towards casein (KUNITZ-test[7]: 1 unit is defined as the amount of enzyme, which, after incubation for 20 min at 37 °C, caused an increase in absorbancy of the trichloro-acetic acid-filtrate of 0.001); moreover, the second one hydrolyzed BAEE also, measured according to the spectrophotometric method of SCHWERT and TAKENAKA[8]. For testing the kinin-releasing activity 0.5 ml of 3% globulin solution (bovine plasma fraction, precipitated between 0.3 and 0.5 fold ammonium sulphate saturation) in 0.06 *M* phosphate buffer pH 8.0 were incubated with 0.1 ml of enzyme fraction for 3 min at 37 °C and an aliquot amount tested on the isolated guinea-pig ileum preparation (in 10 ml aerated Tyrode-solution with 1 mg atropin and 0.1 mg Avil (Farbwerke Höchst AG, Germany) per litre at 37 °C); synthetic bradykinin (Sandoz AG, Basel, Switzerland) was used as standard. Only the BAEE-hydrolyzing fraction showed kinin-releasing activity. Casein- and BAEE-hydrolysis of this enzyme, as well as its release of kinin from plasmaglobulins, is completely inhibited (enzyme-inhibitor ration 1:5, w/w, 30 min preincubation) by soybean- (Serva) and ovomucoid-trypsin inhibitor (Calbiochem), Trasylol (Bayer AG, Germany), N-tosyl-L-lysyl chloromethane (Calbiochem) and phenylmethylsulfonyl fluorid (Serva, specific for serine-enzymes), but not by N-tosyl-L-phenylalanyl chloromethane (Calbiochem, specific for chymotrypsin). Preincubation of the enzyme with different amounts of BAEE resulted in inhibition of the kinin-release, probably due to competitive inhibition (Figure 2). In contrast to the observations of REIS et al.[1], none of the enzyme fractions possessed hydrolytic activity towards N-acetyl-tyrosine ethyl ester (ATEE).

Our results confirm the suggestion that the kinin-releasing activity of pronase can be referred to a trypsin-like enzyme.

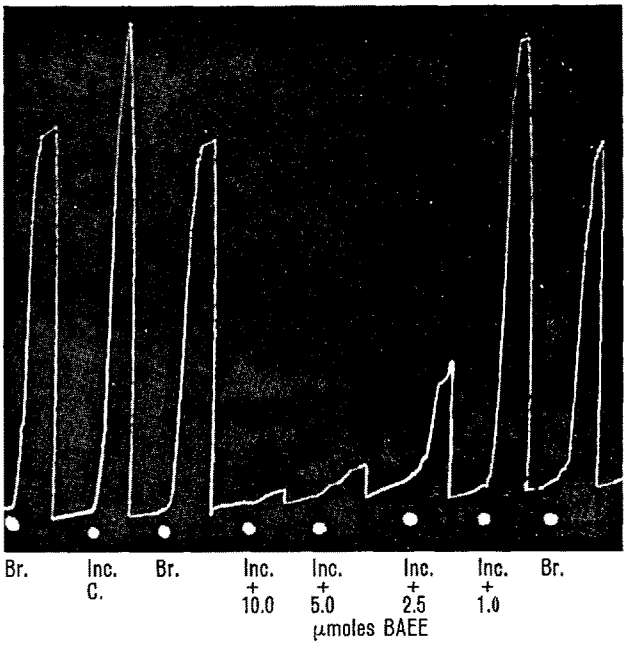

Fig. 2. Inhibitory effect of BAEE on the kinin-releasing activity of the trypsin-like enzyme from pronase, tested on the isolated guinea-pig ileum. Br = synthetic bradykinin 0.02 μg/10 ml Tyrode solution; Inc. = 0.05 ml of the following incubate: 0.1 ml enzyme (30 μg) were preincubated with BAEE for 1 min at 37 °C, than 0.5 ml 3% plasmaglobulin-solution added and incubated for 3 min. C = control, incubate without BAEE.

Zusammenfassung. Die Kinin freisetzende Eigenschaft von Pronase, einem Proteasen-Gemisch von *Streptomyces griseus*, ist auf ein Trypsin ähnliches Enzym zurückzuführen.

D. MEBS

Institut für Rechtsmedizin der Universität, Kennedyallee 104, D-6 Frankfurt a.M. (Germany), 4 August 1970.

1 M. L. REIS, M. C. MEDEIROS and M. ROCHA E SILVA, Abstr. III. Int. Pharmac. Congr., No. 485 (São Paulo 1966).
2 Y. NARAHASHI, K. SHIBUYA and M. YANAGITA, J. Biochem., Tokyo *64*, 427 (1968).
3 S. WÄHLBY, Biochim. biophys. Acta *151*, 394 (1968).
4 M. TROP and Y. BIRK, Biochem. J. *116*, 19 (1970).
5 Y. NARAHASHI and J. FUKUNAGA, J. Biochem., Tokyo *66*, 743 (1969).
6 L. JURASEK, D. FACKRE, L. B. SMILLIE, Biochem. Biophys. Res. Commun. *37*, 99 (1969).
7 M. KUNITZ, J. gen. Physiol. *30*, 291 (1947).
8 G. W. SCHWERT and Y. TAKENAKA, Biochim. biophys. Acta *16*, 570 (1955).

Identification of Clones of Mammalian Cells by Isoenzyme Distribution Patterns

In the field of cell culture, a continuing problem is that of identifying the cell line being studied and of knowing if and when changes in that cell line have occurred[1]. Isoenzyme distribution patterns (zymograms) have been used to differentiate between samples of the same tissue from different species[2–6], different tissues from the same species[2,5,7–9] or different cell lines[1,10,11]. The work presented here describes the use of this technique to distinguish among different clones from the same cell line.

Materials and methods. Cell strains and cloning technique. Cultures of the LM cell line and a clone, Cl 1, from this cell line were maintained serum- and antibiotic-free in spinner culture on medium 199 peptone. Clone 1 was recloned by a one-step dilution method: 10-μl samples from the spinner were mixed with 10 ml of 199P containing 20% horse serum, 500 units penicillin, and 500 μg streptomycin. From this pool, 100 μl of the cell suspension were placed in Falcon tissue-culture-grade plastic petri dishes containing 10 ml of the above medium and incubated in a 5% CO_2 atmosphere. At 4 weeks, 15–35 colonies (15–35% plating efficiency) were observed; and from these, clones (Cl 1–1, 1–2, 1–3, 1–9) were picked at random. The clones were isolated with glass tubing (6 mm) wells, aspirated from the surface of the petri dish with fresh cloning medium, and placed in Falcon T-30 flasks. Cells from the monolayer cultures of the individual clones were harvested, resuspended at 1×10^7 cells/ml in fresh medium containing 5% dimethyl sulfoxide, and stored at liquid nitrogen temperatures.

Growth of the cells and preparation of cell-free extracts. Cultures were obtained by placing the contents of a

Rf values for isoenzyme bands from the various cell cultures

Enzyme substrate	Rf values for isoenzyme bands																													
α-Naphthyl acetate																														
LM	0								12	15			24	29	31	34	39	42		49	51	59		64						
Cl 1	0	4				10		15	19	21	24				35	38	44			52	58									
Cl 1–1	0										23	27	31	34	38	41		49		56										
Cl 1–2	0											27	31	35	40		46	48		54										
Cl 1–3	0								12	14			23	28	30		39	42		49		57								
Cl 1–9	0	3	5			11		13	15				24	28	31		38	42		48	51	55		60						
Nathyl propionate																														
LM	0								11	15		21	23			35	39		43		52	57								
Cl 1	0								10	15		21	24			33	39	42	44		52	58								
Cl 1–1	0	3							11	14	16	19	22			36	38		46											
Cl 1–2	0			9					12	14	17	19	24	26			38		43	48										
Cl 1–3	0								11	15	17		23			34	40		44		52	57								
Cl 1–9	0								10	15	18		23	28		36	39		46			55								
α-Naphthyl butyrate																														
LM	0								10	15	17	19	24				39	41	42	47	51									
Cl 1	0			3	5	7			12		17	19							44	49	53									
Cl 1–1	0								10		16		22				38		43		51									
Cl 1–2	0									13	18	20	22	27	31			42	45		51									
Cl 1–3	0	2		3					10	15	16	18	23				39	41	43	46	53									
Cl 1–9	0								11		17		23	27				42	45		58									
β-Naphthyl acetate																														
LM	0								12	15		19	22	27			38	43		49		57								
Cl 1	0			6						15		21	23	25		34	38	43												
Cl 1–1	0												23	26		29	36	40			54									
Cl 1–2	0	3		5								22	24	25		29						56	59	64	73					
Cl 1–3	0												23			34	39	44			52									
Cl 1–9	0								11				22				38	42		48		56		61						
Protein																														
other clones	0	2	3	5	7	8	10		15	18	19	22	26		31	35	38	43	50	53	60	70								
Cl 1–9	0	2	3	5	7		10	12	15	18	20	23	27						49	54	61	71								

frozen ampoule into 50 ml of serum- and antibiotic-free 199P medium containing 0.12% methylcellulose (15 CPS, Fisher Scientific Co., Silver Spring, Maryland) and placing this in a 200 ml spinner. Suspension cultures were grown at 37 °C for 5–6 days until the count reached $1.5–2.0 \times 10^6$ cells/ml. The cells were sedimented by centrifugation (10 min at $500 \times g$), washed once with saline, resuspended in 1.0 ml water[5] and then stored at − 20 °C until disrupted by sonic oscillation. A Branson sonifier, Model W, was used (15 watts, 30 sec) while the cells were in a 4 °C water bath. The sample was frozen and thawed, and the supernatant liquid, after a single centrifugation at $1000 \times g$ for 10 min, was used for disc electrophoresis. This freeze-thaw cycle yielded clearer supernatant liquids than those obtained from a direct centrifugation following sonic disruption. Protein concentration was determined spectrophotometrically by the difference in absorption at 215–225 nm[12].

Disc electrophoresis. Polyacrylamide gel disc electrophoresis was performed by the technique of ORNSTEIN[13], DAVIS[14] and CLARKE[15], as described by Canal Industrial Corp. (5635 Fisher Lane, Rockville, Maryland 20752). Twice the indicated concentration of N,N,N′,N′-tetramethylethylene diamine (Temed) was used and the small pore gel stock solution contained 34 g Acrylamide and 0.85 g *Bis*-Acrylamide. Ammonium persulfate was used as catalyst for both gels. All samples (0.1 ml) contained 0.2 mg protein and at least 15% sucrose. A thin cap of large pore gel was placed on top of the sample and buffer on top of this. Separation was achieved either at constant current (50 mamp/24 gels) or constant voltage (80 V/24 gels), and the brom-phenol-blue tracking dye migrated 55 mm in 3 h. The gels were cut at the tracking dye so that band position could be determined by Rf value, washed for 5 min in cold 0.02*M* *tris*-HCl buffer, pH 7.1, and then placed in the various staining solutions at 37 °C. Precautions were taken to avoid exposing staining solutions to light.

[1] S. M. GARTLER, Nature, Lond. *217*, 750 (1968).
[2] P. FORTELIUS, Acta path. microbiol. scand., Suppl. *164*, 3 (1963).
[3] L. GROSSBARD, M. WEKSLER and R. T. SCHIMKE, Biochem. biophys. Res. Commun. *24*, 32 (1966).
[4] C. L. MARKERT and F. MOLLER, Proc. natn. Acad. Sci., USA *45*, 753 (1959).
[5] J. PAUL and P. F. FOTTRELL, Biochem. J. *78*, 418 (1961).
[6] R. L. SEARCY, S. HAYASHI, J. E. BERK and H. STERN, Proc. Soc. exp. Biol. Med. *122*, 1291 (1966).
[7] P. CUATRECASAS and S. SEGAL, Science *154*, 533 (1966).
[8] C. L. MARKERT and R. L. HUNTER, J. Histochem. Cytochem. *7*, 42 (1959).
[9] E. E. SMITH and A. M. RUTENBURG, Science *152*, 1256 (1966).
[10] L. BECKMAN and J. D. REGAN, Acta path. microbiol. scand. *62*, 567 (1964).
[11] R. YASIN and G. J. GOLDENBERG, Nature, Lond. *211*, 1296 (1966).
[12] W. J. WADDELL, J. Lab. clin. Med. *48*, 311 (1956).
[13] L. ORNSTEIN, Ann. N.Y. Acad. Sci. *121*, 321 (1964).
[14] B. J. DAVIS, Ann. N.Y. Acad. Sci. *121*, 404 (1964).
[15] J. T. CLARKE, Ann. N.Y. Acad. Sci. *121*, 428 (1964).

Isoenzyme staining procedures. Esterase substrates were dissolved (1 g/100 ml acetone) and dye-substrate mixtures (5 mg/gel) were prepared by combining 150 μmoles *tris*-HCl buffer, pH 7.1, 0.12 ml substrate, and 2.6 mg fast blue RR. Lactic dehydrogenase activity was determined in a solution containing 100 μmoles *tris*-HCl buffer, pH 7.1, 70 μmoles KCN, pH 7.5, 500 μmoles substrate, 1.5 mg NAD, 1.5 mg nitro blue tetrazolium (NBT), and 0.07 mg phenazine methosulfate (PMS). Hydroxy acid oxidase activity (α-hydroxy butyric, α-hydroxy caproic, α-hydroxy valeric, α-hydroxy phenyl propionic, and β-hydroxy butyric) was determined by a modification of the method of ALLEN and BEARD[16]. The reaction mixture contained 200 μmoles potassium phosphate buffer, pH 7.5, 500 μmoles substrate, 2 mg NBT, 2 mg NAD, and 0.1 mg PMS. After staining, the gels were fixed for several hours[2] and then stored in 10% acetic acid in stoppered test tubes. Protein was stained for 1 h in a solution of 2 g Amido Schwarz/100 ml methanol : water : acetic acid (5:5:1) and then destained in several changes of methanol : water : acetic acid (4:10:1).

Results. Identical patterns were obtained when a large batch of cells was disrupted by sonic oscillation and the cell-free extract divided into 3 portions and subjected to disc electrophoresis on days 1, 3, and 7 after sonication and storage at −20 °C. In the absence of substrate (α-naphthyl acetate or lactic acid) no bands appeared.

Each clone as well as the parent cell line gave a reproducible and characteristic zymogram for esterase activity (Figure and Table). The longer the ester-linked side chain became, the lower was the total relative esterase activity. Consequently, the incubation time had to be adjusted; however, no new bands appeared upon prolonged incubation. None of the clones showed any difference in hydroxy acid dehydrogenase activity, and all patterns resembled that obtained for lactic dehydrogenase (4 bands). As was true with esterase activity, the relative enzyme activity decreased as chain length increased.

These patterns were reproducible in duplicate runs on the same sample and remained constant during continuous subculture over a six-month period. One of the clones, Cl 1–1, has been maintained continuously for 1 year with no observable changes in pattern.

Discussion. Maximum cell population is not a reliable criterion for clone identification, however, in 4–9 replicate growth experiments the parent and all subclones with the exception of Cl 1–9 grew to a maximum cell density of $1.42–1.75 \times 10^6$ cells/ml with a standard deviation of $0.24–0.44 \times 10^6$ cells/ml, while in 5 experiments clone 1–9 grew to $0.69 \pm 0.005 \times 10^6$ cells/ml. Zymograms from Cl 1–9 for α-naphthyl acetate, α-naphthyl butyrate and protein were markedly different from those observed among the other clones (Table).

All of the esterase isoenzymes observed in these cells appear to be arylesterases[17], as the reaction rate decreased as the length of the side chain increased and as the naphthyl linkage changed from α to β. MARKERT and MOLLER[4] have proposed that all α-hydroxy acids are oxidized by the same dehydrogenase and the zymograms appear to support this conclusion. As was the case with the esterases, activity decreased as chain length increased.

Although the clones all possessed identical hydroxy acid zymograms, they could be readily distinguished by their esterase zymograms (Figure and Table).

GARTLER[1] surveyed 20 heteroploid human cell lines and found identical zymograms for glucose-6-phosphate dehydrogenase and phosphoglucomutase. After showing that changes did not occur as a result of his method for in vitro culturing, he concluded that all the cultures had been contaminated by HeLa cells. Our work, with PPLO-free L-cell clones, shows no differences among many of the dehydrogenase zymograms but recognizable and reproducible differences among the esterase zymograms. In view of these results, GARTLER's conclusion might be modified to include the possibility 1. that certain enzymes are more prone to phenotypic and/or genotypic variation than others or 2. that mode of culture profoundly influences zymogram pattern. The effect of environment upon cell physiology is such that many instances of supposed contamination by a second cell line or selective growth of a particular cell type may, in fact, have been due to changes in the environment (unpublished observations).

When grown under constant environmental conditions, the zymogram is apparently a stable characteristic of a particular cell line[1,10]. We have found that the zymogram patterns observed for each clone are stable through consecutive subcultures over a six-month period. Recently, we had occasion to determine the zymogram patterns from one of the clones which had been frozen for $2^1/_2$ years. The patterns were identical with those observed previously. The zymograms from individual clones grown in suspension culture can therefore be used to identify and distinguish among the clones. When combined with studies to monitor enzyme specific activities, these procedures should allow for continual and rapid study of the effects of nutritional, environmental, and genetic changes upon the metabolic capability of cells in culture.

Résumé. Nous avons déterminé les zymogrammes d'estérase de cultures de cellule de ligne L–M en suspension, de son clone (Cl 1) et quatre sous-clones de ce clone. Ces zymogrammes sont reproductibles. Ils offrent des différences caractéristiques et peuvent être utilisés pour l'identification et la distinction des divers clones. Aucune différence n'a été observée dans les modèles d'acide hydroxy deshydrogénase.

D. C. FISH, J. P. DOBBS
and R. C. CARTER

Department of the Army, Process Development Division, Fort Detrick, Frederick (Maryland 21701, USA), 23 April 1970.

Characteristic zymogram for α-naphthyl propionate esterase from the various clones.

[16] J. M. ALLEN and M. E. BEARD, Science *149*, 1507 (1965).
[17] D. J. KOMMA, J. Histochem. Cytochem. *11*, 619 (1963).