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16. It is possible that a different immunization procedure (for example, a single injection of alum-precipitated antigen, followed by a single bleeding) might give more homogeneous antibody. However, comparable electrophoretic patterns of heavy and light chains have been obtained from purified antibody to arsanilic acid isolated from a single bleeding of a rabbit immunized with edestin–arsanilic acid coated on acrylamide beads. Antigen (3.3 mg) was injected intravenously on day zero, and additional antigen (2.2 mg) was given by the same route on days 5, 10, and 15, and the rabbit was bled on day 21.
17. We thank James Boone and June Hyepock for the excellent technical assistance.

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Transformation of Auxotrophic Mutants of Group H Streptococci

Abstract. *Stable auxotrophic mutants of a group H streptococcus (strain Challis) were isolated on a modified Mickelson defined medium after exposure to* N-*methyl*-N′-*nitro*-N-*nitrosoguanidine. Such mutants were transformed to both prototrophy and streptomycin-resistance and hence may be used as nutritional markers in the study of streptococcal genetics.*

A number of investigators have reported the transformation of some streptococci to streptomycin-resistance (*1*) and have described conditions for competence (*2*), but to our knowledge nutritional markers have not been used for transformation studies in bacteria of this genus. By use of the chemical mutagenic agent *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (NTG) (*3*) in a procedure not yet reported (*4*), we have succeeded in isolating auxotrophic mutants from cultures of a group H streptococcus, strain Challis (*5*). In brief, rapidly growing cultures of Challis H were exposed to NTG (100, 250, and 500 μg/ml) for 30 minutes and were then plated on brain-heart infusion agar (Difco). After 3 days of incubation at 37°C, the smallest colonies were isolated and checked for nutritional deficiencies in a chemically defined medium. This medium, designated MS9 by us, is a modification of the one described by Mickelson (*6*) and is shown in Table 1. It supports excellent growth (more than 10^9 cells per milliliter) of the parent Challis H strain, which was initially adapted to grow rapidly in this basal medium after 10 to 15 daily transfers.

Cultures of auxotrophs were tested for transformability by the following method in liquid suspensions: 4.5 ml of brain-heart infusion broth, supplemented with 4 percent inactivated horse serum (heated for 30 minutes at 60°C) were inoculated with 0.5 ml from overnight cultures of the auxotrophs. The tubes were incubated from 60 to 90 minutes or until the original optical density doubled as measured with a Coleman Junior spectrophotometer set at 570 nm. As a rule 0.5 ml of these cultures (about 5×10^8 cells) was added to duplicate tubes containing 1.0 ml of brain-heart infusion broth supplemented with inactivated human serum. Deoxyribonucleic acid (20 μg) was added to one tube; a mixture of DNA and deoxyribonuclease was added to the other tube as a control. The DNA used was isolated by Marmur's method (*7*) from a wild-type culture of group H streptococci, strain SBE:12, resistant to 2 mg of dihydrostreptomycin per milliliter (*8*). The tubes were incubated for 60 minutes, diluted in physiological saline, and plated. All incubations were done at 37°C.

For the recovery of transformants to prototrophy the samples were plated on MS9 medium reinforced with 1 percent brain-heart infusion broth. After plating on MS9 medium the plates were overlaid with about 10 ml of plain Bacto agar. The transformants to either marker were counted after 2 or 3 days of incubation of the plates.

The conditions for transformation found to be optimum for the parent strain Challis H were used for all the auxotrophs, and no attempts were made to find the optimum conditions for each auxotrophic mutant. Table 2 shows the results obtained with several stable auxotrophs (stable after 10 to 15 serial transfers in complex medium)

Table 1. Components of chemically defined medium MS9. The medium was sterilized by filtration through a Millipore filter (0.45 μm) or by autoclaving at 2 atm for 15 minutes, glucose being added aseptically.

Component	Amount (g/liter)	Component	Amount (g/liter)	Component	Amount (g/liter)
L-Amino acids		*Purines and pyrimidines*		*Salts*	
Arginine	0.6	Adenine	0.01	K_2HPO_4	12.0
Methionine	0.2	Guanine	0.01	KH_2PO_4	5.2
Valine	0.2	Uracil	0.01	$(NH_4)_2SO_4$	2.0
Leucine	0.2			$MgSO_4 \cdot 7H_2O$	0.2
Isoleucine	0.2	*Vitamins*		$MnSO_4 \cdot H_2O$	0.01
Lysine	0.2	Niacinamide	0.01	$FeSO_4 \cdot 7H_2O$	0.01
Serine	0.2	Thiamine	0.01	Na-Acetate	10.0
Tyrosine	0.4	Riboflavin	0.01	Glucose	10.0
Cysteine	0.2	Ca-Pantothenate	0.01	Water (double distilled) to make 1 liter	
Histidine	0.2	Biotin	0.003		
Cystine	0.05	*p*-Aminobenzoic acid	0.002	*p*H	7.0
Phenylalanine	0.2	Pyridoxal·HCl	0.002		
Glycine	0.2	Pyridoxamine	0.002		
Tryptophan	0.2	Folic acid	0.002		
Glutamic acid	0.5				

Table 2. Transformation of group H streptococci to either prototrophy or streptomycin-resistance. Frequencies of transformation were obtained by dividing the number of transformants to either marker by the number of colony-forming units of the recipient cultures (usually 5×10^8). Control samples with a mixture of DNA and deoxyribonuclease gave rise to less than 100 colonies per milliliter of sample when placed on MS9 medium or brain-heart infusion agar containing dihydrostreptomycin (300 μg/ml).

Auxotrophic mutant	Frequency of transformation (%)	
	Streptomycin-resistance	Prototrophy
M-308 (asparagine⁻)	1.2	0.4
M-40 (proline⁻)	0.6	0.2
M-475 (alanine⁻)	2.1	0.6
M-37 (unidentified)	3.0	0.2
Challis H (wild type)	3.5	

and with the parent Challis H strain.

The auxotrophs transformed well to either marker. The frequencies of transformation to streptomycin-resistance were higher than those to prototrophy. We did not score for the presence of transformants to both markers; since we used an excess of DNA, it is likely that some double transformants were obtained. The control sample with the DNA and deoxyribonuclease mixture gave rise to less than 100 colonies per milliliter of sample when plated on MS9 medium or brain heart infusion agar plates containing dihydrostreptomycin (300 μg/ml).

In addition to the stable auxotrophs transformed as reported here, several others are under investigation, and many other auxotrophs were isolated but reverted to prototrophy after several serial transfers. Niederman *et al.* have also reported (*9*) the isolation of an asparagine-requiring auxotroph from *Streptococcus bovis*. These workers used this auxotroph for microbiological assay of asparagine, but they have not tested it for its ability to be transformed. However, the production of some stable auxotrophs and their ability to be transformed permits a start in mapping the streptococcal chromosome.

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10 March 1966

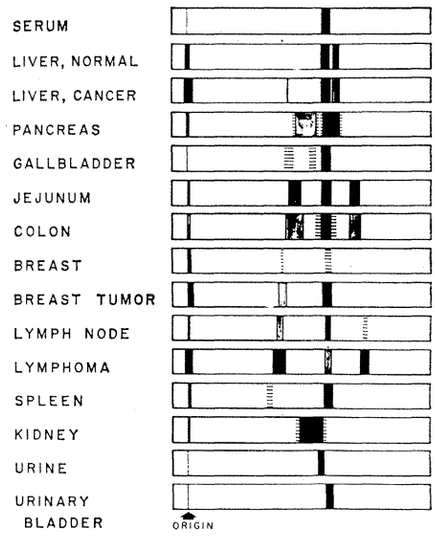

Fig. 1. The electrophoretic distribution on starch gel of naphthylamidases from various human tissues. The liver cancer tissue was obtained from a Carcinoma of the pancreas metastatic to the liver.

Starch-Gel Electrophoresis of Human Tissue Enzymes Which Hydrolyze L-Leucyl-β-Naphthylamide

Abstract. *Electrophoretically distinct forms of the enzyme or enzymes which catalyze the hydrolysis of* L-*leucyl-*β*-naphthylamide were demonstrated in various human tissues. There were characteristic patterns for different tissues. Unlike the enzymes found in normal serum, some tissue-specific enzymes were unaffected or activated by* L-*methionine.*

With L-leucyl-β-naphthylamide as substrate, amino acid naphthylamidases (*1, 2*) have been identified in several electrophoretically distinct forms (presumably isoenzymes) in the serums of patients with various diseases (*3*). We now report the existence of electrophoretically and, in certain cases, biochemically distinct forms of the enzyme in different human tissues.

Specimens of liver, kidney, pancreas, colon, breast, breast adenocarcinoma, heart and skeletal muscle, gallbladder, urinary bladder, jejunum, spleen, lymph node, malignant lymphoma, and adenocarcinoma metastatic to the liver were obtained at operation or immediately after death. Extracts were prepared by homogenizing the tissue in approximately five times its weight of distilled water (20°C) first in a VirTis-45 tissue grinder and subsequently in a Potter-Elvhejem ground glass homogenizer. The homogenates were centrifuged at 900*g* for 30 minutes, and the supernatants were centrifuged again at 20,000*g* for 20 minutes. This supernatant was the extract studied. Each extract was assayed for naphthylamidase activity (*4*) and examined electrophoretically as described below.

Fifty microliters of the extract were subjected to electrophoresis on a vertical starch gel by the method of Smithies (*5*), with a borate-gel buffer, *p*H 8.7, and a borate-bridge buffer, *p*H 8.2. Electrophoresis was carried out at room temperature (23°C) and 4.5 volt/cm for 16 to 17 hours. After electrophoresis a thin layer was sliced from the gel, and the exposed surface of the remaining portion was assayed for the localization of naphthylamidase activity by incubation for 5 hours in a solution of L-leucyl-β-naphthylamide hydrochloride ($6.8 \times 10^{-4}M$) in 0.1*M* phosphate buffer, *p*H 7.0. After incubation, the substrate solution was decanted, and the gel was exposed for 10 minutes to a solution (150 ml) of a stabilized diazonium salt (Garnet GBC; 1 mg/ml). This procedure resulted in conversion of enzymatically released β-naphthylamine to an insoluble red azo dye, which delineated the areas of enzymatic activity. The gel was washed with water and cleared in glycerol overnight.

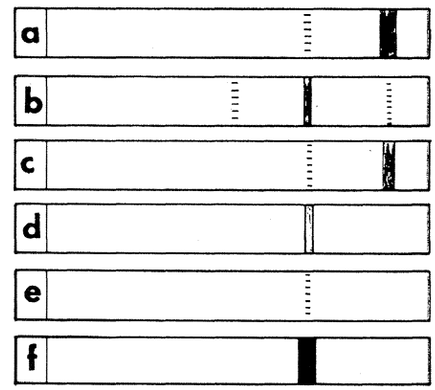

Fig. 2. The effect of 0.01*M* L-methionine on the naphthylamidases of serum and heart and skeletal muscle. (*a*) Skeletal muscle plus methionine; (*b*) skeletal muscle; (*c*) heart muscle plus methionine; (*d*) heart; (*e*) serum plus methionine; (*f*) serum.

Since L-methionine competitively inhibits hydrolysis of the substrate by