with Coe virus(11,12,13). Therefore, it was considered worthwhile to test HEA for its ability to prevent or modify an experimental Coe infection in man. Preliminary results of HEA treatment of such an experimental Coe infection were recently reported(14) and will be described later.

Summary. The compound N^6-(2-hydroxyethyl)adenine (HEA) was effective in favorably modifying experimental Coe virus infection in mice. HEA showed antiviral activity when a single injection, approximately one-tenth the lethal dose, was given from 24 hours before to 17 hours after virus inoculation. The toxicity, but not the antiviral activity of HEA, was partly reversed by simultaneous treatment with a xanthine oxidase inhibitor.

1. Lennette, E. H., Fox, V. L., Schmidt, N. J., Culver, J. O., Am. J. Hyg., 1958, v68, 272.

2. Schmidt, N. J., Fox, V. L., Lennette, E. H., Proc. Soc. Exp. Biol. and Med., 1961, v107, 63.

3. Underwood, G. E., Wisner, C. A., Weed, S. D., Gray, J. E., Am. J. Hyg., 1962, v76, 124.

4. Buthala, D. A., J. Bact., 1963, v86, 1356.

5. Gray, J. E., Underwood, G. E., Mann, K. M., Soc. Toxicol. Mtg., Williamsburg, Va., Mar. 9-11, 1964.

6. Bauer, D. J., St. Vincent, L., Kempe, C. H., Downie, A. W., Lancet, 1963, v2, 494.

7. Bauer, D. J., Brit. J. Exp. Path., 1955, v36, 105.

8. Philips, F. S., Thiersch, J. B., Bendich, A., J. Pharmacol. Exp. Therap., 1952, v104, 20.

9. Elion, G. B., Callahan, S., Nathan, H., Bieber, S., Rundles, R. W., Hitchings, G. H., Biochem. Pharmacol., 1963, v12, 85.

10. Wyngaarden, J. B., Rundles, R. W., Metz, E. N., Ann. Int. Med., 1965, v62, 842.

11. Parsons, R., Bynoe, M. L., Pereira, M. S., Tyrrell, D. A. J., Brit. Med. J., 1960, v1, 1776.

12. Patel, N., Buthala, D. A., Walker, J. S., J. Infect. Dis., 1964, v114, 87.

13. Couch, R. B., Cate, T. R., Gerone, P. J., Fleet, W. F., Lang, D. J., Griffith, W. R., Knight, V., J. Clin. Invest., 1965, v44, 535.

14. Walker, J. S., Buthala, D. A., Evaluation of the Efficacy of An Antiviral Agent in Volunteers. Am. Therapeutics Soc., June, 1965 (Oral presentation).

Received February 1, 1966. P.S.E.B.M., 1966, v122.

Alkaline Phosphatases of the Chick. Partial Characterization of the Tissue Isozymes.* (31083)

S. S. KUAN, W. G. MARTIN AND H. PATRICK

Department of Agricultural Biochemistry, West Virginia University, Morgantown, West Virginia

Evidence obtained from *in vivo* studies with diseased and injured bone(1,2) on the effect of biochemical inhibitors(3), immunochemical and histochemical demonstrations(4, 5,6) suggested that serum alkaline phosphatase is derived from bone. Observations from liver studies suggested a hepatogenous origin of the increased serum alkaline phosphatase found in jaundice and hepatic duct ligation (7,8). It has also been concluded that whereas the major fraction of the serum alkaline phosphatase originates from bone, a small portion may be produced by liver cells(9,10). By observing the enzyme content in the serum and other tissues after fasting and fat injection(11) and also the inhibitory effect of this enzyme *in vitro*(12), an intestinal origin of serum alkaline phosphatase has been generally accepted. A diverse origin was also suggested after carbohydrate ingestion(13). Water dosing elevates the serum alkaline phosphatase(14,15).

In lieu of the diverse results obtained under a variety of nutritional and physiological conditions, studies were initiated whereby changes in the alkaline phosphatase level of the chick were observed under more normal conditions. The source of the serum enzyme was sought during these investigations.

In recent years, chromatographic techniques have become available to study the difference between functionally similar enzymes(16,17,18). The combined use of chromatographic techniques and isotopic Ca^{45}

* The data in this paper are from a thesis submitted by the senior author in partial fulfillment of the requirements for the degree of Master of Science. This manuscript is published with the permission of the Director of West Virginia University, Agri. Exp. Station, Morgantown, as Scientific Paper No. 870.

administration should help to indicate the source and role of the increased serum alkaline phosphatase. The object of the study reported here was to investigate the properties and similarity of the alkaline phosphatases from various tissues under normal conditions in order that some of the questions concerning the origin of serum alkaline phosphatase may be answered.

Methods and materials. Day-old White Leghorn male chicks obtained commercially were fed *ad libitum* the experimental ration as reported by Martin and Patrick(15). The chicks were sacrificed on the 3, 7, 10, 14 or 17 day of age and the blood collected, pooled and stored in the refrigerator until coagulation occurred. Serum was then separated by centrifugation at 2000 rpm and kept in a low temperature freezer until assay, usually within a few days of sampling. Liver and intestines were removed immediately after sacrifice and washed thoroughly with tap water followed by several times with distilled water to remove clotted blood and intestinal contents. The organs were weighed and then homogenized in a blendor with 10 volumes of cold distilled water for 10 minutes. Homogenates were stored in a refrigerator at about 2°C for 48 hours. The tibiae were crushed into very small pieces with a stainless steel crusher and placed in 10 volumes of distilled water under chloroform at room temperature for 10 days. Homogenates and bone filtrates were centrifuged at 7000 rpm for 5 minutes and the supernatants (designated crude extract) were retained for assay. Alkaline phosphatase was determined colorimetrically and reported as Sigma units (amount of p-nitrophenol liberated in 1 hour per ml of serum or per gram of tissue).

A column (1.5 × 30 cm) was packed by gravity with 2 g (dry weight) of Ecteola.† The Ecteola was prepared for the column by immersing 3 g of dry Ecteola in 100 ml 1 N NaOH and washing with distilled water until pH 7.5 was reached. A 3% Ecteola suspension was made and the column prepared by using 66 ml as follows. An amount of the enzyme solution equal to about 90% of its calculated exchange capacity was mixed with three-fourths of the Ecteola and stirred frequently for 10 minutes. The remaining one-fourth was packed on the column before the Ecteola and sample mixture was applied on the column. Stepwise elution of alkaline phosphatase activity from the column was accomplished with 0.01, 0.05, 0.1 and 0.05 N NaCl solution at the rate of 2-3 ml per minute. The fractions were collected and assayed for protein and enzyme activity. The protein was determined by the Folin-Ciocalteu colorimetric method using egg albumin as reference substance and these results were spot-checked at 280-260 mμ in a Beckman DU spectrophotometer.

† Ecteola (Epichlorohydrin triethanolamine) in an intermediate base anion exchanger obtained from Carl Schleicher & Schuell Co.

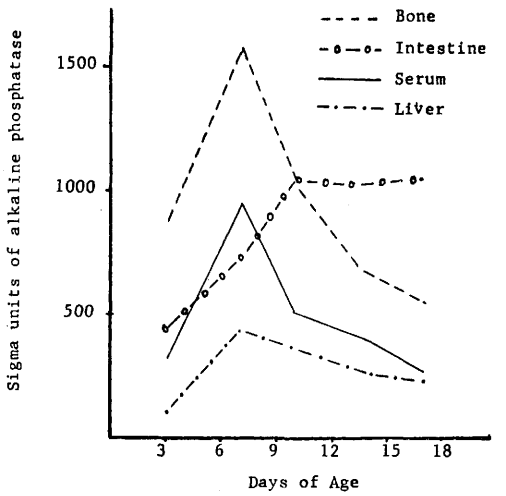

FIG. 1. Alkaline phosphatase activity in various tissues from normal chicks 3 to 17 days of age. The activity of this figure is in units per ml or g.

Results and discussion. Changes in alkaline phosphatase activity of various tissues at different ages. Ten chicks were randomly selected and sacrificed at 3, 7, 10, 14 and 17 days of age and crude enzyme extracts were prepared as described and duplicate assays made. Serum, bone, and liver alkaline phosphatase increased rapidly after hatching until it reached the peak at one week of age, and then continued to decrease with advancing age. Intestinal phosphatase rose from a low level beginning at 3 days of age to the peak at 10 days of age and then leveled off (Fig. 1). The highest liver, serum, and bone en-

TABLE I. Effect of Dosing 10- to 21-Day-Old Chicks with 1 ml Water on Level of Alkaline Phosphatase of Various Tissues.

Tissue	Treatment (min)	Age			
		10 days	14 days	17 days	21 days
		Units per g tissue or per ml serum			
Liver	0	320	435	470	490
	15	370	435	450	530
	30	340	420	450	450
	45	300	410	330	390
	60	370	500	435	380
Intestine	0	1480	1890	1860	1580
	15	1400	1890	1800	1980
	30	1040	1620	2040	2460
	45	1360	1590	2080	2320
	60	1800	1800	2160	2060
Serum	0	320	230	160	145
	15	280	186	120	145
	30	440	310	330	160
	45	340	260	240	170
	60	400	250	230	145
Bone	0	930	920	816	410
	15	754	820	540	470
	30	745	685	510	460
	45	765	800	460	400
	60	880	820	565	420

zyme activity occurring at one week appeared to be related to rapid body growth and bone formation(19), while the gradually increasing intestinal phosphatase activity might be regarded as merely a digestive function to meet the increased amounts of food digestion, absorption, and detoxification of the accumulated phosphate compounds. From these data, it looks unlikely that the increased amount of serum alkaline phosphatase at one week of age was originated from the liver or intestine since the average liver phosphatase level was found to be 440 units/g of liver, the total activity of this organ was considerably less than would account for the increase in the blood which at this age is about 8% of the body weight. Similar reasoning led us to doubt the intestinal origin of serum alkaline phosphatase. Besides, if the intestine was responsible for the increased serum enzyme, it might be expected that its lower enzyme level would be first elevated to a maximum to be diverted at once to the blood. Therefore, the possibility still remains that the increased serum enzyme originates from the bone which is about 20-25% of the body weight and possesses the highest enzyme activity at the same age.

Effect of water dosing on change of alkaline phosphatase activity in various tissues. At the age of 10, 14, 17 and 21 days, 25 chicks were orally dosed with 1 ml Ca^{45} solution. Twenty-four hours after Ca^{45} dosing, they were dosed with 1 ml of distilled water and sacrificed at 15-minute intervals. Five chicks were subsequently sacrificed without water dosing and were designated as controls (0 min). The effect of water dosage on the enzyme activity in various tissues is shown in Table I. An increased serum enzyme activity was associated with a decreased bone enzyme activity in all cases during the experiment. The peak of serum alkaline phosphatase appeared 30 minutes after dosing at 7, 10, and 14 days of age. Liver and intestinal phosphatase fluctuated in the treatments of different ages. The change of tibia Ca^{45} was also well associated with serum Ca^{45} and alkaline phosphatase activity (Table II).

The increase in serum alkaline phosphatase activity after water dosing appears to have some relationship to the dilution of body fluid(15). Evidence from isotopic Ca^{45} studies showed clearly that the change of alkaline phosphatase activity in serum and bone is associated with calcium metabolism.

TABLE II. Effect of Dosing 10- to 21-Day-Old Chicks with 1 ml Distilled Water on Distribution of Ca^{45} Between Bone and Serum.

Treatment (min)*	Age							
	10 days		14 days		17 days		21 days	
	Bone	Serum	Bone	Serum	Bone	Serum	Bone	Serum
	Counts per g of bone and ml of serum							
0	11295	36	6045	40	3222	28	1337	36
15	11070	40	5031	30	2662	26	1170	34
30	9719	68	4692	45	2217	21	985	58
45	9842	60	4830	30	2620	26	984	21
60	10500	80	4195	60	2475	12	1187	40

* min = minutes after water dosing.

TABLE III. Ammonium Sulfate Fractionation of Crude Enzyme Extract from Various Tissues.

Tissue	Saturation (%)	Volume (ml)	Enzyme activity (ml)	Total units	Protein (mg/ml)	Specific activity	Recovery (%)
Serum	0	20	450	9000	.078	5800	100
	0–20	—	—	—	—	—	—
	20–40	10.7	28	297	.023	1200	3.3
	40–60	14.5	278	4013	.042	7000	44.8
	60–80	7.0	14	98	.010	1400	1.1
Intestine	0	200	78	15620	.041	1900	100
	0–20	6.7	109	734	.027	4030	4.37
	20–40	22.0	103	2269	.031	3320	14.56
	40–60	38.0	113	4317	.030	3770	27.81
	60–80	14.0	188	3632	.012	15766	16.94
Liver	0	200	27	5400	.067	400	100
	0–20	21.0	21.6	454	.040	540	8.5
	20–40	26.7	90.0	2404	.132	680	44.8
	40–60	17.5	26.4	462	.094	250	8.6
	60–80	16.5	16.0	264	.076	210	4.9
Bone	0	150	52.4	7860	.083	630	100
	0–20	6.5	57.0	378	.013	4380	4.7
	20–40	13.3	120.0	1596	.031	3870	20.2
	40–60	12.3	356.0	4300	.044	8100	54.7
	60–80	6.8	171.0	1160	.028	6100	14.8

Rapid absorption of water into blood after dosing could increase the total blood volume thereby lowering the blood calcium concentration as well as other nutrients. Accompanied by increased alkaline phosphatase, a withdrawal of the storage calcium from the skeleton into blood could then be started in order to reestablish homeostasis of the blood. An increase of serum phosphatase accompanied by a decline of bone phosphatase also suggests that this enzyme seems to disappear from bone as a result of metabolic functions. Reabsorption of serum calcium and recalcification seemed to start approximately 30 minutes after water dosing. The higher serum Ca^{45} activity that appeared at 60 minutes after dosing may be due to the increased calcium concentration following restoration of normal body fluid level and before complete reabsorption.

Ammonium sulfate fractionation and chromatographic behavior of alkaline phosphatase from different tissues. The crude enzyme filtrates of various tissues from 10 chicks at 14 days of age were fractionated with ammonium sulfate at 0-20%, 20-40%, 40-60%, and 60-80% of saturation, since preliminary studies indicated that only a very small amount of precipitate and enzyme activity could be obtained at 80-100% saturation following the other precipitations. The precipitate from each fraction was dissolved in a small amount of distilled water and dialyzed against cold distilled water at 2°C for 24 hours with 3 changes of water. Upon completion of dialysis, enzyme activity and protein concentration were determined (Table III). The highest recovery of serum, bone, and intestinal enzyme activity was found to be present in fractions at 40-60% saturation and at 20-40% saturation for the liver enzymes. Markedly higher specific activity of serum and bone was concentrated in fractions of 40-60% saturation and liver at 20-40% saturation, while the specific activity of intestinal enzyme appeared to be evenly distributed in the fractions 0-20%, 20-40%, and 40-60% of saturation. The closer properties of serum and bone phosphatase again suggested osseous origin of serum alkaline phosphatase. The low recovery of phosphatase activity possibly showed that a loss of major alkaline phosphatase activity may have occurred during fractionation and dialysis. The question arises whether the loss of activity is due to the loss of some components which are necessary for enzyme activity or some other smaller enzyme molecules. Both questions remain unanswered. The fractions which have the highest specific activity were used

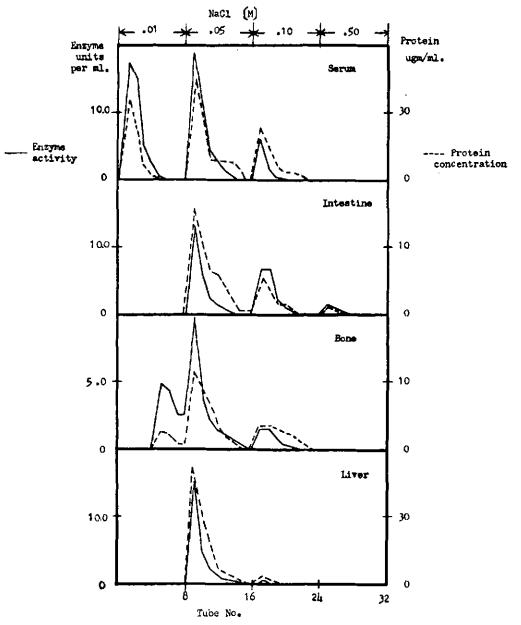

FIG. 2. Stepwise elution diagrams from Ecteola cellulose of crude extracts of alkaline phosphatases from different tissues of chicks.

for chromatographic separation. The elution pattern of these tissues is shown in Fig. 2. The enzyme preparations from different tissues were successfully separated into 2 or 3 components. The major component of all tissues appeared at about the same position, but the minor components were found to have different distribution patterns. The enzyme activity ratio of major to minor components of the same tissue ranged from 2:1 to 10:1 from tissue to tissue. A marked difference in specific activity was also found in different components (Table IV). The closer similarity in elution pattern indicates more evidence of the osseous origin of serum alkaline phosphatase.

TABLE IV. Specific Activity of Chromatographic Components of Alkaline Phosphatase from Different Tissues of Chicks on Ecteola Column.

Tissue	Component	Tube No.*	Enzyme activity (units/cc)	Protein (mg/cc)	Specific activity
Serum	A	1– 3	9.27	.043	218
	C	9–12	5.99	.020	298
	D	17–19	1.95	.016	195
Intestine	C	9–12	5.80	.0093	624
	D	17–19	2.70	.0043	628
	E	25–27	0.61	.0007	924
Bone	B	5– 7	2.70	.003	881
	C	9–12	4.90	.006	891
	D	17–19	0.44	.003	147
Liver	C	9–12	2.30	.018	125
	D	17–19	0.97	.007	139

* Fractions of the tube listed were pooled to make up the chromatographic components labeled.

Phosphatase studied in this experiment does not show direct evidence that the bone cells secrete and liberate phosphatase directly into the blood stream, but suggests the fact that the skeleton is the most likely source. Other techniques such as substrate specificity, inhibitors and activators, heat stability, electrophoresis, and tissue culture studies are in progress in hopes that the exact role and origin of this enzyme may be found.

Summary. The results in this experiment suggest that the increased serum alkaline phosphatase does not appear to be of liver or intestinal origin but may be of bone origin. Evidence in support of this finding has been obtained by the following: changes in alkaline phosphatase activity of various tissues during growth, measuring the change of enzyme activity and Ca^{45} concentration of various tissues after water dosing, enzyme properties of these tissues during ammonium sulfate fractionation and dialysis, and the chromatographic behavior of this enzyme from various tissues. It was also observed that the change of alkaline phosphatase activity in bone and blood is associated with the calcium metabolism.

1. Kay, H. D., J. Biol. Chem., 1930, v89, 249.
2. Stearns, G., Warweg, E., Am. J. Dis. Child., 1935, v49, 79.
3. Pollard, W. O., Shorb, M. S., Greek, R. D., Proc. Soc. Exp. Biol. and Med., 1963, v112, 487.
4. Schlamowitz, M., Bodansky, O., J. Biol. Chem., 1959, v234, 1433.
5. Pritchard, J. J., J. Anat., 1952, v86, 259.
6. Heller-Steinberg, M., Am. J. Anat., 1951, v89, 347.
7. Bodansky, A., Jaffe, H. L., Proc. Soc. Exp. Biol. and Med., 1933, v31, 107.
8. Gutman, A. B., Hugg, B. M., Olson, K. B., ibid., 1940, v44, 613.
9. Gutman, A. B., Am. J. Med., 1959, v27, 875.
10. Chandussi, L., Greene, S. F., Sherlock, S., Clin. Sci., 1962, v22, 425.
11. Gould B. S., Arch. Biochem., 1944, v4, 175.
12. Wilson, H. R., Wilcox, F. H., Proc. Soc. Exp. Biol. and Med., 1963, v113, 413.
13. Bodansky, A., J. Biol. Chem., 1934, v104, 473.

14. Martin, W. G., Patrick, H., Poultry Sci., 1961, v40, 1360.
15. ———, ibid., 1962, v41, 916.
16. Crossberg, A. L., Harris, E. H., Schlamowitz, M., Arch. Biochem. Biophys., 1961, v93, 267.
17. Moss, D. W., Nature, 1963, v200, 1206.
18. Behal, F. J., Center, M., Arch. Biochem. Biophys., 1965, v110, 500.
19. Jeffree, G. M., Proc. First European Bone and Tooth Symp., 1963, Pril, 299.

Received January 12, 1966. P.S.E.B.M., 1966, v122.

Mechanism of Gelatin Inhibition of Reticuloendothelial Function.* (31084)

JAMES P. FILKINS AND N. R. DI LUZIO
Department of Physiology and Biophysics, University of Tennessee Medical Units, Memphis

The functional activity of the reticuloendothelial system (RES) is commonly evaluated by measuring the intravascular clearance of a variety of inert colloids. In addition, RES hypofunction or "blockade" is often experimentally induced by injection of massive doses of particulate matter. Foremost among the agents used to measure and depress RES function are gelatin stabilized colloids such as carbon or gold(1).

Previous studies have demonstrated that high concentrations of gelatin in preparations of chromic phosphate(1,2), carbon(3), or gold(4) markedly retarded intravascular removal of the respective colloid. Prior adminjstration of gelatin also depressed the clearance of subsequent test doses of colloid *in vivo* (5,6,7) as well as in the isolated perfused rat liver(8).

Several explanations have been offered for the mechanism by which gelatin inhibits RES function. It has been postulated that gelatin may retard particle clearance because it either competes with the particle for phagocytic clones or saturates the phagocytic capacity of the RES(3,6). However, it may also inhibit clearance by interaction with, or depletion of, plasma factors or "opsonins" essential for phagocytosis(5,8,9,10). In addition gelatin may directly inhibit the RES(2,7). This study presents evidence that gelatin inhibits colloidal radiogold uptake by rat liver slices due to its interaction with a plasma opsonic system.

Methods. The proceduce employed in evaluating phagocytosis by rat liver slices was essentially as previously described(11). In brief, 300-400 mg slices of liver tissue from male Holtzman rats (300-330 g) were incubated in 3 ml of pooled heparinized (100 U.S.P. units/ml) plasma containing 400 μg of either colloidal radiogold (Abbott Laboratories, North Chicago, Ill.) or 1-5 μ human albumin aggregates labeled with radioiodine (E. R. Squibb and Sons, New Brunswick, N. J.). After 30 minutes of agitated incubation at 37.5°C the liver slices were removed, washed, weighed, and their accumulated radioactivity measured. The uptake was calculated as a per cent of the added dose per 100 mg of liver tissue.

Gelatin was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, and was prepared and neutralized in 0.9% saline immediately prior to use.

Heparinized plasma (100 U.S.P. units/ml) was also obtained from mice (A/J males, Jackson Laboratories, Bar Harbor, Maine), rabbits (3 kg, male, New Zealand Whites), and dogs (5 kg, male mongrels).

Results. Effect of gelatin on colloidal gold and denatured albumin uptakes by rat liver slices. To ascertain whether gelatin would inhibit phagocytosis in the liver slice system, a series of experiments was performed in which varying amounts of gelatin were added to heparinized plasma prior to the addition of either the gold or albumin aggregates and the liver slices. As indicated in Fig. 1, gelatin markedly inhibited gold uptake with practically complete suppression occurring at 320 μg/ml; however, a gelatin dose as low as 10 μg/ml produced a 35% depression in colloidal

* This investigation was supported in part by the Atomic Energy Commission.