mean cloning efficiency was 19.1%. Sub-clones were obtained with a mean frequency of 23%.

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Tryptophane Transport in Cultures of Human Fibroblasts.* (31424)

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Cystinuria(1), Hartnup Disease(2) and certain forms of hyperglycinuria(3) appear to involve defects in amino acid transport. It is possible that the mutations responsible for these biochemical lesions are expressed only in certain specialized cell types, such as the renal tubular epithelium and the intestinal mucosal cells. An alternative hypothesis is that all somatic cells are affected, although possibly to varying degrees. An investigation of amino acid transport in cultured skin fibroblasts from patients and controls would provide a simple test of this hypothesis. In this paper we describe a method suitable for such studies, together with the results of tryptophane transport studies on a group of control cultures.

Materials and methods. Cell strains. Human skin fibroblast cultures were established by explant techniques and maintained in Waymouth's medium(4) supplemented with 10% newborn calf serum (heated to 56°C for 30 minutes) and penicillin (50 units/ml). Table I lists the strains employed in this study. All of the subjects were caucasian. The biopsy of CHAM was obtained 18 hours after this child died of a bronchopneumonia; skeletal anomalies of the skull and pachygyria were observed. Chromosome studies have not yet been carried out on these strains. With the possible exception of CHAM, they are almost certainly diploid, since numerous cytogenetic studies of such strains developed in our laboratory have revealed stable diploidy with a small (less than 4%) population of tetraploids in most cultures.

Preparation of monolayers. Trypsinized cells(5) (approximately 100,000 cells/ml × 2 ml) were allowed to attach to 22 mm square coverslips ("Gold Label," No. 2 thickness, Clay Adams Co., New York) in 35 mm sterile plastic petri dishes ("Blue Label," Falcon Co., Los Angeles, Calif.). After a 24-hour period of incubation at 37°C in a continuous flow carbon dioxide incubator (5% CO_2, 95% air), the coverslips were transferred to Columbia staining jars containing 10 ml fresh medium and permitted to incubate for an additional 24 hours. The use of staining jars provided a common medium for replicate cultures(6) with better temperature control during subsequent manipulations of the cultures. At 48 hours, when pulsing experiments were performed, the monolayers were still subconfluent with the exception of small confluent patches in the para-central areas of the coverslips.

Pulsing technique. Immediately before incubation in radioactive medium, the monolayers were rinsed in Hanks' balanced salt solution(7) buffered with 0.02 M tris (hy-

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droxymethyl) aminomethane-HCl (pH 7.4 at 37°C)(5) instead of bicarbonate. Two types of radioactive pulsing media were prepared, to each of which tritiated L-tryptophane was added (specific activity 3 curies/mM, lot 6501, Schwarz BioResearch, Orangeburg, N. Y.). *Competitive* medium consisted of serum-free Waymouth's medium minus non-radioactive tryptophane and minus bicarbonate, to which was added tris-HCl buffer (0.02 M, pH 7.4 at 37°C), penicillin (100 units/ml), puromycin dihydrochloride (0.36 mM, Nutritional Biochemicals, Cleveland, Ohio, lot 6432) and L-tryptophane-H^3 (2 microcuries/ml × 10 ml). Non-competitive medium consisted of serum-free Waymouth's medium minus all amino acids, minus glutamine and glutathione and minus bicarbonate, to which was added tris-HCl buffer (0.02 M, pH 7.4 at 37°C), penicillin (100 units/ml), puromycin dihydrochloride (0.36 mM) and L-tryptophane H^3 (1 microcurie/ml × 10 ml). The coverslips were pulsed by incubation in Columbia staining jars containing 10 ml of radioactive medium for periods of 1, 2, 4, 8 and 16 minutes. Immediately after incubation in the radioactive medium, the coverslips were washed in 4 consecutive rinses in 4 staining dishes containing 150 ml of the tris-buffered Hanks' balanced salt solution described above. After a total of 6 coverslips had been processed, the first rinse was discarded and the container washed with 150 ml of distilled water. This container was then refilled and employed as the final rinse; the previous final rinse became the third rinse; the previous third rinse the second rinse, and so on. After rinsing, the coverslips were air-dried, excess fluid being removed by touching the edges of the coverslips to tissue paper. Puromycin was used in the pulsing medium to prevent protein synthesis during the incubation period(8,9,10). The concentration employed was the same as that used by Riggs and Walker in their *in vitro* studies of amino acid transport in Ehrlich mouse ascites tumor cells(10). Thus, the radioactivity measured can be largely assumed to reflect the transport of free amino acid into the cell, together with small amounts of labeled amino acid incorporated into short, incomplete polypeptide chains and complexed with transfer RNA.

Measurement of radioactivity. A windowless gas-flow counter (Model D47, Nuclear Chicago, with automatic sample changer and T_3 time delay valve) was employed to measure the radioactivity of the dried coverslip monolayers. The radioactivity measured was 10 to 1,000 times the background.

Protein assays. The protein content of the dried monolayers was determined by a modification(11) of the method of Oyama and Eagle(12). The specific radioactivity was calculated as counts per minute per microgram of protein.

Results and discussion. The kinetics of uptake for the 5 presumably normal diploid fibroblast strains are given in Fig. 1 (non-competitive medium) and Fig. 2 (competitive medium). The points represent mean values for duplicate or triplicate assays. In the case of non-competitive medium, which results in specific activities more than 10 time greater than those obtained with competitive medium, approximately linear kinetics obtained during the first 8 minutes of incubation, so that slopes of initial maximum velocities could be calculated for the various strains. Under the conditions of the experiments it can be assumed that these rates are measures of the amino acid transferase activities of these cell cultures. The rate of uptake for strain WARR (slope = approximately 1.125 in non-competitive medium) was almost twice the mean rate (slope = 0.625) for the 4 other strains. The latter rates clustered together with a variation no greater than the intra-strain variation obtained with independent experiments on strain MART. If such strain variation proves to be genetically determined, the method should prove useful as a marker for somatic cell genetic studies *in vitro*.

In autoradiographic experiments quite similar to those reported here(13 and unpublished) essentially no uptake of tritiated proline was observed following heating of the coverslip monolayers at 80°C for 5 minutes. This was interpreted as evidence indicating the participation of an active transport mechanism, presumably enzymic, in amino acid uptake by these cells in culture. Preliminary experiments with tritiated cystine have given

results quite comparable to those obtained with tryptophane. Thus 1) linear kinetics of uptake were observed in non-competitive medium during the first 8 minutes of incubation and 2) there was an 8.5- to 18.5-fold increase in the amount of labeled cystine taken up by cells in non-competitive as compared with competitive medium.

The authors are greatly desirous of having patients with Hartnup Disease and cystinuria brought to their attention since such patients are known to have defects in amino acid transport. Cystinuria involves a defect in the transport of the 4 dibasic amino acids(1).

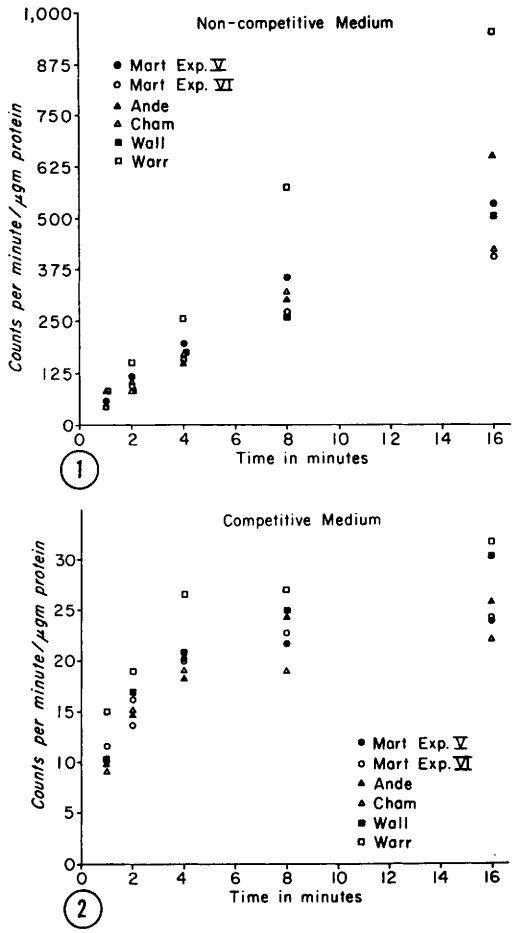

FIG. 1. Uptake of L-tryptophane H^3 by monolayers of human fibroblasts in presence of puromycin and in absence of other amino acids ("non-competitive medium").

FIG. 2. Uptake of L-tryptophane H^3 by monolayers of human fibroblasts in presence of puromycin and other amino acids ("competitive medium").

TABLE I. Strains of Human Fibroblasts Which Were Investigated.

Name	Sex	Age	Diagnosis	Tissue
MART	♂	37	Normal	Forearm skin
CHAM	♂	1.5	Pneumonia	Pectoral "
WARR	♀	30	Normal	Forearm "
WALL	♂	88	"	" "
ANDE	♀	19	"	" "

Hartnup Disease involves a defect in the uptake of the neutral amino acids(2). Thus, we may assume that 2 different types of amino acid transferase are involved in these diseases. It would be of great interest to discover whether or not such mutations are expressed in cultured diploid fibroblasts from such individuals. In carrying out such studies, it would be important to investigate uptake in competitive as well as non-competitive medium, since structural defects in transferase proteins might manifest as quantitatively or qualitatively unusual inhibitions by other amino acids.

Summary. A method is described which permits kinetic studies of amino acid transport in cultures of human fibroblasts. Approximately linear kinetics of uptake were found to obtain during the first 8 minutes of incubation in medium containing puromycin and L-tryptophane H^3, in the absence of other amino acids ("non-competitive medium"). Specific activities of monolayers pulsed in such media were more than 10 times those which were obtained in the presence of competing amino acids. The rate of uptake (in non-competitive medium) for one strain was almost double the mean rate obtained for 4 other presumably normal diploid strains, suggesting that the method might be useful as a marker for *in vitro* somatic cell genetic studies. The potential usefulness of the method for investigations of cystinuria and Hartnup Disease are discussed.

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Anti-Staphylococcal β-Hemolysin Antibodies in Humans with Neurological Disease. (31425)

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The hot-cold β-hemolysin is one of many diffusible substances produced by *Staphylococcus aureus*. It is called "hot-cold" because hemolysis of red blood cells (RBC) is strongest when the system is incubated at 37°C followed by refrigeration. β-hemolysin acts strongly on sheep red cells while α-hemolysin acts on both sheep and rabbit red cells, and delta hemolysin on sheep, rabbit and human RBC. The β-hemolysin bears no antigenic relationship to the other two hemolysins(1). Reports on chemical and physical characteristics of β-hemolysin are available(2-6).

Doery *et al*(2) reported that β-hemolysin-producing strains of *Staphylococcus aureus* hydrolyze sphingomyelin. This phospholipase C-like activity was confirmed in this laboratory and was shown to be specific for sphingomyelin (Eng *et al*, personal communication). Thus, the possibility was entertained that β-hemolysin might be implicated in the pathogenesis of neurological diseases in which destruction of sphingomyelin is a prominent feature. Individuals subjected to repeated exposure to β-hemolysin-producing staphylococci and incapable of producing adequate antibodies against the hemolysin, could be assumed to develop such conditions. Consequently, a study of anti-β hemolysin antibodies in patients with multiple sclerosis and other neurologic disease was undertaken.

Methods. A beef heart infusion broth was employed in the preparation of the β-hemolysin filtrate(7). *Staphylococcus aureus* strain No. 31, which exhibits strong hot-cold hemolysis on sheep blood agar and weak action on human and rabbit blood agar plates or strain J32,* which produces only β-hemolysin was used as inocula. The inoculated medium was incubated for 96 hours at 37°C in a 25-30% carbon dioxide environment. The culture was centrifuged at 2,000 × *g* for 30 minutes and the supernatant Seitz filtered. The filtrates were stored at —4°C until use.

β-Hemolysin titration. Bacterial filtrates were heat treated according to Smith and Price(5) and 2-fold serial dilutions made in phosphate-buffered saline(4). To each tube was added 1.5 ml of a 1% suspension of thrice-washed sheep red cells. A 50% hemolysis control was prepared by adding one drop of a 1% aqueous solution of saponin to 0.75 ml of the RBC suspension in 2.25 ml of diluent. Appropriate controls were included. The mixed contents of all tubes were incubated for 80 minutes at 37°C followed by 30 minutes in an ice-bath(3). Remaining intact red cells were settled by centrifuging at 2,000 × *g* for 5 minutes and 2 ml of supernatant were pipetted into Coleman cuvettes.

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