even the dystrophic animals continued to eat and show a weight gain until their sacrifice.

It is recognized that there is a disturbance of creatine metabolism in Vit. E-deficient animals. As early as 1932(9) attempts were made, without success, to treat progressive muscular dystrophy by supplementation with glycine on the assumption that there was a disturbance in formation of creatine. Dinning and Fitch(10) have recently reported that rate of creatine synthesis and turnover rate of skeletal muscle creatine is increased in dystrophic animals. Nevertheless that glycine decreases in most tissues of dystrophic animals and dietary glycine appears to prevent a decrease in tissue phosphatase, even though this enzyme has not been implicated with the dystrophic state, suggests that glycine is in some unknown way implicated in the dystrophic state.

Summary. 1. Acid and alkaline phosphatase activity of 6 tissues of normal and Vit. E-deficient rabbits was determined. 2. These activities were decreased in heart, spleen, liver, kidney and brain of dystrophic animals. 3. Acid phosphatase decreased slightly in muscle, but no change occurred in alkaline phosphatase activity. 4. 1% glycine in the dystrophic diet prevented reduction of tissue phosphatase.

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Enzyme and Nucleic Acid Content of Thrombocytes from Normal and Thrombocytopenic Calves.* (23935)

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Hypoplastic or aplastic anemia can be elicited in the bovine by feeding of trichloroethylene-extracted soybean oil meal (TCE-SOM)(1,2). Development and course of this anemia indicate that in this species the bone marrow is the primary site of action of the toxic agent. Among circulating derivatives of bone marrow, the composition of thrombocytes has apparently not previously been studied in any hypoplastic anemia.

Calves with induced hypoplastic anemia provided sufficient blood for preparation of thrombocyte concentrates in which the presence of nucleic acids and the activity of some hydrolytic enzymes could be investigated. Study of the latter was selected because sensitive analytical methods could be adapted for this purpose and because their activity in leukocytes is affected in other types of blood dyscrasias(3).

Methods. Young female Holstein calves used for other purposes(2) served as blood donors. Normal control calves were fed

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alfalfa, hexane-extracted soybean oil meal and milk while the others were rendered thrombocytopenic by feeding of TCESOM. A. *Preparation of thrombocyte concentrates.* All glassware which came in contact with blood was silicone-coated with Desicote.† Blood was collected at slaughter, by exsanguination, in an equal volume of anticoagulant solution (25 g sodium citrate, 9 g sodium chloride, 1000 ml distilled water). This mixture was centrifuged at 900 g for 5-10 minutes, or until a distinct boundary of erythrocytes had just formed. The turbid supernatant containing thrombocytes was siphoned into another container and centrifuged at 2000 g for 20 minutes. The supernatant was discarded and the sediment suspended in anticoagulant solution and centrifuged at 50 g for 5-10 minutes. The thrombocyte-rich supernatant was removed and its leukocytes, erythrocytes and thrombocytes were counted. An arbitrary value of not more than one erythrocyte/5000 thrombocytes was chosen as the minimum standard of purity, and when the erythrocyte contamination exceeded this number, the process of centrifugation at 50 g and removal of supernatant was repeated until the desired degree of purity was attained. Contamination from erythrocytes always exceeded that from leukocytes. The final thrombocyte concentrates were kept at −20°C until analyzed in duplicate. B. *Phosphatase determination.* The phosphatase method was adapted from that of Valentine and Beck(4). To release the enzyme, one volume of thrombocyte concentrate was treated for 30 minutes with 2 volumes of aqueous 2% saponin. To find the optimum pH for enzyme activity, substrates were prepared in the range from pH 2 to 11 by dissolving 0.5 g sodium 2-glycerophosphate and 0.42 g sodium diethylbarbital in water, adjusting the pH with 1 N acetic acid, citric acid or sodium hydroxide, and diluting to 100 ml with water. A mixture of 2.25 ml of substrate and 0.05 ml of 0.05 M $MgCl_2$ solution was warmed 10 minutes at 37°C, and 0.20 ml of thrombocyte-saponin mixture was added. After incubation at 37°C for 60 minutes, the phosphatase was inactivated by addition of 0.5 ml of 30% trichloroacetic acid. In control experiments trichloroacetic acid was added to the substrate before addition of the thrombocyte-saponin mixture. Inorganic P was determined in 2.50 ml of clear, centrifuged supernatant by the method of Berenblum and Chain(5). Phosphatase activity was expressed as micromoles of inorganic P liberated/hour/10^{10} thrombocytes. C. *Beta-D-glucuronidase determination.* This was done by adaption of the method of Fishman *et al.*(6). A mixture of 0.1 ml of thrombocyte concentrate, 0.8 ml of 0.1 M acetate buffer and 0.1 ml of 0.01 M phenolphthalein monoglucosiduronic acid‡ was incubated at 37°C for 24 hours. Then 1 ml of 10% trichloroacetic acid and 2.5 ml of an alkaline reagent mixture (4 volumes of 0.4 M glycine buffer, pH = 10.45, plus 1 volume of 1.0 N NaOH) were added, the volume was adjusted to 5 ml, and the suspension centrifuged. The clear supernatant was decanted and its absorbancy read in a Beckman spectrophotometer at 555 mμ. Tubes containing only thrombocyte concentrate and buffer served as controls. For determination of optimum pH of beta-glucuronidase activity, a series of acetate buffers (pH 3.4-5.4) was prepared. After addition of trichloroacetic acid, calculated amounts of 0.04 M NaOH or 0.04 M acetic acid were added to adjust the pH, before development of the color, to that obtained when the mixture contained acetate buffer of pH 4.5. D. *Arylsulfatase determination.* The method was adapted from that of Rutenberg *et al.*(7) for histochemical demonstration of arylsulfatase. One ml of dilute (1:3) thrombocyte concentrate was incubated 24 hours at 37°C with 1 ml of substrate (25 mg 6-benzoyl-2-naphthyl-sulfate§ in 80 ml hot aqueous 0.85% NaCl and 20 ml of 0.5 M acetate, pH 5.7). Then 0.25 ml of tetrazotized diorthoanisidine (100 mg in 100 ml 0.05 M phosphate buffer pH 7.6) and 3 minutes later 0.25 ml 40% trichloroacetic acid were added. The mixture was shaken vigorously with 5 ml of chloroform and centrifuged.

† Desicote, Beckman Instruments, Inc., Pasadena, Cal.

‡ Prepared from cinchonidine salt according to Fishman *et al.*(6).

§ Obtained from Dajac Laboratories, Leominster, Mass.

The chloroform layer was drawn off, shaken with a little anhydrous sodium sulfate, centrifuged and the absorbancy of the clear supernatant was read in the Beckman spectrophotometer at 540 mμ against standards prepared from 6-benzoyl-2-naphthol. Blanks were prepared by addition of thrombocyte suspension after that of trichloroacetic acid. E. *Esterase determinations.* Aliesterase activities were determined by adaption of the method of Hardin *et al.*(3). Substrates of 2-naphthyl acetate, 2-naphthyl laurate and 2-naphthyl stearate were synthesized(8). Buffer-substrate solutions were prepared immediately before use as follows: 2-naphthyl acetate: 0.5 ml of 2-naphthyl acetate solution (0.5% in acetone) was added with stirring to 17.5 ml of 0.05 M tris-(hydroxymethyl)aminomethane and 2 ml of water; 2-naphthyl laurate and 2-naphthyl stearate: 1 ml of solution (2.0% 2-naphthyl laurate in acetone, or 0.2% 2-naphthyl stearate in acetone), was added with stirring to 19 ml of 0.05 M tris(hydroxymethyl)aminomethane. For determination of aliesterase activity 1 ml of buffer-substrate, warmed to 37°C for 5 minutes, was mixed with 1 ml of diluted thrombocyte concentrate (diluted 1:15 when 2-naphthyl acetate was used, or diluted 1:4 with the other esters) and incubated 30 minutes (2-naphthyl acetate) or 2 hours (2-naphthyl laurate and stearate). Then, 0.25 ml of 0.4% tetrazotized diorthoanisidine was added to all mixtures, and 3 minutes later, 0.25 ml of 40% trichloroacetic acid. The azo dye was extracted by vigorous shaking with 6 ml of chloroform, which was removed after centrifugation and clarified by shaking with a little anhydrous sodium sulfate. The absorbancy of the clear chloroform solution was read at 540 mμ. In the blanks the thrombocyte suspension was added after the trichloroacetic acid. Aliesterase activity was expressed as μmoles of 2-naphthol liberated/10^{10} thrombocytes/hour. The optimum pH for each of the substrates was determined with buffers covering the range of pH 5 to 9. Acetylcholinesterase activity was determined by the method of Glick(9), using an incubation period of 24 hours. Activity was calculated in terms of μmoles of acetic acid liberated/24 hours/10^{10} thrombocytes. Pseudocholinesterase activity was measured by modification of the method by Kalow and Lindsay (10). Two ml of benzoylcholine chloride solution (2.44 mg in 100 ml of M/15 phosphate buffer, pH = 7.4) was warmed to 37°C for 5 minutes, 0.5 ml of thrombocyte concentrate (diluted 1:10 with M/15 phosphate buffer, pH = 7.4) was added and the mixture incubated 30 minutes. Then, 1.7 ml of 1 M $HClO_4$ was added and after centrifugation, the absorbancy of the supernatant was read at 240 mμ. Control analyses were made simultaneously in which the thrombocytes were added after the $HClO_4$. F. *Nucleic acids.* Thrombocyte concentrates were examined for deoxypentosenucleic acid by ultraviolet light absorption of perchloric acid extracts(11), deoxypentosenucleic acid P analyses, the indole reaction(12), and the Feulgen reaction made on smears(13). The presence of the pentose-

TABLE I. Enzyme Activities of Bovine Thrombocyte Concentrates.

Enzyme	Range and mean ± S.E. Normal calves*	Thrombocytopenic calves†
Acid phosphatase‡	.87–1.61 1.29 ± .083	.77–1.64 1.23 ± .071
Beta-D-glucuronidase§	12.2–27.7 20.2 ± 1.45	8.8–34.2 19.8 ± 1.86
Arylsulfatase‖	1.2–5.2 2.9 ± 1.4	1.4–4.6 2.6 ± .3
Aliesterase¶		
2-naphthyl acetate	10.2–22.0 17.2 ± 1.20	5.1–49.1 25.1 ± 3.68
" laurate	.34–3.90 1.14 ± .36	.40–3.20 1.74 ± .21
" stearate	.08–.44 .22 ± .04	.08–1.70 .55 ± .12
Acetylcholinesterase**	10.2–49.2 24.4 ± 4.7	7.2–40.4 28.2 ± 3.0

* Values from 9 calves fed hexane-extracted soybean oil meal. Mean blood thrombocyte count was 664,000 ± 30,000/mm³.

† Values from 17 calves rendered thrombocytopenic by feeding of trichloroethylene-extracted soybean oil meal. Mean blood thrombocyte count was 269,000 ± 37,800/mm³.

‡ μmoles of inorganic phosphorus formed/10^{10} thrombocytes/hr.

§ μmoles of phenolphthalein formed/10^{10} thrombocytes/hr.

‖ mμmoles of 6-benzoyl-2-naphthol formed/10^{10} thrombocytes/hr.

¶ μmoles of 2-naphthol formed/10^{10} thrombocytes/hr.

** μmoles of acetic acid liberated/10^{10} thrombocytes/24 hr.

nucleic acid was investigated by ultraviolet light absorption of perchloric acid extracts (11), pentosenucleic acid P analyses, the orcinol reaction(14), and the methyl greenpyronin and ribonuclease method on smears(13).

Results. Table I is a summary of results of determinations of enzyme activities. As indicated by the relatively large standard errors of the means, the enzyme activities of thrombocytes from different normal or thrombocytopenic individuals varied greatly. There was no statistically significant difference, as determined by the "t" test(15), of the means of the 2 groups of animals and, among the thrombocytopenic specimens no correlation between the degree of thrombocytopenia and enzyme activity.

Phosphatase was most active at pH 5.0, a value similar to those reported for phosphatase in equine(16), rabbit(17) and human (16,18,19) thrombocytes. In contrast to Salvidio's(19) findings with human thrombocytes no alkaline phosphatase could be detected.

Bovine thrombocytes contained a beta-D-glucuronidase with optimum pH of 4.5. With the same substrate, Fishman(6) found no difference in beta-glucuronidase activities of thrombocyte-rich and thrombocyte-poor plasma and concluded, in contrast to Alexander (18), that human thrombocytes did not contain beta-glucuronidase activity. Arylsulfatase had a relatively low activity and an optimum pH of 5.7.

Recent work indicates that tissues of the rat(20), liver of the ox(21,22,23) and several tissues of man(24) contain arylsulfatases which can be differentiated through solubilities, affinities for various substances, and pH optima.

With the same substrate as used in these studies, Rutenberg *et al.*(7) could not detect arylsulfatase in any of the blood cells of the rat by a histochemical procedure. The pH optimum for aliesterase activity was 7.3 with 2-naphthylacetate as substrate and 6.9 with the other 2 esters and the activity decreased with increasing chain length of the acyl groups. Because of the lack of substrate specificity among esterases(25), the observed activities may reflect a difference in affinity of the same enzyme for various substrates or the presence of more than one aliesterase in thrombocytes. In contrast to pancreatic lipase which is more active in the presence of 0.1 M sodium taurocholate(26), but like the esterase of rat liver(26), the aliesterase activity of bovine thrombocytes was inhibited by this reagent, to the extent of 83% with the acetate, 73% with the laurate and 58% with the stearate.

Our data confirm the recent report of Zajicek(27) of the occurrence of low cholinesterase activity in normal bovine thrombocytes. Moreover, both cholinesterase and aliesterase activity were inhibited to the extent of about 50% when, for other purposes, 6 calves were injected intramuscularly with 1.4 to 5.6 mg diisopropylphosphorofluoridate‖/100 kg body weight 90 minutes before collection of the thrombocytes.

Nucleic acids. Measurements of ultraviolet absorption or nucleic acid P in specimens containing 2-3 x 10^9 thrombocytes from normal or thrombocytopenic calves indicated the presence of only traces of pentosenucleic acid or deoxypentosenucleic acid. Tests for these components by the orcinol and ribonuclease reactions or the indole and Feulgen reactions respectively gave negative results. This is in accord with those of Maupin(28) and of Bestetti *et al.*(29,30) and with the widely accepted theory(31) that thrombocytes represent cytoplasmic fragments of megakaryocytes in which deoxypentosenucleic acid is largely confined to the nuclear structure.

The results of others suggesting that human (32) or equine(33) thrombocytes contain appreciable amounts of nucleic acids could be due to specie difference or to presence of relatively large amounts of adenosinephosphate which Bestetti and Crosti(30) found in human thrombocytes and which could be associated with protein.

Summary. 1. Bovine thrombocyte concentrates contained acid phosphatase, beta-D-glucuronidase, arylsulfatase, aliesterase (active with three fatty acid esters of carbon chain lengths: C_2, C_{12} and C_{18}) and acetylcholinesterase but no alkaline phosphatase. Benzoylcholine was not hydrolyzed by throm-

‖ Generously supplied by Merck, Sharp and Dohme Research Laboratories, West Point, Pa.

bocytes. 2. There was no significant difference in these enzyme activities of thrombocytes from normal or thrombocytopenic calves calculated in terms of activity per 10^{10} thrombocytes. 3. There was no significant quantity of deoxypentosenucleic or pentosenucleic acid in bovine thrombocyte concentrates from either normal or thrombocytopenic calves. 4. Thrombocytes released by hypoplastic bonemarrow could not be differentiated from normal thrombocytes by the tests used.

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Relationship of Blood Heparin Levels to Serum Lipoproteins and Cholesterol Levels in Fasting Subjects.* (23936)

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In recent years much of the investigative effort on atherosclerosis has centered about

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