recorded also at time of sacrifice were hematocrit, hemoglobin, body weight, and wet tumor weight.

Results. The alteration in body weight from the time of tumor implantation to the time of sacrifice is shown in Fig. 3. It is apparent that during the first 10 days there was no significant wasting as a result of tumor implantation and growth. The increase in tumor weight as a function of time is shown in Fig. 4. Tumor weight increased markedly from about the third day to the 12th, and then decreased. It was also observed that at about the 12th post-implantation day, many tumors ulcerated and much of their mass was ingested by the animals, leaving an open shell which was often cast off or resorbed. Such ingestion undoubtedly accounts for the drop in tumor weight during this period; while toxemic effects of the necrotizing tumor may well account for the body weight loss during this period. It should be pointed out that ulceration and tumor ingestion do not occur in all the mice. Many tumors after the 12th day

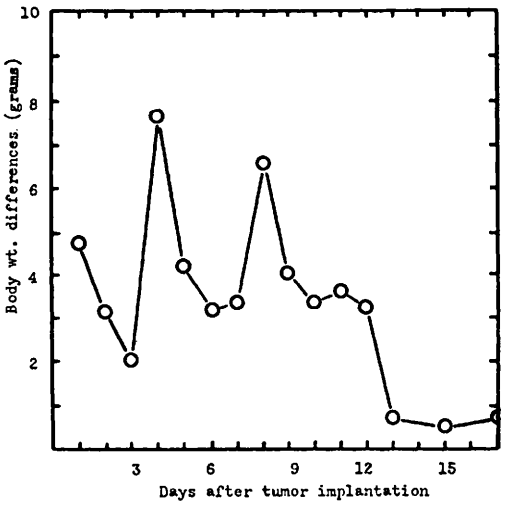

FIG. 3. Body Wt Changes in Mice Following Tumor Implantation. Points represent the differences between the mean body wt for all the mice (75) at the beginning of the experiment and the mean for each group of 5 sacrificed daily for blood determinations.

3. Zahl, Paul A., and Drasher, M. L., *Cancer Res.*, 1947, v7, 605.

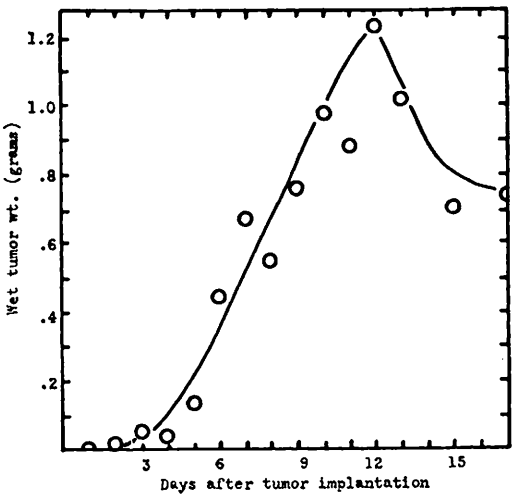

FIG. 4. Wet Wt of Tumors Plotted Against Days After Implantation. Each point represents the mean for 5 animals.

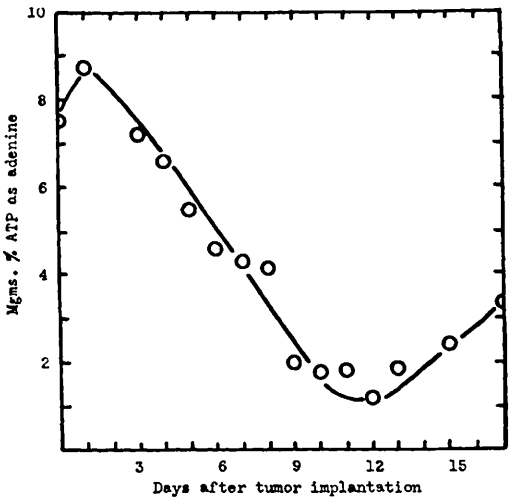

FIG. 5. ATP in the Blood of Mice Sacrificed at Daily Intervals Following Tumor Implantation. Each point represents the mean for 5 animals.

grow to massive proportions before the animal succumbs. The drop in the tumor weight curve after the 12th day obviously reflects the mean weights of both the partially ingested tumors and the non-ingested large ones.

No significant change was observed in the total nucleotide blood level during the observed 17 days of tumor growth. These data are not presented because their range and mean values were not significantly different from those in Table I for the irradiated and control mice. As to known nucleotides, there were only occasional trace amounts of AA or ADP, and these traces showed no consistent relation to one animal group or another. The bulk of the known nucleotide material was in the form of ATP and is presented as such in Fig. 5. After the first post-implantation day there appears to be a slight rise in blood ATP, followed by a sharp decrease which parallels the increase in tumor weight for this period. It appears, further, that the ATP level of the blood begins to increase again at the time when the tumor and whole body weights begin to decrease.

ATP in the blood is normally found in the red cells, not in the plasma. One may therefore argue that the decrease may be related to the hematocrit. That there is no relationship between blood ATP and hematocrit values until at least the 10th day is shown in Fig. 6. At the 10th, 11th, and 12th days when the ATP is lowest, the hematocrits are not significantly different from what they were before tumor implantation; a significant drop in the hematocrit begins with the 13th day along with the drop in body weight. There is too much variation in the hemoglobin values (Fig. 7) to justify any correlation, although the drop after the 13th day appears to be a consistent one.

If blood ATP is related to tumor growth, as would be supposed from the data of Fig.

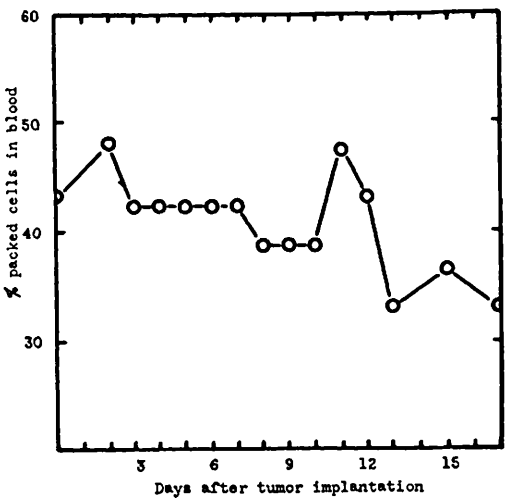

FIG. 6. Hematocrit Readings for Mice Sacrificed at Daily Intervals Following Tumor Implantation. Each point represents the mean for 5 animals.

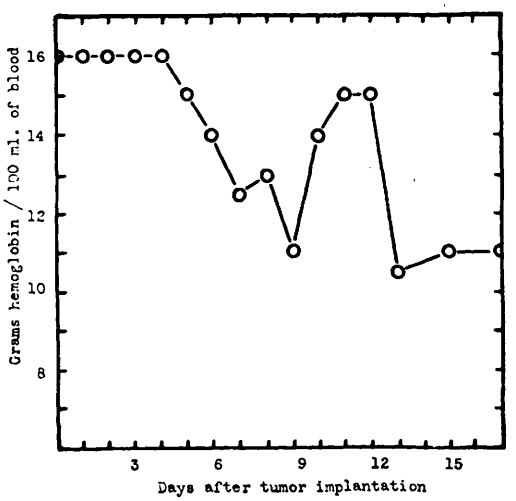

FIG. 7. Hemoglobin (Haden-Hausser) Readings for Mice Sacrificed at Daily Intervals Following Tumor Implantation. Each point represents the mean for 5 animals.

4 and 5, then tumor removal might also be expected to result in a rise of the level of blood ATP. This was tested. A technic was developed for removing five 10-day-old tumors without excision and with minimum trauma. This consisted of a single basal ligature which instantly stopped all vascular interchange between tumor and host body. Details of the technic are described elsewhere(4). One experiment consisted of extirpating 7-day tumors. After a recovery period of 2 days, the animals were sacrificed and their blood analyzed. Another experiment consisted similarly of extirpating 10-day tumors. The animals were sacrificed after a 5-day recovery period, and their blood analyzed. Not much alteration in ATP level occurred in the 2-day extirpation experiment, but considerable return toward the normal ATP level was apparent in the 5-day animals (Table II).

Discussion. We have observed no change in the nucleotide content of blood of mice undergoing post-irradiation wasting, a condition in many respects resembling shock. Green(5) reported a similarity between shock produced by injected myo-adenosine triphosphate and that produced by trauma. Meyer *et al.*(6) and McShan *et al.*(7) have proposed that the induction of shock may be favored by exhaustion of the energy store in blood and tissues represented by these purine derivatives. Kalckar and Lowry(8), on the basis of adenosine levels in the blood of traumatically shocked rabbits, concluded that adenylic acid components play no primary role in the etiology of shock; in addition, they found that the injection of adenosine deaminase, adenyl-pyrophosphatase, and alkaline phosphatases—materials expected to counteract any toxic action of ATP—did not alleviate traumatic shock.

In a further effort to illuminate the hypothesis that adenosine derivatives are shock-inducing, we injected ATP as sodium salt (purity: >90%) into mice. Parenteral doses of as high as 50 mg in 20 g animals failed to elicit overt symptoms of shock; indeed were wholly without observable toxicity. All mice so treated survived indefinitely. Intravenous doses of 20 mg were acutely lethal, but 10 mg doses were easily tolerated and did not induce shock-like symptoms. Higher parenteral doses were not attempted as being beyond the range of meaningful physiological experiment. These observations together with our failure to observe changes in the nucleotide content of blood of mice undergoing post-irradiation wasting, leads us to doubt that adenosine derivatives released into the blood stream by

TABLE II. ATP Levels in Whole Blood from Control Mice, Tumor-Bearing Mice, and Mice after Tumor Extirpation.

Type of animal	No. of animals	ATP mg % (mean)
Control	8	7.3
With 7-day tumor	7	5
With 7-day tumor; 2 days after extirpation	7	5.3
With 10-day tumor	8	3.4
With 10-day tumor; 5 days after extirpation	8	5.9

4. Zahl, Paul A., *Science,* in press.

5. Green, H. N., *Lancet,* 1943, 147.

6. Meyer, R. K., McShan, W. H., Goldman, A., and Shipley, E. G., *Am. J. Physiol.,* 1946, v147, 66.

7. McShan, W. H., Potter, R .V., Goldman, A., Shipley, E. G., and Meyer, R. K., *Am. J. Physiol.,* 1945, v145, 93.

8. Kalckar, H. M., and Lowry, O. H., *Am. J. Physiol.,* 1947, v149, 240.

injured tissues constitute a primary factor in shock, at least in so far as irradiated mice are concerned.

As to the marked ATP drop in the blood of tumor-bearing mice, little may at the present time be ventured in the way of interpretation. Whether this change in blood composition specifically reflects the tumor growth process, or whether it is merely a non-specific reaction to toxic materials released by a necrotizing lesion in the course of tumor growth, remains to be demonstrated. On such demonstration will depend the possible usefulness of our observation as a diagnostic tool. Also moot and as yet uninterpretable is the fact that total nucleotide levels during tumor growth do not change, whereas those of known nucleotide (ATP) do.

Summary. No changes were observed in the blood levels of total nucleotide or of adenylic acid, adenosine diphosphate, or adenosine triphosphate during the wasting period that follows whole-body X-ray irradiation of mice. Massive injected doses of ATP failed to induce overt symptoms of shock in mice.

In mice bearing implanted tumors there is a continuous decrease in blood adenosine triphosphate until about the 10th post-implantation day, followed thereafter for several days by a slight but significant rise in the ATP curve. The first phase of this curve parallels, on the time scale, the increase in tumor weight. Mice whose tumors were extirpated at stated intervals after implantation show a tendency to return to the normal ATP blood level. There is no apparent relation between changes in ATP blood levels and body weight, cell-fluid ratio in the blood, or hemoglobin, at least up to the 10th post-implantation day. No significant changes were observed in the total nucleotide values for blood during tumor growth; nor was the adenylic acid or the adenosine diphosphate picture different from that of normal mice.

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Encephalitis in Midwest IV. Western Equine Encephalomyelitis Virus Recovered from Nestling Wild Birds in Nature. (18788)

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Neutralizing antibodies to the virus of Western Equine Encephalomyelitis (WEE) have been demonstrated many times in the sera of wild and domestic birds(1,2). This virus has been recovered only once from wild birds. Cox, Jellison and Hughes(3) isolated it from the brain and spleen of a prairie chicken shot August 27, 1941, near Rugby, N. D. The present paper reports 3 isolations of the WEE virus from the circulating blood of nestling wild birds in Weld County, Colo.

As part of an extensive search for the natural reservoirs of encephalomyelitis viruses, 1941 blood specimens were collected from nestling birds of 20 species (Table I). Collection of these was begun the first week of May and continued throughout the summer.

Technic of taking blood samples was as follows: Blood was obtained from nestlings 4 or more days old. A blood sample consisted of approximately 0.1 ml of blood withdrawn by cardiac puncture or from a wing vein into a syringe containing an equal volume of 10% inactivated normal rabbit serum in buffered saline. Each sample was placed separately

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1. Howitt, B. F., and Van Herick, W., *J. Inf. Dis.*, 1942, v71, 179.

2. Reeves, W. C., Proc. of the 49th Ann. Meet. of the U. S. Livestock Assn., p150. Dec. 1945.

3. Cox, W. R., Jellison, W. L., and Hughes, L. E., *Pub. Health Rep.*, 1941, v56, 1905.