mice failed to produce inhibitory effect on DPN-synthesizing enzyme of the blood of these animals. Information is also being gathered on the effect of transplanted tumors on DPN synthesis by tissues other than liver or blood. Growing tumors may exert their influence on DPN synthesis by systemic metabolic competition with other tissues. On the other hand, the thesis that the tumor contains an inhibitor which is transmitted through the blood and which blocks its synthetic capacity to form DPN is also being explored. Search is being made for a humoral factor either in tumor tissue or in tissues from tumor-bearing hosts which may inhibit the synthesis of DPN from NMN.

Summary. 1. Homogenates of liver from mice bearing transplanted tumors (Sarcoma 37, Lymphoma 1, Lymphoma 2, Leukemia L 1210, Hepatoma 129 and Leukemia L-4946) exhibited a drop of 30 to 50% in capacity to synthesize DPN. 2. Surgical removal of Sarcoma 37 restored the DPN-synthesizing capacity of the livers to the level of livers from control mice. 3. Within one day after inoculation of tumor into mice, ability of the blood to synthesize DPN dropped about 50%.

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Proteolytic Enzymes. III. Circulating Esterase Activities after Parenteral Trypsin and Chymotrypsin in Rabbits.* (22619)

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The hydrolysis of *p*-toluenesulfonyl-L-arginine methyl ester (TAMe) by trypsin, and of acetyl-L-tyrosine ethyl ester (ATEE) by chymotrypsin was first reported in 1948(1,2). The method used, based on titration of acid liberated by hydrolysis of the ester, was rapid, relatively accurate, and required low enzyme concentrations. We were interested in measuring circulating enzyme activity after parenteral administration of trypsin and chymotrypsin and chose the esterase method because of these advantages.

Materials and methods. Crystalline preparations of trypsin (Armour D-531-157) and chymotrypsin (Armour D-530-165) were employed. The respective hemoglobin activities (3) of these lyophilized preparations were 1.47 and 0.78 μg tyrosine μg/minute. Rabbit plasma was prepared by mixing 9 parts of blood with 1 part of 3.2% sodium citrate, followed by centrifugation. Imidazole buffer was prepared by adding 1.75 g imidazole to 90 ml of 0.1 *N* HCl. The pH was adjusted to 7.4 and the volume brought to 100 ml with distilled water. This solution was diluted 1:10 with saline for use. TAMe, the substrate for trypsin, was present in the final reaction mixture at a concentration of 0.02 M. For chymotrypsin, ATEE was used in a concentration of 0.006 M.

Esterase determinations were carried out separately at 37°C in a small temperature-controlled water bath. Reaction volumes were constant at 4.0 ml, and mixing was continuous with a magnetic stirrer. All determinations were run at pH 7.4. One ml imidazole buffer was added to the reaction mixture except when plasma was present. Saline was used as the diluent for all reagents. Rate of substrate hydrolysis was measured by determining time required at pH 7.4 for liberation of an amount of acid neutralized by 0.01 ml of 0.1 *N* NaOH.

Results. In vitro studies. In the absence of plasma, reaction velocity was linearly re-

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lated to enzyme concentration. The reaction rates, expressed in μM acid/min./μg enzyme, were 0.45 for trypsin and 0.52 for chymotrypsin. Successive readings for any one determination showed rate to be constant with time, indicating a zero-order reaction.

The inhibiting effect of plasma on esterase activities of the enzymes was determined as follows for both trypsin and chymotrypsin. One part of a saline solution of the enzyme was incubated at 37°C with 5 parts (v/v) of plasma. After a definite time, a 0.6 ml aliquot was transferred to an otherwise complete reaction mixture for determination of esterase activity. Fig. 1 and 2 show the results obtained with increasing levels of trypsin and chymotrypsin, respectively, after a 2-minute incubation between enzyme and plasma. Comparative curves for the enzymes in the absence of plasma are also shown.

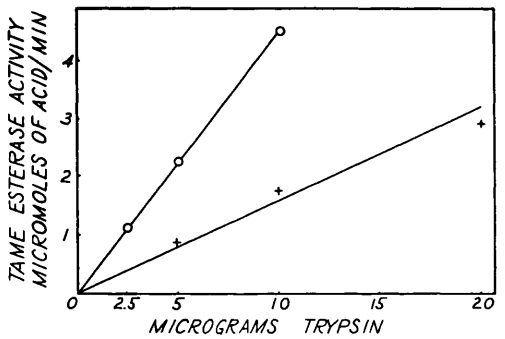

FIG. 1. Relation between trypsin concentration and TAMe esterase activity: (○) in absence of plasma, and (+) in presence of 0.5 ml rabbit plasma.

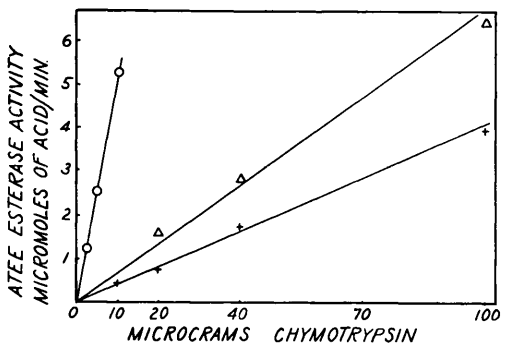

FIG. 2. Relation between chymotrypsin concentration and ATEE esterase activity: (○) in absence of plasma, and (△, +) in presence of 0.5 ml plasma samples from 2 different rabbits.

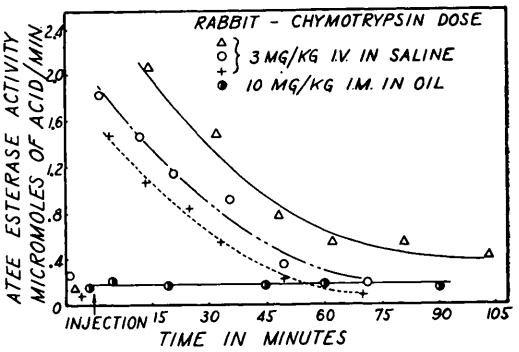

FIG. 3. ATEE esterase activity of 0.5 ml plasma samples withdrawn from rabbits treated parenterally with chymotrypsin.

The percentage inhibition appeared to be nearly constant at the different enzyme concentrations studied with any given plasma sample. However, the inhibition of trypsin varied from 58 to 68% using plasma samples from 6 rabbits. For chymotrypsin, the range was 85 to 93% with plasma samples from 4 rabbits.

The effect of increasing incubation time between enzyme and plasma was also investigated. No significant decrease in esterase activity was found to occur within the 2- to 18-minute interval studied for either enzyme.

In vivo studies. Circulating esterase activity was investigated in rabbits treated with chymotrypsin and trypsin. Chymotrypsin in a concentration of 1 mg/ml in saline was administered intravenously to 3 rabbits at a dose of 3 mg/kg. Blood samples (1.8 ml blood + 0.2 ml 3.2% sodium citrate) were taken by heart puncture before, and at intervals after injection. These were immediately centrifuged for separation of the plasma. Determination of the esterase activity present in 0.5 ml plasma was begun approximately 3 minutes after the heart puncture. Assay conditions were the same as described previously for chymotrypsin.

One rabbit was treated intramuscularly with 10 mg/kg of a commercial preparation of chymotrypsin in oil (Chymar). Circulating esterase levels were measured as described above.

The results for intravenous and intramuscular administration of chymotrypsin are shown in Fig. 3. Intravenous administration re-

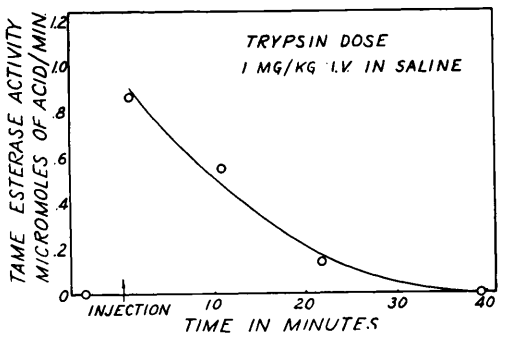

FIG. 4. TAMe esterase activity of 0.5 ml plasma samples withdrawn from a rabbit following an intravenous injection of trypsin.

sulted in a high level of circulating esterase activity which decreased with time. Approximately 50% of the initial activity was lost by 30 minutes after the injection. A small amount of spontaneous activity was measured in plasma samples collected from rabbits prior to injection. An intramuscular injection of a relatively high dose of chymotrypsin in oil did not detectably raise this level of activity.

Circulating esterase activity following administration of trypsin was also investigated. One rabbit was treated intravenously with a dose of 1 mg/kg in a concentration of 0.33 mg/ml saline. One-half ml plasma samples were prepared and assayed for TAMe activity using methods already described. The result is shown in Fig. 4.

Intramuscular administration of trypsin was studied in 3 rabbits. Doses of 1.5 and 7.5 mg/kg in saline were given to 2 rabbits while a third received 7.5 mg/kg in oil. In no instance was any TAMe esterase activity detected in blood samples withdrawn from these animals.

Discussion. *In vitro* experiments showed the TAMe esterase activity of 2.5 to 20 μg trypsin to be 58 to 68% inhibited by 0.5 ml plasma samples from several rabbits. Parallel experiments with 10 to 100 μg chymotrypsin showed 85 to 93% inhibition of esterase activity. These results indicate low inhibition of esterase activity compared to inhibition of protease activity of these enzymes by plasma reported by other workers(4,5,6,7). Innerfield(8) reported that trypsin administered intramuscularly was clinically effective as an anti-inflammatory agent. This observation has since been confirmed by other workers(9,10). Later, Miechowski and Ercoli(11) working with mice, rats, rabbits and guinea pigs found intramuscular chymotrypsin to be a highly effective anti-inflammatory agent. The mechanism of action whereby these enzymes produce a systemic anti-inflammatory effect is not known. On one hand, it seems possible that the mechanism could involve a prolonged systemic adsorption of the enzymes. Alternatively, the anti-inflammatory effect may result from systemic absorption of local digestion products produced by enzymatic hydrolysis at site of injection. Because of these possibilities we studied circulating trypsin and chymotrypsin levels following injection of these enzymes by the intramuscular route in rabbits. The results showed that no detectable systemic absorption of trypsin or chymotrypsin occurred within the interval studied, even though relatively high doses were used. While this does not prove that systemic absorption of the enzymes is not involved in the anti-inflammatory effect, it must tentatively be considered as evidence against this possibility.

Summary. Inhibition of trypsin and chymotrypsin by plasma has been studied using ester substrates. The amount of inhibition measured was low compared to that reported in the literature using protein substrates, and permitted the measurement of enzyme concentrations in plasma of the order of 10 μg/ml. Intravenous injection of trypsin and chymotrypsin to rabbits resulted in measurable circulating esterase activity, which persisted, in the case of chymotrypsin, up to an hour after injection. No activity was detected following intramuscular trypsin, however, and no detectable increase over a small spontaneous esterase activity was noted following intramuscular chymotrypsin. Thus, it appears that the anti-inflammatory effect noted following intramuscular injection of these enzymes is probably not mediated by their systemic absorption.

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Propagation of Strain L (Earle) Cells in Agitated Fluid Suspension Cultures.* (22620)

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Several workers, using various technics for measuring increases in cell populations, have shown that a characteristic growth curve exists for mammalian tissue cells cultivated *in vitro*(1,2,3,4). As is true with microbial populations, this growth cycle can be separated into 4 major phases: 1) lag phase, 2) logarithmic growth phase, 3) stationary phase, and 4) logarithmic death phase(2,5). The study reported here made use of the rate of change of growing cell populations to demonstrate the effects of inoculum size, pH and serum concentration on strain L mouse fibroblasts in agitated fluid suspension cultures. It was hoped that an analysis of the growth curve would make it possible to establish a more efficient system for cultivation of these cells.

Materials and methods. Strain L mouse fibroblasts(8) were used in this study. The stock strain was grown in stationary cultures on the surface of bottles with a capacity of 250 ml and a surface area of approximately 56 cm^2. Each culture contained 10 ml of a nutrient medium composed of 20% horse serum, 60% modified synthetic solution 199† (9), 20% Hanks balanced salt solution, and 100 units per ml each of penicillin and streptomycin. The fluid suspension culture method (6,7) was used in these studies. In this type of culture, 80 ml of medium of the same composition as previously described, and containing the desired number of cells, was placed in a 250 ml Erlenmeyer flask stoppered with a #6 rubber stopper. During incubation such cultures were placed on a New Brunswick rotary shaker, modified to give a speed of 100 rpm. Daily or twice daily, counts were made from a small sample using a hemocytometer. Since the cells in such cultures remain in a homogenous suspension, no treatment was required to disperse the cells prior to making the counts. The pH of samples taken for counting was determined with a Beckman Model G pH meter. For other cultures used as controls, cells were harvested from a stock culture by removing the growth from the wall of the bottle with a rubber scraper. These cells were evenly suspended in the medium by repeated pipetting. A series of replicate cultures was set up with an inoculum of approximately 200,000 cells per ml by pipetting 10 ml of the cell suspension into each of 10-15 bottles, as described for stock cultures. The initial pH was adjusted to approximately 7.4. Two or 3 of these cultures were harvested each day, and cell counts were made using a hemocytometer.

Results. When the logarithm, to the base 2, of the cell number was plotted against time in

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† Purines and pyrimidines omitted and medium sterilized by autoclaving.