manent protective action in all the stilbestrol treated rats with normal values in blood sugar and glycosuria, in contrast with the increase of these values observed in non-injected controls.

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Some Effects of Protein Depletion and 2-Acetylaminofluorene on Activity of Rat Plasma Pseudocholinesterase.* (17669)

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McCance, Widdowson and Hutchinson(1) demonstrated that starvation resulted in a considerable decline in the pseudocholinesterase activity of human plasma. Mendel, Hawkins and Nichikawara(2) reported similar results in rats. The following experiments were planned to determine whether or not this decrease in pseudocholinesterase activity was associated with depletion in protein stores. The activity of this enzyme was also studied in animals fed 2-acetylaminofluorene, where depletion in protein and other signs of inanition have been noted incident to the development and growth of tumors.

Methods. A. *Enzyme Assay.* The Warburg manometric method of pseudocholinesterase determination as described by Ammon(3) and modified by Mendel and Mundell(4) was used to estimate enzyme activities. All determinations were done in duplicate; 3 readings were taken at 20 minute intervals and, after summing, the activities were expressed as μl CO_2/ml plasma/hr. The buffer system was bicarbonate-CO_2 at pH 7.4. The substrate was benzoylcholine chloride (Hoffmann-LaRoche) at a final concentration of 0.006 M. Blood was obtained from ether anesthetized animals by heart puncture with a lightly oxalated syringe.

B. *Liver Protein Assay.* Liver protein was determined by applying the factor of 6.25 to micro-Kjeldahl N determination. The liver was removed from the animal immediately upon sacrifice and a sample homogenized for analysis.

C. *Diets and Animals.* Male Wistar rats were used throughout. Control animals were fed a semi-synthetic diet compounded as follows, the values being expressed as percentages. Casein 18, sucrose 13.4, glucose 20.2, dextrin 18.2, lard 24.2, Wesson's salt mixture(5) 1.7, agar 3.3, cod liver oil 1, water added equal to 1.4 times the dry weight. Vitamin supplements, as mg/kg wet weight of diet were, choline chloride, 1000 mg; inositol, 100; riboflavin, 3.2; thiamin, 2; ascorbic acid, 4; pyridoxine HCl, 1.6; 40 mg each of Ca Pantothenate, niacin, p-aminobenzoic acid and α-tocopherol and 0.2 each of 2-methyl-1, 4-naphthoquinone biotin and folic acid. Water was added so that the diet might be fed as a gel, thus reducing scattering and allowing a more accurate estimation of uneaten food. For animals to be fed the protein-free diet, glucose was substituted isocalorically for the casein of the control diet. For animals to be fed the "drug" diet, 0.05% 2-acetylaminofluorene was added to the control diet.

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2. Mendel, B., Hawkins, R., and Nichikawara, M., *Am. J. Physiol.,* 1949, v154, 495.

3. Ammon, R., *Pflug. Arch. ges. Physiol.,* 1933, v233, 486.

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TABLE I.
Plasma Pseudocholinesterase Activities and Liver Protein Concentrations of 4 Protein-starved Rats Compared with 4 Pair-fed Controls.

	42 days protein-free diet	Pair-fed controls
Pseudocholinesterase activity (μl CO_2/ml/hr)	14, 41, 34, 23 (28)*	79, 64, 75, 54 (68)* † t = 9.5; P = <0.01
Liver protein conc. (g protein/100 g liver)	11.5, 12.4, 11.0, 9.6 (11.1)*	19.3, 19.0, 19.2, — (19.2)*

* Means.
† "t" and "P" values are in relation to the pseudocholinesterase values obtained for the two groups.

Results. A. *Characterization of Rat Plasma Pseudocholinesterase.* Since rat plasma pseudocholinesterase specifically has not been characterized, it was deemed necessary to establish the identity of the rat and dog enzymes, the latter having been thoroughly studied(6). Accordingly, curves relating enzyme activity to *pH, enzyme concentration,* and *substrate concentration* were obtained, using the pooled plasma of 22 rats. These curves were in good agreement with the data recorded for the dog plasma enzyme by Mendel, Mundell and Rudney(6). Since Warburg determinations to be described were extensive and spread over a period of approximately 14 days, plasma was used in many cases, that had been stored in the frozen state. To test enzyme stability, plasma was pooled from 5 rats and divided into 5 equal portions. Portion 1 was assayed for pseudocholinesterase activity immediately upon being taken from the animal. Portions 2 and 4 were stored at 4°C and portions 3 and 5 were stored frozen, at –19°C. After 7 days, portions 2 and 3 were assayed for peudocholinesterase activity and, after 14 days, portions 4 and 5 were assayed. The values obtained, for the 5 portions respectively were: 119.6, 121.7, 119.0, 118.0, and 131.1. The plasma is therefore very stable (whether stored at 4°C or frozen at –19°C) with respect to enzyme activity.

B. *Protein Depletion.* Eight rats, ages approximately 6 months, weights 200-250 g were divided into 2 groups. The first group was fed the protein-free diet for 6 weeks while the second group was pair-fed on the control diet. At 42 days all rats were sacrificed, their plasmas assayed for pseudocholinesterase activity and their livers for protein concentration. The data in Table I show that the fall in liver protein induced by protein starvation is accompanied by a decrease of plasma pseudocholinesterase activity to approximately 50% of control level. The average protein concentration in the livers of control rats agrees well with data from the literature(7) and with data obtained from similar experiments in this laboratory. Likewise, evidence of severe depletion in the animals fed the protein-free diet is given when the protein concentration of their livers is compared with values reported in similar experiments by Kosterlitz(7). Thus it would seem that a depletion in liver protein is correlated with a fall in the level of rat plasma pseudocholinesterase activity.

C. *2-Acetylaminofluorene.* Eight groups of rats were placed on experiment for a period of 32 weeks. Four groups were maintained throughout on the control diet. The remaining 4 groups received the "drug" diet for a period of 14 weeks and thereafter were given the control diet, free of the drug. At 8, 14, 24 and 32 weeks respectively, a group of the drug-fed animals and a group of the control rats were sacrificed. Plasmas were assayed for pseudocholinesterase activity, liver proteins were determined and the incidence of tumors noted. Table II indicates that after 8 weeks on a diet containing 2-acetylaminofluorene, the level of liver protein in the drug-fed animals did not differ significantly from the corresponding level in pair-fed controls. Plasma pseudocholinesterase activity, on the

6. Mendel, B., Mundell, D. B., and Rudney, H., *Biochem. J.*, 1943, v37, 473.

7. Kosterlitz, H. W., *J. Physiol.*, 1947, v106, 194.

TABLE II.
Liver Protein, Pseudocholinesterase Activity of the Plasma and Hepatoma Incidence in Rats Fed 2-acetylaminofluorene and in Pair-fed Controls. Four animals in each group—a control group and a drug group sacrificed at the end of each period.

Wks	Liver protein conc. g protein/100 g liver		Pseudocholinesterase activity μl CO_2/ml/hr		*"t"	*"P"	Hepatomas, %	
	Controls	Drug†	Controls	Drug†			Controls	Drug†
8	18.7	17.5	92	193	7.9	<.01	0	0
14	16.7	17.4	71	169	3.3	.015	0	50
24	19.2	15.9	68	160	7.4	<.01	0	75
32	18.7	12.4	86	110	2.5	.04	0	100

* "t" and "P" values refer to the relationship of the pseudocholinesterase values obtained for the drug-fed animals to those obtained for the control animals for each period.
† Drug withdrawn after 14 wks.

other hand rose to a level approximating twice that of control values. After 24 weeks, at which time the drug-fed animals displayed the initial evidence of decrease in liver protein concentration, pseudocholinesterase activity values were still significantly higher than control values. After 32 weeks, when liver protein concentration was quite low in the animals fed 2-acetylaminofluorene, plasma enzyme activity remained slightly higher than corresponding control levels. It should be noted however, that the total liver protein increased in the drug-fed animals as a result of an increase in size of the liver. This increase in size was associated with abnormally high water and fat concentrations but with an abnormally low protein concentration.

The mechanisms by which plasma pseudocholinesterase is stimulated by the feeding of 2-acetylaminofluorene remains obscure at this time. Attention is drawn, however, to the fact that the rise in plasma pseudocholinesterase activity precedes any gross evidence of hepatoma, and that the activity of the enzyme does decrease significantly following this rise,—a decrease which may be associated with depletion. These observations are receiving further study.

Summary. Evidence is presented for the identity of the plasma pseudocholinesterase of dog and rat and that the latter enzyme is stable when stored at low temperatures. Protein starvation is accompanied by a decrease in rat plasma pseudocholinesterase activity. The activity of this enzyme has been shown to increase markedly in rats fed 2-acetylaminofluorene before any gross evidence of hepatoma can be detected. As the hepatoma develops and liver protein concentration declines, the pseudocholinesterase activity of the plasma decreases from this high level.

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Methylene Blue Potentiation of Alloxan Diabetes.* (17670)

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Highet and West(1) reported that the intraperitoneal injection of methylene blue (1 cc of a 1% solution per rat), administered 30 minutes prior to alloxan protected

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1. Highet, Doris H., and West, Edward S., *J. Biol. Chem.*, 1949, v178, 521.