that dietary methylene blue which protects E deficient chicks against the exudative diathesis and brown coloration of adipose tissue also affords E deficient rats a marked, though not complete, protection against hemolysis of erythrocytes in dialuric acid. Antioxidants, other than alpha-tocopherol, may also be able to reverse the susceptibility of the red blood cells of premature infants to hydrogen peroxide.

Summary. Hemolysis of red blood cells in dilute hydrogen peroxide was determined for 23 premature infants. Three infants received evaporated cows' milk, and the remainder partially-skimmed cows' milk feeding mixtures. A significant degree of *in vitro* hemolysis was found and this persisted as long as the infants were observed without supplementation of their diets with tocopherol. In each of 9 trials in 8 infants the *in vitro* hemolysis was reversed by feeding alpha-tocopherol.

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Dephosphorylation of Adenosine Triphosphate by Concentrates of the Virus of Avian Erythromyeloblastic Leucosis.* (19409)

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The virus of avian erythromyeloblastic leucosis(1) occurs in high concentration in the plasma of chicks affected with the disease. Concentration and purification of the agent have been effected(2,3) by the application of ultracentrifugal procedures to such plasma passed through earthen filters. The morphology of the virus and the characters of the concentrates obtained in this way have been described(3). In the course of studies of the chemical constitution and properties of the virus, inquiry was made into any possible activity which the virus might exert on adenosine triphosphate. It was learned, that the virus concentrates and other preparations containing the virus possessed the capacity to liberate inorganic phosphorus from this material. The importance of adenosine triphosphate as a source of metabolic energy(4) and the significance of its enzymatic dephosphorylation, possibly, by the virus in the absence of living tissue *in vitro* would appear to merit the present report of findings thus far encountered.

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Materials and methods. Experiments have been made with plasma from presumably normal chickens and from chicks diseased with erythromyeloblastic leucosis and with virus preparations obtained by ultracentrifugal fractionation of plasma from diseased birds. The character and origin of the strain of leucosis investigated have been described(5). All plasma was drawn into citrate, freed of cells by centrifugation, filtered through Celite and through Selas filters in the same way as that obtained for the purification processes. All preparations likely to contain free phosphorus were dialyzed against calcium-free Ringer solution in the cold. For *purification* of the virus, in a typical experiment, a pool of blood was collected by heart puncture into 6% sodium citrate in the proportion of one volume of citrate to 9 volumes of blood. The 53 donor birds with the disease, as diagnosed by blood smears(5), had been inoculated intravenously with infectious plasma 46 days previously at the age of 3 days and fasted for 24 hours before bleeding. The blood was centrifuged at 2,000 X g in 50 ml tubes in the horizontal centrifuge for 20 minutes for removal of the cells. The plasma was then recentrifuged under the same conditions, frozen in a dry ice-alcohol mixture and stored at about −14°C for 8 days. At the time of the experiment, the plasma was thawed in running tap water and centrifuged as described above to remove any clotted material. A 3 mm mat of Celite No. 512 was laid over a coarse filter paper, and 5 g of Celite No. 512 were added to each 120 ml of plasma. The plasma was filtered through the mat under suction and then passed through an 8″ Selas 02 filter (No. FP-88, Selas Corp. of America, Philadelphia, Pa.) under 4 lb of positive pressure. A volume of 420 ml of this clarified plasma was spun in the ultracentrifuge at 20,000 X g for one hour, and the resulting pellets were suspended in 42 ml of calcium-free Ringer solution containing 1% by volume of heparin (Liquaemin, 'Organon', 1 ml = 10 mg). The concentrate was centrifuged in the horizontal centrifuge at 2,000 X g for 10 minutes and the sediment discarded. The concentrate was spun at 20,000 X g for 30 minutes and resuspended in Ca-free Ringer solution containing heparin. This was repeated twice more for a total of 4 sedimentation cycles. Samples of each concentrate and supernatant fluid were taken for measurement of enzymatic activity and for nitrogen determination. The data obtained with each cycle are given in Table I.

TABLE I. Total Nitrogen Content and Dephosphorylating Activity on Adenosine Triphosphate of Plasma from Birds Diseased with Erythromyeloblastic Leucosis and of Ultracentrifugal Purification Fractions Derived from it in Experiment of Fig. 1.

	N/ml of reaction mixture, γ	Phosphate liberated/min/ml reaction mixture, μMol
Plasma pool	1775	.188
1st cycle concentrate		.061
supernatant	1900	.035
2nd cycle concentrate	.77	.054
supernatant	11.89	.011
3rd cycle concentrate	.57	.038
supernatant	.21	.004
4th cycle concentrate	.50	.031
supernatant	.04	.001

A study was made also with the purified virus preparation spun in an intermediate ultracentrifugal field. In this case, a concentrate obtained as described was placed in a 5 ml collodion tube, spun rapidly up to 10000 X g and held there for 3 minutes. The opalescent supernatant fluid was removed by pipetting. There was no real pellet at the bottom of the tube; instead, there was a small, somewhat opaque deposit on the wall. This was taken up in the volume of the concentrate initially used for this process. For the *measurement of enzymatic activity,* 1.8 ml of the virus preparation or plasma were mixed with 1.8 ml of adenosine triphosphate solution (adenosine triphosphate, disodium salt, Pabst Laboratories, Milwaukee, Wis.) brought to pH 7.2 with NaOH containing 3 mg of the salt per ml. Reaction took place at room temperature; at intervals 0.4 ml samples of the mixture were added to 4.6 ml of N perchloric acid. If necessary, the protein precipitate was removed by centrifugation. Phosphate determination was based on the method of Berenblum and Chain(6). The clarified

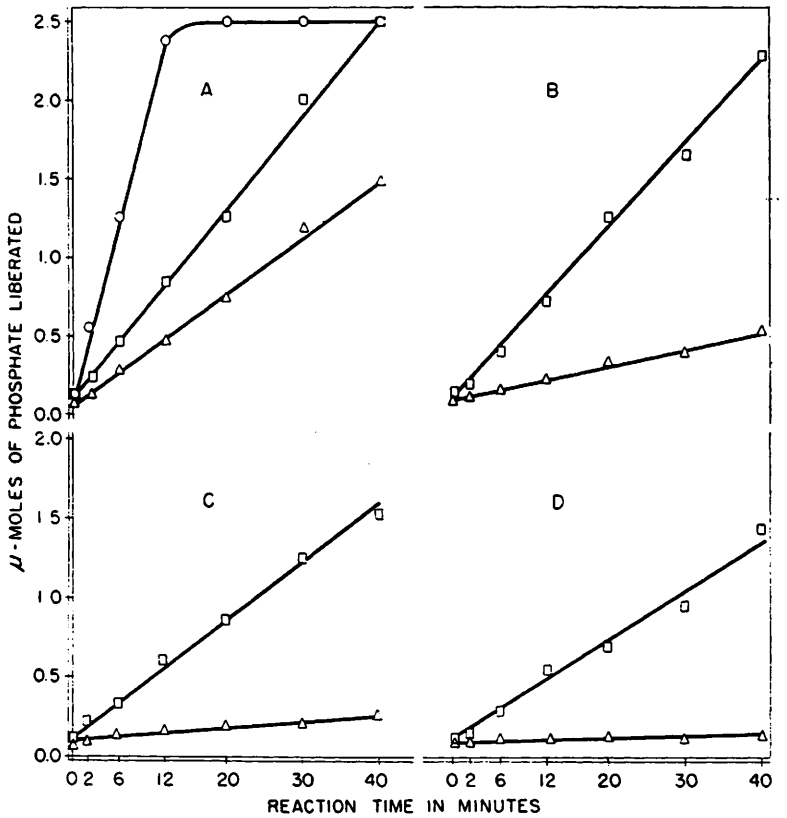

FIG. 1. Dephosphorylating activity on adenosine triphosphate of plasma from birds with erythromyeloblastic leucosis (circles of A) and of the concentrates (squares) and supernatant fluids (triangles) in 4 cycles (A, B, C, D) of sedimentation of the virus from this plasma.

sample was removed to a separatory funnel, followed by 2 ml of water washings, 2 ml of 5% ammonium molybdate, and 8 ml of isobutyl alcohol. The mixture was shaken for 1½ minutes. the aqueous layer was removed, and the alcohol layer was washed twice with 5 ml of N H_2SO_4, followed by 13 ml of stannous chloride solution (30 seconds shaking for each washing). The alcoholic solution was run into 10 ml volumetric flasks followed by the alcoholic wash fluid to volume. Absorption was read with a No. 600 filter in an Evelyn colorimeter.

Results. In Fig. 1 there is shown the character of the results obtained with the plasma from diseased birds and with the ultracentrifugal fractions resulting from the purification process through four sedimentation cycles. As seen in Fig. 1 A, the reaction with plasma was rapid, reaching completion in about 12 minutes. The pellet of the first sedimentation cycle resuspended in a volume proportional to that of the plasma was likewise exceedingly active, and the activity of the supernatant fluid of this cycle, the results with which are shown also in Fig. 1 A, was considerably less. Repetition of the sedimentation cycles resulted, Fig. 1 B, C, and D, in successive concentrates of relatively high, though progressively diminishing, activity, while all of the activity of the supernatant fluids was lost in the same procedures. This distribution of dephosphorylating activity in the concentrates and supernatant fluids closely parallels the partition of the infectious capacity of analogous fractions tested in repeated titrations in 3-day-old chicks. This is not meant to imply that the activity is associated only with infectious virus; instead, it is evident here that enzymatic activity is to be found in those frac-

tions in which characteristic particulate material and virus activity reside. Consideration of the maximum amount of phosphorus freed at the equilibrium point indicates that, under the conditions of these experiments, only the third phosphorus is liberated with conversion of adenosine triphosphate to adenosine diphosphate.

The relation of nitrogen to enzymatic activity is shown in Table I. Here again it is evident that activity is associated with the particulate material rather than substances not susceptible to the centrifugal field employed. It is seen, also, that activity per unit of nitrogen in the concentrates is exceedingly high. Some of the activity of the original plasma was lost in the first sedimentation cycle, due in part to the considerable amount of material eliminated in the low speed centrifugation of the concentrate (see above) of this cycle.

Essentially identical results have been obtained with 7 different batches of plasma from chickens with erythromyeloblastic leucosis and 4 preparations of purified virus. Results such as those described above were obtained with freshly purified virus; experiments made daily on the same batch of the purified agent kept in the refrigerator at 2-4°C showed high activity for about 7 days, whereupon activity declined rather suddenly. This result is likewise compatible with the stability of the infectivity of this virus which declines rapidly even in plasma stored, frozen, at about –14°C.

Ultracentrifugation of the virus concentrate at 10000 X g, which would be expected to sediment a portion of the virus as well as masses larger than the virus, resulted in the elimination of about one-fourth to one-third of the enzymatic activity. Electron microscopy revealed only dispersed virus particles in the supernatant fluid. The sediment revealed an entirely different picture, containing not only some dispersed virus but many amorphous masses in which small numbers of virus particles seemed incorporated.

Experiments made with 4 different batches of plasma from normal chickens showed no evidence of enzymatic activity.

Discussion. The present experiments have revealed a highly potent enzymatic activity associated with preparations containing the virus of erythromyeloblastic leucosis in the dephosphorylation of adenosine triphosphate. Consideration of the problem is concerned not with the magnitude of the reaction but with the question of the identity of the enzymatic component. The fact that nothing in normal chicken plasma is effective reveals a specific association of the enzyme with the erythromyeloblastic disease. Several possible origins of such an enzyme seem obvious: the material might be elaborated into the circulation by the tissues of the host, by the affected bone marrow or the specific primitive cells or other tissues not directly affected by the virus; cell fragments remaining in the plasma after filtration might act as units or contain incorporated enzymes; or the reaction might be specific to the virus itself.

It is notable that the constituent of plasma carrying the activity must be associated with particles of the size of the virus since the enzymatic effects are carried through those processes designed to select the virus. This would rule out materials of small molecular size that might be freed by tissues into the circulation. It is recognized that the plasma contains amorphous masses larger than the virus which pass through the filter and appear in the concentrates of the virus as masses or aggregates up to about 7 μ in diameter. In the purification procedures such masses are easily eliminated by brief ultracentrifugation in fields of 10,000 X g. It is evident, then, that the enzymatic activity is associated, in all of the experiments thus far made, with particles essentially identical in sedimentation characters with the particles which are regarded as the virus. This is clearly shown in Fig. 1 in which the partition of enzymatic activity is parallel, within the limits of biological assay, with the distribution of virus infectivity among the ultracentrifugal fractions, as shown in titration experiments which will be described in another report. It is not inconceivable that the virus preparations contain a particulate component the size of the virus derived through possible breakdown of the primitive cells in the circulation. At this point, it is well to recall the experiments of Kielley and Meyerhof(7) who isolated from

the muscle of the rat a particulate material with which there was associated the capacity to cause dephosphorylation of adenosine triphosphate. The sedimentation and other properties of this material seem, at least superficially, similar to analogous properties of the virus concentrates. While the possible contribution of such particles cannot be ruled out entirely at the moment, it can be said that in every respect in which the purified particulate material specific to erythromyeloblastic leucosis can be interpreted as the virus so can these same particles be regarded as the source of the enzyme freeing phosphorus from adenosine triphosphate.

The implications of these results, if fully established in further work, are of much significance. Evidence of enzymatic and metabolic activity of viruses in the absence of cells is so limited that this lack in constitution is regarded, in general, as a deficiency characteristic of this group of infectious agents. It is notable in this respect that while vaccinia elementary bodies give no evidence of such metabolic activity(8), the virus shows the presence of copper(9), flavin(10) and biotin (11), suggesting the existence of a limited respiratory system. Other enzymes found (12,13) in association with vaccinia virus have been viewed(13) as possible adsorbed contaminants carried through the purification procedures. Intensive investigation has shown that the influenza virus possesses the capacity to alter the physical structure(14) of the egg white inhibitor of hemagglutination and to liberate constituents(15,16) of this and other inhibitors of the reaction. It is noteworthy that this activity of influenza virus is likely to be of limited significance in comparison with that of enzymatic constituents which may be involved in physiological and reproductive processes of viruses. The capacity of the virus of erythromyeloblastic leucosis to react with adenosine triphosphate *in vitro* suggests possession by the virus of the immediate means for providing the energy, not only for utilization of intracellular materials but for making its entrance into the cell. It scarcely needs saying that the present experiments are open to criticism that the enzyme may be adsorbed on the virus particles. Such possible adsorption assumes considerable significance in itself, however, for it is evident that it must have occurred within the host cell since none of the enzyme of small molecular dimensions was present in the plasma. These possibilities make necessary further investigation, which is already underway.

Summary. A highly potent capacity for the dephosphorylation of adenosine triphosphate has been observed with preparations containing the virus of avian erythromyeloblastic leucosis. This enzymatic reaction occurred with the filtered plasma of birds diseased with the virus and with virus concentrates obtained from the plasma by ultracentrifugal procedures. The partition of enzymatic activity in these preparations has closely paralleled the distribution of virus infectivity of analogous materials measured in other work by titration of the virus in susceptible host chicks. No evidence of the reaction was obtained with the plasma from normal chickens. These experiments demonstrate the specific relationship of the dephosphorylation of adenosine triphosphate with avian erythromyeloblastic leucosis and indicate that the activity is a property of the etiological virus.

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Are Foamy Cells in Atheromata of Reticulo-endothelial Origin? (19410)

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Although plausible reasons have been advanced for believing that cholesterol esters are carried into the atherosclerotic vessel wall by macrophages(1-3), more recent studies support another view. Kuntz and Sulkin(4) concluded that foam cells of atheromata arose *in situ* from vascular endothelium, and in rabbits with early atherosclerosis given India ink or lithium carmine intravenously thrice at 2-day intervals they observed pigment granules in foam cells in the liver and spleen, but not in atheromata. The interval between the last injection and death of the animals was not stated, but presumably was short. Duff and McMillan(5) gave Thorotrast to atherosclerotic rabbits at intervals of 5 minutes to 4 days before death. Thorotrast was found in foam cells in the superficial part of the plaques and in some endothelial cells overlying the plaques. Simonton and Gofman(6) administered Thorotrast to rabbits, and subsequently began cholesterol feeding. Thorium-containing phagocytes in the liver and spleen engulfed India ink injected 24 hours before death. Radioactivity in atheromata was low, and the authors concluded that macrophage migration was not a significant source of foam cells of atheromata.

In view of the brevity of the interval between labeling of the reticulo-endothelial cells and death in the experiments cited, and since thorium-laden cells may perhaps migrate less readily than others, our experiments may be of interest.

Methods. Atherosclerosis was induced by incorporation in the diet of young adult rabbits of 3% of a 10% suspension of cholesterol in cottonseed oil. After 2 weeks, weekly intravenous injection of 3 cc of 17% Higgins India ink in normal NaCl solution was instituted. The duration of cholesterol administration was as follows: one rabbit, 3 months; 4 rabbits, 6 months; and one rabbit, 10 months. Two control rabbits were given ink for 10 months.

Results and discussion. There was a small intimal plaque in the ascending aorta of each control rabbit. The aorta of the rabbit that received cholesterol for 3 months was normal. The remaining animals had severe atherosclerosis. The reticulo-endothelial cells of all rabbits were laden with carbon. Although cells with foamy cytoplasm were abundant in the atheromata, no carbon-containing phagocytes were present. Absence of pigmented phagocytes from the aorta of the controls should be expected, since Leary(1) stated that macrophages in man and in experimental animals in which a great variety of forms of particulate matter have been engulfed exhibit no tendency to invade arterial walls.

While few animals were used, it was obvious that use of larger numbers would be futile. The results are more in accord with the view that cholesterol esters enter blood vessel walls by diffusion than with that that they are transported therein within migrating reticulo-endothelial cells.

Summary. Atherosclerosis was induced in rabbits by administration of cholesterol while the reticulo-endothelial cells were being laden