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Selection of Temperature-sensitive Activating Enzyme Mutants in *Escherichia coli*

SAMUEL KAPLAN AND DEBORAH ANDERSON
Department of Microbiology, University of Illinois, Urbana, Illinois 61801

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A method based on temperature-conditional resistance to thymineless death has been designed to facilitate the isolation of amino acid-activating enzyme mutants. This method may be modified to obtain a greater or lesser proportion of activating enzyme mutants. In the latter instance, an increased proportion of temperature-sensitive macromolecule mutants of other types is obtained. Additional uses of this procedure are discussed.

Regulation of amino acid biosynthesis and ribonucleic acid (RNA) biosynthesis in *Escherichia coli* has been the subject of numerous investigations. Earlier work provided substantial evidence that free amino acids were involved in both small molecule and macromolecule control (1-6, 8, 11-13, 17).

Studies by Neidhardt (12), Yanif and Gros (Proc. 3rd Federation Europ. Biochem. Soc., 1966), and Roth and Ames (16) have implicated either the aminoacyl-adenosine monophosphate (AMP) or the aminoacyl-transfer ribonucleic acid (tRNA) derivatives of the free amino acids in both the control of RNA synthesis and the end-product repression of amino acid formation. Although alternative interpretations are possible, it is clear that in some cases the inability to activate an amino acid, although the amino acid per se is present, is sufficient to produce derepression of the enzymes in the biosynthetic pathway which synthesize that amino acid. Likewise, these conditions are sufficient to stop or greatly reduce RNA biosynthesis.

It would seem desirable to isolate many different conditionally lethal activating-enzyme mutants to expand the observations and conclusions already made and to study further the molecular means whereby aminoacyl derivatives might affect end-product repression and RNA synthesis.

The present communication describes a method of mutant selection which can be used to isolate, at high frequencies, mutants with temperature-sensitive activating enzymes.

MATERIALS AND METHODS

Media. The minimal salts medium used throughout contained (grams per liter): Na_2HPO_4, 5.8; KH_2PO_4, 3.0; NaCl, 0.5; NH_4Cl, 1.0; and $MgSO_4$, 0.12. Supplementation, when employed, consisted of: L-amino acids, 40 μg/ml; Vitamin Free Casamino Acids, 0.5%; thymidine, 40 μg/ml; and glucose or glycerol, 0.5%.

CA medium designates Casamino Acids in minimal salts medium containing L-tryptophan and glycerol.

CAT medium is the same as CA medium with the addition of thymidine. Enriched CA or CAT medium contained 40 μg/ml each of adenine, guanine, cytosine, and uracil. In plating medium, 1.5% agar was used.

Strains of E. coli employed. D_2, a stable thymine-requiring mutant obtained from Sydney Brenner, was used in addition to the following conditional lethal mutants: 5A1, 6C5, 9D3R3, and 9D5. The conditional mutants, with the exception of 9D3R3, were isolated from D_2 as described below, after *N*-methyl-*N*-nitroso-*N'*-nitroguanidine mutagenesis. 9D3R3 is a spontaneous partial revertant of 9D3.

Amino acid-activating enzyme assays. Assays were performed by the method of Eidlic and Neidhardt (4), with minor modification.

Preparation of RNA and protein determination. Protein was determined by the method of Lowry et al. (10), with bovine serum albumin as standard. tRNA (*E. coli* K-12) was a commercial preparation purchased from Sigma Chemical Corp., Freehold, N.J.

Thymineless death curve. Two methods for measuring thymineless death were employed.

The first method involved death in a liquid medium. A culture of the thymine-requiring D_2 strain or mutants derived therefrom were grown to mid-log phase on enriched CAT at 30 C. The cells were harvested by filtration on membrane filters (0.45-μ pore size; Millipore Corp., Bedford, Mass.), washed with enriched CA, and suspended in enriched CA medium to a density of 10^8 cells/ml, at either 30 or 42 C. Samples were removed at zero-time and at regular intervals, diluted, and plated for survival on enriched CAT plates at 30 C.

A second method measured death on petri plates. It yielded results identical to the method described

above, but was much easier to perform. Cells were grown, washed, and suspended as above, but to a cell density of 10^6/ml. Serial dilutions were made, and 0.1-ml samples were spread on dry, enriched CA plates. The final cell numbers plated were 10^5, 10^4, 10^3, 10^2, and 10^1. The plates were incubated at the appropriate temperature, and at regular intervals plates containing overlapping cell dilutions were overlaid with enriched CAT medium and incubated at 30 C. Survivors were then determined.

Chemicals. Radioactive chemicals (^{14}C-L-proline and uracil-5-^3H) were purchased from Schwarz BioResearch, Orangeburg, N.Y. Specific activites of 1 μc/μmole for ^{14}C-L-proline and 3 μc/μmole for uracil-5-^3H were used in whole-cell incorporation experiments. StanStar (Schwarz BioResearch) amino acids were used at 50 μc/μmole for amino acid activation assays.

N-methyl-*N*-nitroso-*N'*-nitroguanidine was purchased from K and K Laboratories, Jamaica, N.Y.

RESULTS

Evidence has been presented supporting the observation that RNA or protein synthesis (or both) is a prerequisite for thymineless death in both *E. coli* and *Bacillus subtilis* (14, 15). It was therefore reasoned that mutants conditionally resistant to thymineless death, i.e., resistant at 42 C but susceptible at 30 C, would most probably possess lesions directly affecting either protein or RNA biosynthesis (or both).

Mutant selection. An overnight culture of D_2 was washed by centrifugation and concentrated 20-fold into 0.2 M acetate buffer, *p*H 5.0. *N*-methyl-*N*-nitroso-*N'*-nitroguanidine was added to a final concentration of 1 mg/ml. The treated cell suspension was kept at 25 C for 210 min. A sample (0.1 ml) was removed, added to 10.0 ml of enriched CAT medium, and grown to stationary phase at 30 C.

A sample of this culture was used to inoculate fresh enriched CAT, and the cells were grown to 10^8 to 2×10^8/ml at 30 C. The cells were harvested by filtration, washed five times with enriched CA medium, suspended at 5×10^7/ml, and incubated at 42 C.

Figure 1 illustrates the course of thymineless death for strain D_2 under these conditions. After approximately 6 hr, there were 10^3 survivors/ml. At that time, 0.1-ml samples were removed, plated on enriched CAT agar medium, and incubated at 30 C. At the end of 3 days, the colonies were replica-plated onto two enriched CAT plates, one enriched CA plate, and one minimal plate supplemented with glucose and thymidine. One enriched CAT plate was placed at 42 C, and the remaining three plates were incubated at 30 C.

Colonies appearing on both minimal-glucose-thymidine plates and enriched CAT plates at 30 C, but not on enriched CAT at 42 C or enriched CA at 30 C, were selected for further study. The important consideration is that these selected strains, even in the presence of thymidine, did not grow at 42 C.

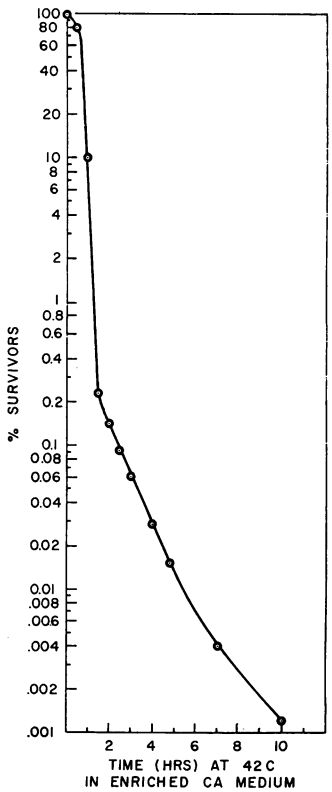

FIG. 1. *Thymineless death curve for Escherichia coil strain D_2 in enriched CA medium at 42 C with aeration. Survivors were determined by the first method described in Materials and Methods.*

By this technique, 60 independent temperature-sensitive mutants were isolated.

Aminoacyl synthetase assay and characterization. Since our primary concern was the isolation of aminoacyl synthetase mutants, 16 of the 60 mutant strains were selected at random for aminoacyl synthetase assays. Each of the 16 strains was spontaneously revertible, at a low but measurable frequency, to the capacity for growth at 42 C. None of the mutant strains had growth rates at 30 C less than half that of the parental strain; most were between 10 and 30% slower.

Of the 16 mutants tested in vitro, 14 were presumptive aminoacyl synthetase mutants, as judged by one of the following criteria: (i) the complete absence of one of the 18 synthetase activities measured at both 30 and 42 C; (ii)

greatly reduced activities of one aminoacyl synthetase at 30 C with no activity at 42 C; (iii) greatly reduced activity of one aminoacyl synthetase at 30 C with lesser activity at 42 C.

No strain presumptively considered to be a synthetase mutant had more than one defective synthetase.

Of the 14 presumptive mutants, 12 were accurately identified. Ten of these were valyl-tRNA synthetase mutants, one was an alanyl-tRNA synthetase mutant, and the other was a phenylalanyl-tRNA synthetase mutant. Two remained presumptive; the first was a possible histidyl-tRNA synthetase mutant and the remaining one was a possible leucyl-tRNA synthetase mutant. The remaining 2 of the 16 mutants, although apparently involving macromolecule biosynthesis, appeared not to be aminoacyl synthetase mutants.

Table 1 shows the results of in vitro synthetase assays for mutants 9D5, 9D3, 9D3R3, and the parental D_2.

Mutant 9D3 was a valine-activating enzyme mutant. At 30 C, mutant 9D3R3, a partial revertant of 9D3, had levels of valine-activating activity mid-way between those of the wild-type strain and the parental strain. At 42 C, mutant 9D3R3 displayed a low growth rate only in the presence of at least 60 μg of L-valine per ml. Mutant 9D5 lacked alanine-activating activity.

Figure 2A shows the heat denaturation profile for the alanine synthetase of D_2 and the residual alanine-activating activity in extracts of 9D5 grown at 30 C. The plateau observed was probably due to the protective effect of very high protein concentrations needed to demonstrate the mutant activity. To assay the wild type under similar conditions, bovine serum albumin was added to raise the protein concentration. Normally, wild-type activity at low protein levels shows no plateau. Nonetheless, it is clear that the residual alanine synthetase activity of 9D5 was more heat-labile than that of D_2.

Figure 2B illustrates the heat denaturation profiles for D_2, 9D3, and 9D3R3. Clearly, the valine-activating activity of D_2 was extremely heat-stable and that of 9D3 exceedingly unstable; between the two extremes is the profile obtained for the partial revertant 9D3R3.

Neidhardt (13) and Yanif and Gros (Proc. 3rd Federation Europ. Biochem. Soc., 1966) observed that macromolecule biosynthesis was severely affected in temperature-sensitive aminoacyl synthetase mutants when they were subjected to a shift from a growing to a nongrowing temperature. As anticipated, protein synthesis terminated rapidly, directly followed by a cessation of RNA biosynthesis. Deoxyribonucleic acid (DNA) generally showed a 40 to 60% increase prior to cessation of synthesis.

TABLE 1. *In vitro synthetase assays*[a]

Amino acid	Strains			
	D_2	9D5	9D3	9D3R3
Alanine	13	3[b]	—	8
Arginine	98	68	86	73
Aspartic acid	103	75	—	96
Glutamic acid	107	103	—	62
Glycine	78	47	—	34
Histidine	115	108	—	74
Isoleucine	63	41	96	35
Leucine	88	67	98	42
Lysine	94	89	—	81
Methionine	79	79	99	99
Phenylalanine	112	97	—	91
Proline	19	17	7	28
Serine	98	104	—	102
Threonine	54	41	—	21
Tyrosine	62	60	—	73
Valine	99	94	36[b]	1

[a] Cells were grown at 30 C, and extracts were assayed at 42 and 30 C. Results show the activity at 42 C as a percentage of the activity at 30 C.

[b] For 9D5 and 9D3, the activities at 30 C represent only 10 and 2.5%, respectively, of the corresponding D_2 activities at 30 C. Therefore, in addition to having considerably lower 42 C/30 C ratios, the absolute amount of 30 C activity is also considerably reduced.

Figures 3A, B, C, and D represent the results for similar experiments performed on strains D_2, 9D5, 9D3, and 9D3R3. As observed by Yanif and Gros, the alanine tRNA synthetase mutant 9D5, when shifted from 30 to 42 C, showed an immediate decrease in the rates of both RNA and protein synthesis (Fig. 3B). However, for a period of at least 4 hr (*unpublished data*), RNA synthesis continued at a rate equal to approximately 3% of the wild-type rate. The RNA formed under these conditions for up to 90 min after the temperature shift of strain 9D5 entered 70*S*, 50*S*, and 30*S* particles, as well as 4*S* material, as determined by sucrose gradient centrifugation. Protein synthesis, measured by ^{14}C-proline incorporation and by colorimetric assay, over this same period of time occurred at 15% the wild-type rate, with the protein content of the cells doubling in slightly over 6 hr. This is something of a paradox, since the heat decay curve for the alanine synthetase suggests its total inactivation after a very short time at the higher temperature. Furthermore, the altered rates of synthesis are assumed immediately after the temperature shift and synthesis continues at these new rates for 6 hr.

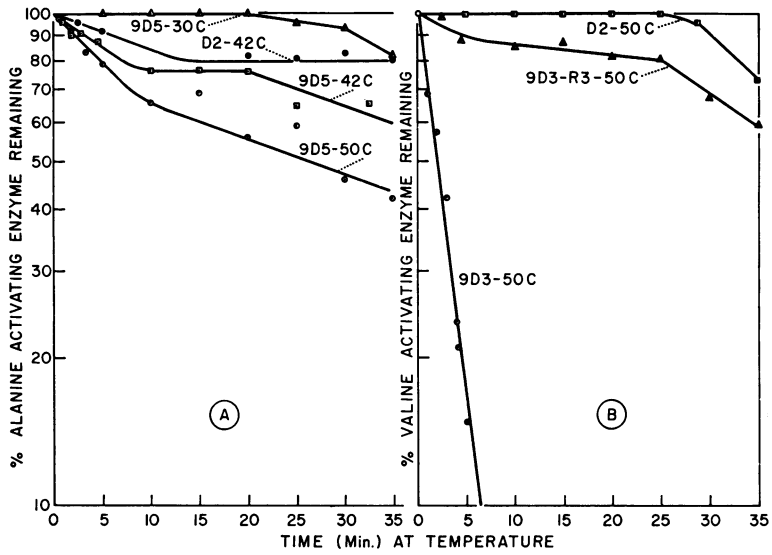

FIG. 2. *Temperature decay profiles of mutant 9D5 and strain D_2 (A) and of mutants 9D3 and 9D3R3 and strain D_2 (B). Cells were grown at 30 C. Extracts were treated with a one-sixth volume of 15% streptomycin sulfate. The supernatant fluid was brought to 80% saturation with $(NH_4)_2SO_4$. The precipitate was triturated in as small a volume as possible and dialyzed against 0.01 M tris(hydroxymethyl)aminomethane-chloride (pH 7.4) containing 0.005 M mercaptoethanol, 0.03 M KCl, and 10^{-4} M magnesium acetate. The concentrated extract was maintained at the temperature indicated; samples were removed and assayed at 30 C for 40 min for alanine-activating activity. The aminoacyl-tRNA formed was precipitated by treatment with an equal volume of cold 10% trichloroacetic acid, and 250 μg of cold carrier bovine serum albumin was added. The precipitates were filtered on Reeve-Angel glass-fiber filters (2.8 cm in diameter), washed three times with cold 5% trichloroacetic acid, and dried. Radioactivity was determined with a Beckman LS 100 scintillation spectrometer by use of toluene-based 2,5-diphenyloxazole, 1,4-bis-2-(5-phenyloxazolyl)-benzene.*

Strain 9D3 showed an abrupt cessation of both RNA and protein synthesis after the temperature shift (Fig. 3C). As might be expected, both RNA and protein synthesis were only slightly affected in the partial revertant 9D3R3 (Fig. 3D).

In all strains, DNA synthesis, when measured colorimetrically, showed a 30 to 60% increase and then synthesis ceased. This is compatible with the evidence of Lark (9), regarding the continued synthesis of a DNA-initiator protein.

All of these data support the conclusion that we are dealing with temperature-sensitive aminoacyl synthetase mutants. Mapping experiments are currently in progress.

Resistance to thymineless death. The data presented above confirm, at least for 14 of 16 isolated strains, that temperature-conditional resistance to thymineless death yields a very high proportion of synthetase-type mutants. It would then be expected that such mutants would show marked resistance to thymineless death at 42 C, whereas at 30 C the removal of thymine should continue to be lethal. Of the 16 strains, 2 were selected for testing, strain 6C5, a valine tRNA synthetase mutant, and strain 5A1, which, as far as is known, involves a lesion other than an altered aminoacyl synthetase. Figures 4A and 4B illustrate the thymineless death curves for 6C5 and 5A1. It is clear that, when the cells were grown at 28 C, all strains were susceptible to thymineless death. At 42 C, strain 6C5 was exceedingly resistant to thymineless death, and 5A1, although highly resistant, was not as resistant as 6C5.

Figures 5A and 5B show the pattern of macromolecule biosynthesis obtained for each strain after a shift from 30 to 42 C. Whereas RNA and protein synthesis terminated rapidly with 6C5, synthesis of RNA, protein, and DNA continued for a substantial period of time in strain 5A1. Although very little work has been done with 5A1, we suspect that the lesion may be involved in cell division. After a temperature shift, the cell number always underwent exactly one doubling, with protein, and RNA increasing at ever decreasing rates thereafter.

However, what is of concern is that the degree of resistance to thymineless death after the temperature shift appears to be related to the residual ability to synthesize macromolecules.

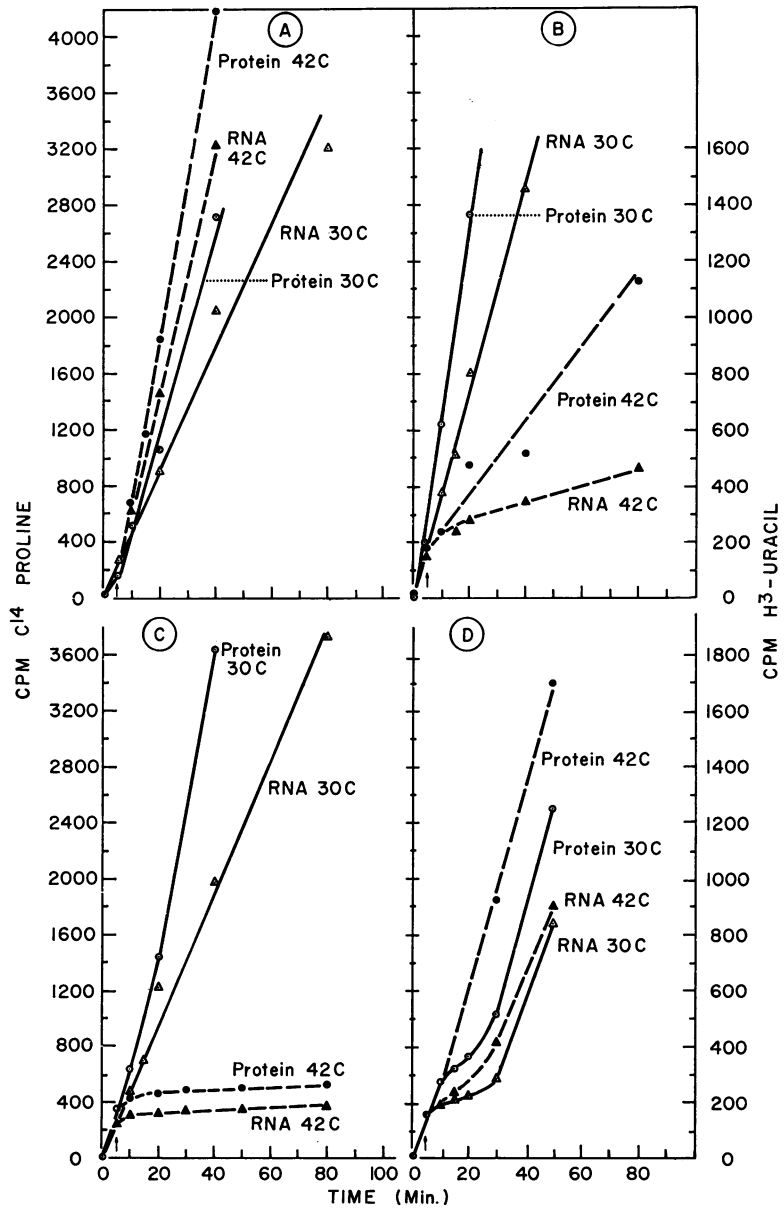

FIG. 3. *Incorporation of ^{14}C-proline and ^{3}H-uracil. Cells were grown at 30 C with shaking. ^{14}C-proline and ^{3}H-uracil (see text) were added 5 min prior to the temperature shift (indicated by arrow). A portion was removed and placed at 42 C. Samples were removed at the indicated times from both the 30 C and the 42 C cultures and were added to an equal volume of cold 10% trichloroacetic acid. The precipitates were filtered on membrane filters (Millipore) and washed three times with cold 5% trichloroacetic acid. The filters were dried, and radioactivity was determined in a Beckman LS-100 scintillation spectrometer by use of toluene-based 2,5-diphenyloxazole, 1,4-bis-2-(5-phenyloxzazolyl)-benzene (A) wild type; (B) 9D5; (C) 9D3; and (D) 9D3R3.*

DISCUSSION

As described above, of 16 mutants selected at random from a group of 60 temperature-sensitive mutants, 14 have been shown to be temperature-sensitive aminoacyl synthetase mutants. On the basis of these data, it seems justified to conclude that the method of selection by temperature-sensitive resistance to thymineless death is highly efficient in obtaining this type of mutant.

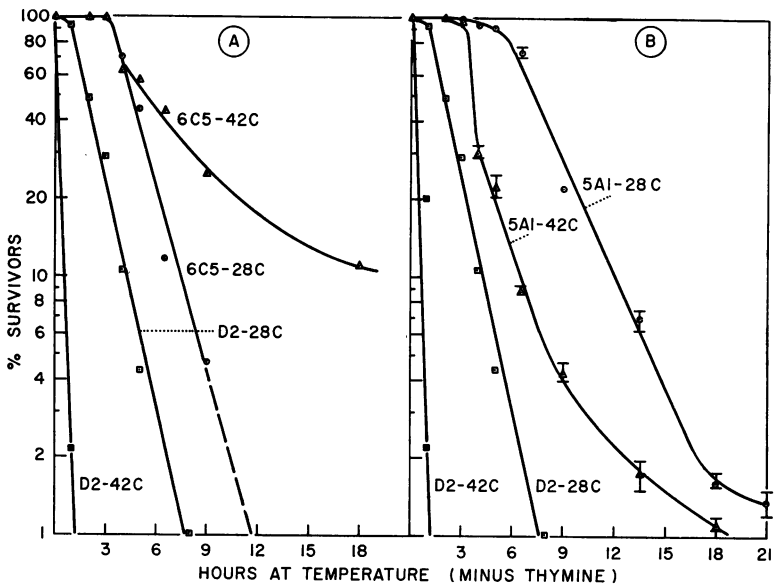

FIG. 4. *Thymineless death curves for D_2, 6C5, and 5A1 at 30 and 42 C on enriched CA medium plates. Survival was determined by the second method described in Materials and Methods.*

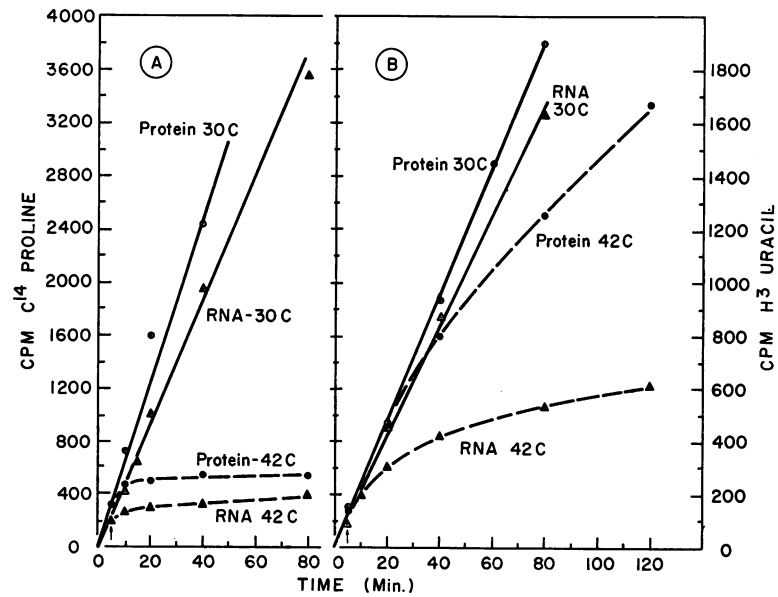

FIG. 5. *Incorporation of ^{14}C-proline and ^{3}H-uracil. Cells were grown with shaking. Radioactive proline and uracil (see text) were added 5 min prior to the temperature shift (indicated by arrow). A portion was removed and placed at 42 C. Samples were removed from both 30 and 42 C. cultures at the indicated times. (A) 6C5. (B) 5A1. For details, see Fig. 3.*

It is possible, however, that making the conditions of selection less stringent, by plating after 3 or 4 logarithms of kill, would result in the more frequent occurrence of macromolecule mutants of other types. Furthermore, the data available indicate that subjecting survivors from one cycle of kill to regrowth and a second cycle of kill should enrich for synthetase mutants to an even greater degree than that observed above.

These conclusions are based upon the data

in Fig. 4 and 5, where it is evident that the degree of residual sensitivity to thymineless death in these mutants merely reflects the residual ability to synthesize protein and RNA.

We have also used this technique for the selection of a variety of amino acid auxotrophs (not of a temperature-sensitive type), as well as for selection of mutants incapable of growing on glucose but able to grow on glycerol. Wachsman and Hogg (18) used a similar approach to isolate doubly auxotrophic mutants in *Bacillus megaterium*.

Preliminary experiments (D. Anderson, *unpublished data*) have indicated that resistance to thymineless death can be employed as a means of selecting for rare transductants of recessive characters, where linkage is unknown. This method appears to be highly effective, owing to the death of the majority of the phage recipients which have not been transduced. The rapid death is apparently a combination of two factors: thymineless death and thymineless induction of lysogens, except in those lysogens where a lesion is present which can prevent protein or RNA synthesis, or both. This is similar to the effect observed by Korn and Weissbach (7) in λ lysogens.

ACKNOWLEDGMENT

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