cervical spinal cord. It was completely abolished by removal of the thoracic and upper lumbar spinal cord in 4 of 6 dogs studied. It was also blocked by localized resection of the fifth to seventh thoracic segments of the spinal cord, but not by removal of more caudal segments. These data indicate that the portion of the spinal cord above the seventh thoracic segment is the only part of the central nervous system which is essential in the dog for the increase in epinephrine secretion produced by insulin-induced hypoglycemia.

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Preparation of Mammalian Cell Cultures with Enzyme from *Aspergillus oryzae*. (28573)

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For quantitative studies with a subline of Earle's L strain cells adapted to grow in a chemically defined medium(1), the need arose for a suitable enzyme that would release cells from glass without injurious effects. Preliminary studies suggested that this need was satisfied by a proteolytic enzyme isolated from an *Aspergillus oryzae* fermentation and designated Aspergillin-O(2). We therefore undertook to determine whether Aspergillin-O was equally effective in preparing primary cultures and cultures from established cell lines. Our findings are reported here.

Materials and methods. Enzyme stock solutions of 1% trypsin (Difco, 1:250) and 0.1% Aspergillin-O were prepared in glucose-potassium-sodium (GKN) solution(3) and sterilized by passage through EO Ertel and UF sintered glass filters, respectively. Stocks stored at −20°C were appropriately diluted in solution GKN and adjusted to pH 7.4 with 7% sodium bicarbonate prior to use.

The Aspergillin-O was from lot #P-73. It was prepared in these laboratories by submerged fermentation of a strain of *Aspergillus oryzae* and was highly purified. The preparation used assayed 24,000 units per mg when tested by a fibrinolytic plate assay(4) which was modified for use in these laboratories. This preparation is many times more potent on a weight basis than the preparation described by Stefanini *et al.*(5).

Preparation of primary cultures. Trypsin (0.25%) or Aspergillin-O (0.01%) was used to prepare cell cultures from 10-day-old chick embryos and from rhesus and African green monkey kidneys according to the technique described by Younger(6). For cultivation, chick cell suspensions were diluted with chemically defined medium CMRL-1066*

* CMRL-1066 is identical with medium 858(7) except that the fat soluble vitamins (A, D, E, and K) and ferric nitrate included in medium 858 have been omitted from CMRL-1066; also, 5 B vitamins that were present in earlier media, but omitted from medium 858, have again been added. (G. M. Healy and R. C. Parker, unpublished experiments.)

TABLE I. Comparative Enzyme Concentrations for Effective Release of Cells Cultivated on Glass.

Cell lines	Cell type	Source*	Medium	Trypsin (%)	Aspergillin-O (%)
MDBK	bovine kidney	A	CMRL-1066-10 FCS	.15	.003
H.Ep. #2	human carcinoma	B	MEM-10 FCS	.15	.005
W. S.	" amnion	C	"	.15	.005
CMRL-HA	" "	D	"	.15	.005
L-929†	mouse fibrosarcoma	D	CMRL-1066	—‡	.002
LLC-MK_2	monkey kidney epithelium	E	"	—	.003

* A—Dr. S. Madin, Naval Biological Laboratory, Univ. of California, Oakland. B—Microbiological Associates, Inc., Bethesda, Md. C—Dr. J. F. Enders, Children's Med. Center, Boston, Mass. D—Connaught Medical Research Laboratories, Toronto, Canada. E—Dr. R. N. Hull, Eli Lilly & Co., Indianapolis, Ind.

† A subline of L-929 cells propagated since Aug., 1956, in unsupplemented defined medium CMRL-1066.

‡ No growth.

supplemented with fetal calf serum (1%), whereas monkey kidney cell suspensions were diluted with Hanks' salt solution containing lactalbumin hydrolysate (0.5%) and fetal calf serum (2%). All media contained penicillin (100 units/ml) and streptomycin (100 μg/ml). One-oz. Brockway bottles were planted with approximately 2 x 10^5 cells per ml of medium and incubated at 37°C.

Preparation of cultures from established cell lines. Enzymatic dispersion of the cells of various lines (Table I) was carried out in 4-oz Brockway bottles by treating twice-washed confluent cell layers with either Aspergillin-O or trypsin. When detachment from the glass was first noted by microscopic examination, most of the enzyme solution was separated from the cell layer by inverting the bottle, and subsequently removed. Medium CMRL-1066 or MEM(8), with or without 10% fetal calf serum, was then added and a well-dispersed suspension of viable cells prepared by careful pipetting. Each suspension was then planted in 2-4 bottles and incubated at 37°C. Cultures prepared in this manner, with 2 or 3 subsequent fluid changes, were ready for subculture 5-10 days later. For comparison, subcultures were also prepared by scraping the cells from the glass.

Observations and results. The data summarized in Table I indicate effective concentrations of trypsin and Aspergillin-O for dispersion of cells of established lines. These enzyme concentrations were chosen for reproducibility of cell "lift-off" from glass rather than for minimal levels. L and LLC-MK_2 cells that had been adapted to unsupplemented, chemically defined medium (CMRL-1066) failed to grow when they were released from the glass with trypsin. In contrast, both cell lines responded favorably to treatment with Aspergillin-O, which released the cells from the glass individually. Without exception, however, treatment with Aspergillin-O caused the cell lines cultivated in a medium containing serum to be released from the glass as small islets of cells which could then be dispersed by careful pipetting.

Fig. 1 and 2 show the appearance of 5-day old cultures of L cells prepared by scraping and by treatment with Aspergillin-O, respectively. After 34 consecutive passages, L cells subcultured with Aspergillin-O were similar to scraped cultures both in morphology and in rate of division. It was noted, however, that epithelial cells of line LLC-MK_2 grew much more uniformly when subcultured with Aspergillin-O (Fig. 4) than when subcultured by scraping (Fig. 3). Kidney tissue from rhesus or African green monkeys yielded similar cultures regardless of whether the cell suspensions were prepared with Aspergillin-O (Fig. 5) or trypsin. Aspergillin-O-dispersed primary chick embryo tissue yielded a mixture of fibroblastic and epithelial-like cells (Fig. 7) whereas a fibroblastic cell growth occurred as a result of trypsin dispersion (Fig. 6).

Discussion. The inability to subculture trypsinized cells that have been previously

adapted to proliferate in unsupplemented chemically defined medium agrees with the findings of Pumper(9) with a line of mouse lung fibroblasts. In contrast, Evans *et al*(10)

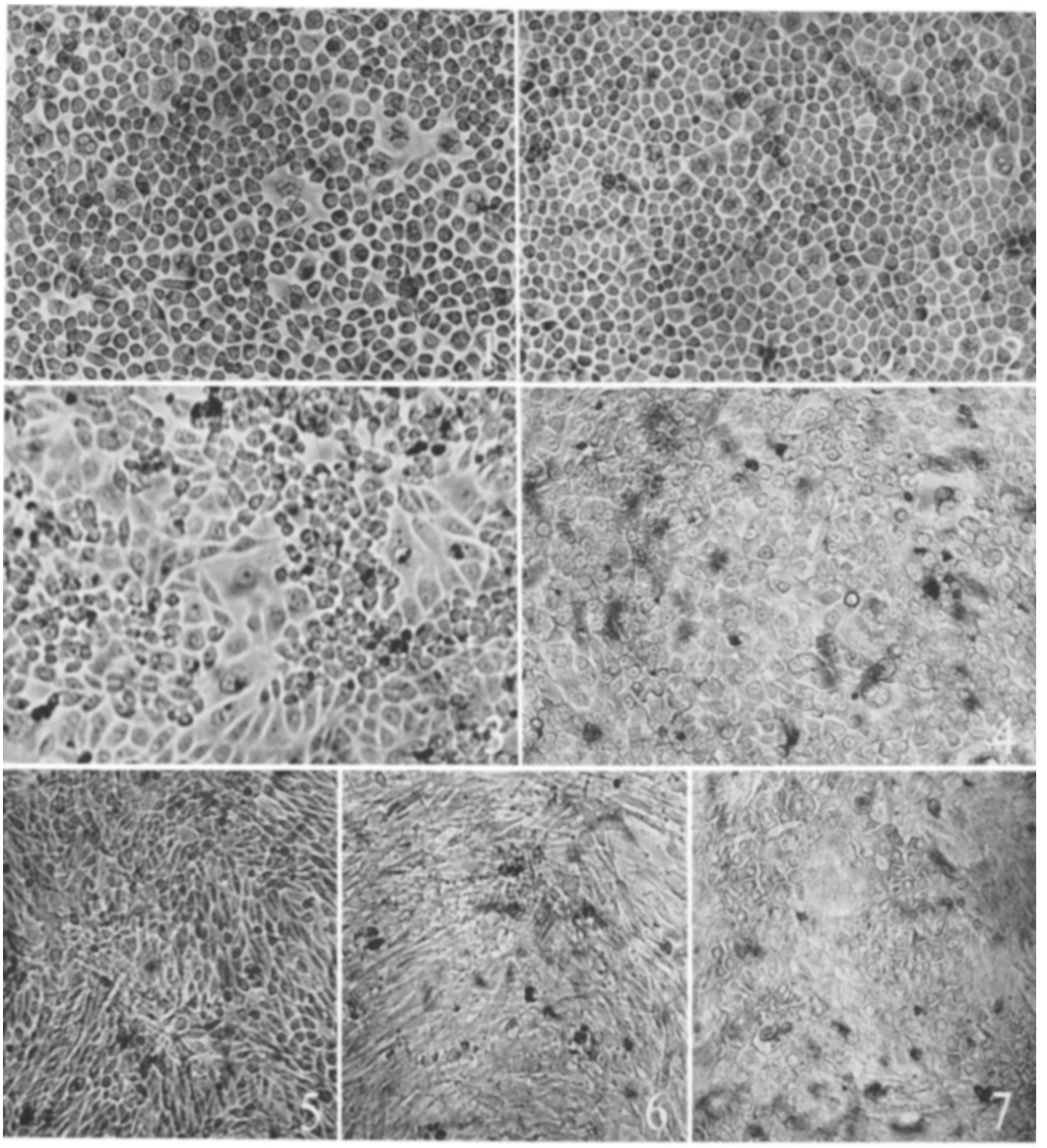

FIG. 1. Five-day culture of 929-L cells prepared from suspension obtained by scraping. Medium: CMRL-1066. × 210.

FIG. 2. Five-day culture of 929-L cells after 34 consecutive passages made with Aspergillin-O. Medium: CMRL-1066. × 210.

FIG. 3. Eight-day culture of LLC-MK_2 cells prepared from suspension obtained by scraping. Medium: CMRL-1066. × 210.

FIG. 4. Eight-day culture of LLC-MK_2 cells after 8 consecutive passages made with Aspergillin-O. Medium: CMRL-1066. × 210.

FIG. 5. Six-day primary culture of rhesus monkey kidney cells dispersed with Aspergillin-O. Medium: Hanks' solution containing 0.5% lactalbumin hydrolysate and 2% fetal calf serum. × 210.

FIG. 6. Four-day primary culture of chick embryo cells dispersed with trypsin. Medium: CMRL-1066 containing 1% fetal calf serum. × 210.

FIG. 7. Four-day primary culture of chick embryo cells dispersed with Aspergillin-O. Medium: CMRL-1066 containing 1% fetal calf serum. × 210.

reported the use of trypsin in the preparation of subcultures of LLC-MK_2-NCTC subline 3206 of monkey-kidney cells cultivated in a chemically defined medium. Recent unreported work in this laboratory shows that in subculturing L cells the obviously harmful effect of trypsin can be suppressed by addition of soybean trypsin inhibitor. The present studies have established that subcultures of L and LLC-MK_2 cells can be prepared enzymatically with Aspergillin-O in the absence of such additives as serum or soybean trypsin inhibitor. Comparison of the effect of trypsin and Aspergillin-O on cell lines cultivated in the presence of serum showed that cells become detached individually, with trypsin, and as small islets with Aspergillin-O. Because little is known about the exact mode of action of Aspergillin-O or trypsin on tissue cells, their activity in releasing cells from glass cannot therefore be explained. If tissue cells incorporate ovomucoids or other enzyme inhibitors from media containing serum, it is possible that these substances might provide a mild protective action against the hydrolyzing activity of Aspergillin-O but not of trypsin. Or, Aspergillin-O may be more specific in its action for substances binding cells to glass.

Experiments are in progress with various cell lines treated with Aspergillin-O to determine whether their sensitivities to a variety of viruses have been altered.

Summary. Aspergillin-O, an enzyme isolated from a submerged fermentation of *Aspergillus oryzae,* has been used to advantage: (a) to prepare subcultures of cell lines adapted to continuous growth in unsupplemented chemically defined medium (CMRL-1066) and (b) to obtain luxuriant primary cultures from fresh tissues.

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Investigations on Extraction of Rat Plasma Phospholipids.* (28574)

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Two of the most commonly used solvent mixtures for extraction of lipids from blood and other animal tissues are ethanol:ether (3:1 v/v)(1) and chloroform:methanol (2:1 v/v)(2).

In lipid work carried out in this laboratory over a number of years, alcohol and ether have been used extensively as primary extractants of lipids from rat tissues. With the advent of methods of separating lipid classes on silicic acid columns by elution with increasing amounts of ethyl ether in petroleum ether (3,4,5), it became convenient to evaporate the primary extract and reextract the lipid into petroleum ether for storage or for application to the column. Although 99% or more of the plasma cholesterol, triglycerides, or free

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