that 6-mercaptopurine decreases incorporation of radioactive acetate(8). This would indicate that the effect of purine analogs on acetate metabolism is not confined to *Tetrahymena*. Also 6-mercaptopurine may act in much the same way as the analogs described above.

No explanation is yet available for the death of cultures containing certain of the purine analogs. It cannot be accounted for on the basis of accumulation of acid as is the case in cultures deficient in either thioctic acid (9) or thiamine(10). Among other compounds which cause "suicide" of *Tetrahymena* are protopine and α-phenylbutyric acid, an analog of acetate (unpublished observations). So far it has not been possible to reverse the inhibition caused by protopine, but all the other "suicidal" compounds or conditions are in some way involved in acetate metabolism.

Summary. Eight purine analogs were compared as to their effects on growth of *Tetrahymena*. A certain degree of correlation was found between type of structural change present in the analog and ability of adenine, acetate, sterol or combinations of these compounds to reverse the inhibition. Analogs which cause "suicide" of cultures do not fit the pattern of other inhibitors.

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An Activator of Plasminogen Produced by Cell Culture. (25229)

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A simplified scheme, modified from Astrup (1) for activation of plasminogen by "activators" or protease, and for demonstration of such activation by clot lysis or casein hydrolysis is shown in Fig. 1. There are many activators of plasminogen. Those of note are streptokinase, particulate "tissue activators," potassium thiocyanate extracted "tissue activators," microsome fractions of animal tissues, urokinase, human milk, trypsin, and chloroform. In addition, spontaneous activation may occur(1). This study describes materials released by metabolizing cell cultures which activate plasminogen(2) or which are proteolytic in the absence of plasminogen(3,4).

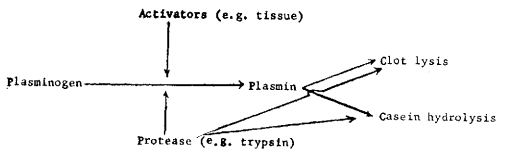

FIG. 1.

Materials and methods. Clarified supernatant medium from cell cultures was used as a source of plasminogen activator and of caseinolytic protease. The supernatant medium was prepared by feeding Medium 199(5) to cell cultures which had been previously washed 4 times with balanced salt solution (b.s.s.). After incubation for 96 hours at 36°C it was decanted and clarified by low speed centrifugation. Supernatant medium

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TABLE I. Activator of Plasminogen, and Protease from Cell Cultures.*

Exp.	Source of plasminogen	Activator	Substrate	Result
1	HS, PP	KB cell culture	SM, BC	+
2 a	HS, PP	KB supernatant	"	+
b	None	" "	"	0
c	HS, PP	None	"	0
d	None	KB supernatant + SK	"	0
3 a	None	MK supernatant	"	+
b	HS, PP	" "	"	++++

* Abbreviations are as follows: HS = human serum; PP = purified plasminogen; SM = skim milk; BC = bovine clot; SK = streptokinase (Varidase® Lederle). + = Proteolysis; 0 = no proteolysis; ++++ = accelerated proteolysis.

was assayed for activator of plasminogen by Christensen's method(6) employing the lysis of bovine fibrin clots and by a method employing the hydrolysis of casein(7) as indicated by the clearing of the opalescence of autoclaved skim milk(2). Duplicate serial 2-fold dilutions of supernatant medium in 0.2 ml amounts were made in Veronal® buffer solution (VBS), pH 8.2, ionic strength 0.05. To one dilution series, used to assay for activator, 0.1 ml of a 1:30 dilution of pooled human serum (HS) was added as the source of plasminogen, and to the other dilution series used to assay for protease, 0.1 ml of VBS was added. Then 0.1 ml of a 1:2 dilution of autoclaved skim milk (SM) was added as substrate to both series. A serum control containing only HS, VBS, and SM substrate served to control for spontaneous activation of plasminogen. Plates of clear vinyl plastic cups, Dispostrays® model 96 CV† were used instead of test tubes. Cups were covered with 0.6 ml sterile heavy mineral oil, incubated at 36°C and the clearing of opalescence of SM observed daily. A preparation of supernatant medium was considered to have a titer of activator or protease in units equal to the reciprocal of the highest dilution which caused clearing at 96 hours incubation.

Results. Activator of plasminogen and protease produced by cell cultures. KB cell culture (KB), derived from a human epidermoid carcinoma(8), caused clearing of skim milk or clot lysis if a source of plasminogen were present (Table 1, Exp. 1). The continued presence of cells was not essential for this reaction since the supernatant medium, free of cells (Table I, Exp. 2), caused proteolysis in the presence of plasminogen. Streptokinase (Varidase,® Lederle Laboratories) plus the supernatant medium did not produce proteolysis, *i.e.,* plasminogen was not present in the supernatant. These observations indicated release of an activator of plasminogen from KB cell culture. Supernatant from primary monkey kidney cell culture (MK)(9) (Table I, Exp. 3) caused proteolysis in absence of plasminogen and accelerated proteolysis when tested in presence of plasminogen. These observations indicate the presence of both protease and an activator of plasminogen in the supernatant medium from MK.

Characteristics of activator from cell culture. To determine the effect of various ratios of concentration of activator, *i.e.,* supernatant medium from KB cell culture, and of HS on the time required to clear SM, one was diluted and incubated with dilutions of the other (box titration). Increased concentrations of activator or HS up to an optimum (final concentration of 0.8% HS and 12.5% KB supernatant) shortened the time of clearing. Further increase delayed time of clearing. This delay may be attributed to inhibitors of proteolysis which have been described in tissue(1) and human serum(10). Human euglobulin(11) and purified plasminogen, "Pro-Actase"®, kindly supplied by the Ortho Co., showed less inhibition at the higher concentrtaions than did HS. Increased concentrtaions of SM substrate (5-20%) delayed time of clearing.

Evidence for hydrolysis of casein was the rise in optical density of the trichloroacetic acid filtrate at 275 mμ when SM substrate was incubated with activator from MK supernatant and euglobulin. This corresponded in time with clearing of the opalescence of SM. Identical evidence was obtained when KB supernatant or streptokinase-streptodornase (Varidase®, Lederle Laboratories) was used as activator for human euglobulin or human, chicken, rabbit and monkey serums. KB supernatant which assayed as 64 units of activator by milk clearing, had activity equal to

† Obtained from Joseph E. Frankle Co., Phila. Pa.

2 units of streptokinase when measured by Christensen's method(6) using clot lysis. Thus, the clot lysis method was not sufficiently sensitive for assay of most lots of unconcentrated supernatant medium.

The activator in supernatant medium from KB cell culture was non-dialyzable, stable to 3 cycles of freezing and thawing, active at pH 7 and pH 8.5, stable on storage at 4°C or −20°C for at least 6 weeks, but showed an inconstant slight drop in titer after 1 week at 36°C or 20°C. Complete inactivation occurred after 10 minutes at 80°C or 3 hours at 56°C, and after filtration through very fine fritted glass or Seitz S3 filters. Activity was sedimented at 18,000 g for 90 minutes (microsome size). Activator from KB supernatant after treatment with 1M KSCN(12), showed no change of titer and showed only a minimal loss of titer after filtration. The activator is thus made soluble and filterable by treatment with KSCN, which is a property described for "tissue activator."(12).

Source of activator produced by KB cell culture. Experiments were designed to determine if activator released from cell culture represents portions of an intracellular store of activator or represents materials being continually produced by metabolizing cells. The supernatant medium was removed daily from cell cultures, the cells washed, and the cells fed with fresh medium. The decanted medium was assayed for activator using SM as substrate. Sixteen units of activator were released daily from a 32 oz. cell culture bottle containing approximately 3 x 10^7 KB cells with 50 ml of Eagle's medium or Medium 199. The release of activator remained constant for 4 days and diminished during the next 3 days as the cell sheet degenerated. Release of activator from cell cultures in lactalbumin hydrolysate medium(13) was diminished and variable. To determine whether activator which was continually released from KB cells came from intracellular stores or was continually produced, intracellular activator content was measured as follows. KB cells were scraped from 32 ounce cell culture bottles and washed 4 times in 30 ml of b.s.s. by low speed centrifugation. After washing, the cells were resuspended in sufficient VBS to give a concentration of 10^7 cells/ml. Aliquots of cells in suspension were disrupted by 3 cycles of freezing and thawing or by sonic oscillation in a 250 watt, 10 kilocycle, Raytheon sonic oscillator at maximum input for 10 minutes. Samples of intact and disrupted cells were extracted with KSCN. The resulting 4 preparations of disrupted cells were assayed for activator and protease using SM as substrate. In no instance were more than 2 units of activator detected in any of these preparations although as many as 3 x 10^7 cells were used. Assays for inhibitors of proteolysis in these preparations indicated that the inhibitor content of KB cells was insufficient to mask appreciable amounts of intracellular activator. The data testifies for production of activator by metabolizing cells rather than for release of active intracellular material by cell breakdown.

Comparison of MK supernatant with KB supernatant. Serum-free Medium 199 from MK was found to contain 32 units of protease when tested without serum and 512 units of activator when tested in the presence of serum, using SM as substrate. The activity as caseinolytic protease was minimally diminished after filtration through very fine glass while the activity as activator of plasminogen was diminished to a greater extent after filtration. Thus, MK supernatant consistently had an activator: protease ratio of 4:1 to 8:1 after filtration, regardless of how great this ratio was prior to filtration. As previously stated, KB supernatant preparations with as much as 512 units of activator showed activity only as activator of plasminogen and the activity was not filterable. An interpretation might be that both KB and MK cells release a microsome-size activator. In addition MK cells release a filterable trypsin-like protease which can activate plasminogen as demonstrated by the 4 to 8 fold increase of titer in the presence of serum.

Summary. Previous investigators observed clot lysis by tissue grown in plasma clots(14, 15). Our results indicate that an "activator" of plasminogen is released from KB cell cultures. Supernatant from MK cell cultures activated plasminogen and was also proteolytic in the absence of plasminogen. These cell cul-

tures had a third property, inhibition of proteolysis. The activator described here resembles "tissue activator" in that it is not proteolytic in the absence of serum and can be dissolved by KSCN. It also resembles activator from excised rat lung(16) in that it is the size of microsomes. The interpretation that release of activator from cell culture is a metabolic process and not from cell breakdown seems warranted since only a small fraction of the activity found in the supernatant can be extracted from the cells.

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Enzymatic Determination of *in vitro* Lysis of Leukocytes by Tuberculin. (25230)

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In vitro lysis of leukocytes by tuberculin, described by Favour(1), generally is determined by microscopic counts of leukocytes before and after incubation in presence and absence of tuberculin. The sensitivity of this method depends on the accuracy of cell counts. O'Neill and Favour(2) reviewed the factors affecting leukocyte counts and concluded that the mechanical errors inherent in the method can be controlled so that an overall error of less than 10% may be obtained. In practice, however, a cytolysis of less than 10% is not considered significant(3,4). Another limiting factor in the method is the time involved in performing large numbers of cell counts. For these reasons, we have been interested in finding a method for determining leukocyte lysis which is more rapid and more sensitive than microscopic cell counts. Kerby (5) described a method to demonstrate leukocyte injury and lysis, based on the fact that certain cellular components are released into the surrounding medium when leukocytes are damaged. Using release of a lysozyme-like enzyme as a measure of cell damage, Kerby demonstrated a harmful effect of certain bacterial derivatives on human leukocytes(6), and later showed that ACTH and cortisone therapy protected human leukocytes against injury by piromen(7). This paper describes our attempts to use release of this intracellular enzyme as a measure of lysis of leukocytes by tuberculin.

Materials and methods. The technic for assay of lysozyme-like enzyme was similar to that described by Kerby(5). However, it

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