creas to a meal of meat under normal circumstances. Studies of the importance of products of digestion of protein and fat as pancreatic secretagogues and the discovery of pancreozymin have demonstrated other physiologic mechanisms capable of stimulating the pancreas during normal digestion. The protective role of the acid-secretin mechanism in instances of gastric hypersecretion seems unquestionable. Data concerning this concept have been reviewed recently by Thomas(4).

Conclusions. 1. When canine pancreatic juice secreted in response to a meal was permanently removed by the method used in these experiments, the total volume of juice secreted was significantly greater than was the case when the juice was replaced in the duodenum. 2. A similar reduction in the volume of postprandial pancreatic secretion occurred when tenth normal sodium bicarbonate solution was instilled into the duodenum in place of pancreatic juice. 3. No similar reduction in secretion occurred when equivalent volumes of isotonic saline solution were substituted for pancreatic juice.

4. Thomas, J. E., *The External Secretion of the Pancreas,* Springfield, Illinois, Charles C Thomas, 1950, chap. 5, pp. 62-83.

Received May 2, 1951. P.S.E.B.M., 1951, v77.

Probable Mechanism of Alizarin Inhibition of Calcification. (18786)

GEORGE H. PAFF AND BENNETT SALLMAN.

From the Departments of Anatomy and Bacteriology, Hahnemann Medical College, Philadelphia, Pa.

Alizarin red S has been found to prevent calcification in embryonic metatarsals without interference with normal cartilage and fibrous connective tissue growth(1). This paper describes our attempts to determine the mechanism of the inhibition by observing the effects of alizarin on the enzyme, phosphatase, as well as on the calcium and phosphate ions.

Experimental. Alizarin and bone phosphatase. Because of the diversity of materials and methods employed, these will be presented along with our experimental results. The effect of alizarin on phosphatase production by many paired metatarsals and fibulas from 10-day-old chick embryos was studied first. One member of each pair was placed on the surface of a hanging drop consisting of equal parts of blood plasma and embryonic extract; the other member was treated similarly save that the medium contained one drop of a 1% Seitz-filtered aqueous alizarin solution to 120 drops of medium. At this dye concentration, calcification is inhibited. After 4 days of cultivation both members of live and fixed (95% alcohol) preparations were placed either in calcium nitrate as controls or in glycerophosphate substrates for phosphatase visualization. The preparations were then treated with silver nitrate, washed with distilled water and mounted in glycerine gelatin. Phosphatase was found abundantly distributed in the paired bones from both the alizarin-containing and alizarin-free media. Thus, no indication of bone phosphatase inhibition by alizarin in cultures was observed. The *in vitro* effect of alizarin on phosphatase was examined next. To chick alkaline bone phosphatase(2), assaying 46.8 Bodansky units per 100 ml(3), alizarin red S was added in an amount (0.6 mg per ml) approximately 6 times that required to inhibit calcification in tissue culture. There was no significant reduction in phosphatase activity (45.2 Bodansky units per 100 ml). Since bone phosphatase is not inactivated by alizarin either

1. Paff, G. H., and Eksterowicz, F. C., *Anat. Rec.,* 1950, v108, 45.

2. Martland, M., and Robison, R., *Biochem. J.,* 1929, v23, 237.

3. Hawk, P. B., Oser, B. L., and Summerson, W. H., *Practical Physiological Chemistry,* 12th ed., Blakiston, Philadelphia, 1949, p. 584.

in tissue culture or in the test tube, some other locus of dye action was sought.

Alizarin and calcium. Since alizarin can form a lake with calcium, the question arose whether the calcification inhibition observed was due to disionization of calcium in the medium. The clotting time of the blood media with and without the presence of dye in calcification-inhibiting concentration was recorded to determine whether this amount of alizarin was removing calcium and hence delaying clotting. The first appearance of fibrin threads on the cataract knife used for mixing was recorded as the clotting time. No difference in clotting times was observed. However, if the dye concentration was increased 5 to 10 times the minimal calcification-inhibiting concentration, clotting was distinctly retarded. Further evidence that alizarin did not disionize the calcium in the medium in order to prevent calcification was obtained by comparing the behavior of 50-hour embryonic chick hearts grown for 2 days on alizarin-containing and dye-free media. New growth appeared from the cut ends of each type of preparation and the average rates of beat of the two types were practically identical (94 times per min. on alizarin; 114 times per min. on alizarin-free media). Since activity of this kind cannot occur unless the calcium ion is present(4), the conclusion is obvious.

Litmus and calcification. Since litmus, like alizarin, is a dye having an affinity for calcium(5), we compared the two for effect on clotting time and calcification. A purified litmus was prepared from the crude dye(6), and a 1% litmus-Tyrode solution made. For both the clotting and calcification experiments, the clots were composed of equal parts of blood plasma and a mixture consisting of Tyrode solution, embryonic extract and the respective dye. The litmus concentration used was approximately 15 times greater than that of alizarin since it was found from preliminary

TABLE I. Effect of Alizarin and Litmus on Paired Embryonic Chick Fibulas Grown in Media Containing the Respective Dyes.

	Alizarin		Litmus	
Pair of fibulas	Total length, mm	Mineralization zone, mm	Total length, mm	Mineralization zone, mm
1	4	.50	4.20	1
2	4	.55	4.25	.75
3	4.05	1.10	4	1.15
4	4	.90	4.10	1.10
5	4.5	.50	4.20	1.20
6	4.40	1.05	4.35	1.25
7	3.60	.50	3.60	.75
Avg	4.08	.73	4.10	1.03

experiments that this concentration of litmus had an effect on clotting time but not on calcification. The average clotting time of 10 litmus drops was 4 min. while that of an equal number of alizarin drops was 2.5 min. Apparently litmus, unlike alizarin, removed sufficient calcium ions from the medium to increase the clotting time. For a comparison of calcification in litmus and alizarin, the same media described for the clotting experiments were used. One member of each pair of a series of femurs, tibias, fibulas and metatarsals from 9-day-old chick embryos was placed on an alizarin clot and the other on a litmus clot and grown for 3 days. After this period, 2 drops of alizarin-Tyrode solution (1 drop of a 1% aqueous alizarin solution to 59 drops of Tyrode solution) were added to each culture, litmus as well as alizarin, in order to visualize the extent of mineralization. After 5 hours, measurements of total lengths and of the lengths of the mineralization zones were made for each bone. Typical data for one such experiment on 7 pairs of fibulas are presented in Table I. It can be seen that average total lengths of the paired fibulas are practically the same when grown in the presence of either dye. As regards the zones of mineralization, however, the fibulas grown on litmus show about 40% more calcification than do those grown on alizarin (1.03 mm as against 0.73 mm). Unlike alizarin, then, litmus will not stop calcification in concentrations which interfere with the clotting process. It appears that, in the medium in general, a higher concentration of calcium ions is necessary to promote plasma clotting than is re-

4. Lewis, W. H., *Carnegie Cont. to Emb.*, 1929, v20, 173.

5. Mann, G., *Physiological Histology*, Clarendon, Oxford, 1902, p441.

6. Clark, W. M., *The Determination of Hydrogen Ions*, Williams and Wilkins, Baltimore, 1928, p68.

quired for calcification.

Alizarin and the medium. To determine whether alizarin inhibits calcification by rendering some constituent of the medium unavailable to the growing bone, bones grown on alizarin medium were transferred to dye-free medium. As a precautionary measure, the bones were washed and placed side by side with the controls in dye-free medium. Over a period of 4 days, calcification was not resumed in the alizarin-stained bone although the cartilage and fibrous connective tissue grew at a normal rate. In contrast, the control bone of each pair always continued to show calcification as well as normal growth of cartilage and connective tissue.

Discussion. These observations demonstrate that calcification can be stopped in tissue culture without inhibiting phosphatase and without removing the calcium and phosphate ions to a significant degree from the medium in general. The effect of alizarin, therefore, must be on some other mechanism or at some specific locus within the hanging drop culture. We are inclined toward the latter supposition and will presently develop it more fully.

Throughout the hanging drop medium there is a uniform and abundant distribution of phosphatase as well as calcium and phosphate compounds. Under these conditions the concept of the operation of a local factor has been introduced to explain why calcification occurs only in one small area of the bone. Robison(7) favored the concept that there is a shift in pH toward the alkaline in regions where calcification is occurring. However, it has been shown that in cultures of embryonic chick femurs more calcification occurs at pH 7.0 than at 8.0(8). This occurs despite the facts that the pH of chicken plasma is 7.8 to 8.0(9) and that the optimal pH for chick bone phosphatase in the test tube is 9.85(10). In addition, bones growing on litmus have occasionally been observed to produce a distinctly reddish color in the region where calcification is occurring. This finding agrees with the report by Rous(11) that the pH of bone in the living animal is 6.5 to 7.2. It has also been suggested that the local factor is a system comprising phosphorylase, glycogen and phosphate ions, whereby the amount of substrate upon which phosphatase can act is increased. We are cognizant that phosphorylase may be active in our preparations but as yet we have been unable to test the action of alizarin upon it.

Let us consider the apparent discrepancy between the results obtained with alizarin and those obtained with litmus. Although both dyes have an affinity for calcium, concentrations of alizarin which do not retard the clotting process can stop calcification in tissue culture; concentrations of litmus which retard the clotting process will not stop calcification. The difference in calcification is probably related to the availability of the calcium ion and the solubility of calcium alizarinate compared to the calcium-litmus complex. Since calcium in combination with alizarin is much less soluble than is the calcium-litmus complex, one would expect a concentration of calcium alizarinate in an area where the calcium ion is most available. This would be within the zone of mineralization if one is willing to accept the not unfounded idea that in this zone there is a drop in pH which is favorable to the increased ionization of calcium. In the case of bones growing in tissue culture, this localized binding of calcium by alizarin has the effect of stopping the process of calcification. The calcium-litmus complex does not produce the same stoppage because it is much more soluble.

The clotting effects obtained with the two dyes are not contradictory in light of our calcification studies. They indicate that, in the medium in general, a higher concentration of calcium ions is necessary for clotting than for calcification. With litmus, clotting is interfered with at a dye concentration which does not inhibit calcification. With alizarin, calcification is stopped at a concentration of

7. Robison, R., *Biochem. J.*, 1926, v20, 388.

8. Paff, G. H., PROC. SOC. EXP. BIOL. AND MED, 1948, v68, 288.

9. Cameron, G., *Essentials of Tissue Culture Technique,* Farrar and Rhinehart, New York, 1935, p64.

10. Motzok, I., *Biochem. J.*, 1950, v47, 193.

11. Rous, P., *J. Exp. Med.*, 1925, v41, 739.

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)

PAUL A. ZAHL AND HARRY G. ALBAUM.
(With technical assistance of Philip L. Cooper, A. N. Nowak, and Richard Scher.)
From the Haskins Laboratories, New York City; and the Biology Research Laboratory, Brooklyn College, N. Y.

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

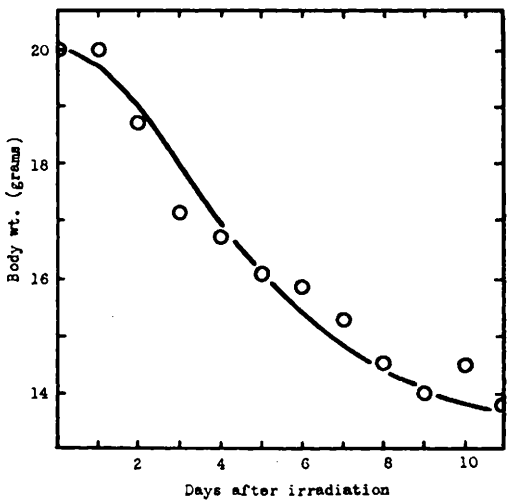

FIG. 1. Mean Body Wt of 20 Male Mice Following a Whole-Body X-ray Dose of 800 r, Plotted Against Time.

* 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.