SOME PHYSIOLOGICAL AND GENETIC PROPERTIES OF A STRAIN OF *ESCHERICHIA COLI* REQUIRING THYMINE, ARGININE AND URACIL

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SUMMARY

A polyauxotrophic mutant of *E. coli* strain 15 has been studied. The organism requires thymine, uracil, and arginine for normal growth and multiplication. The formation of citrulline and ureidosuccinate is blocked almost completely. Nevertheless, significant although depressed amounts of both ornithine and aspartate transcarbamylases are readily detectable in cell-free extracts of the organism.

The bacterium can be induced to synthesize xylose isomerase in the presence of exogenous arginine and in the absence of exogenous uracil, as in strain $15T^-U^-$. Relatively extensive peptide synthesis is obtained under these conditions. Analysis of the observed RNA metabolism suggests a turnover of part of the RNA and a greater conservation of the bases than of the ribose. Most of the turnover appears to depend on an exogenous supply of arginine, as does the function of enzyme formation.

Reversion to arginine and uracil independence occurs in a single genetic step. Thymine starvation increases the percentage of revertants in the culture before the bacteria die as a result of unbalanced growth.

INTRODUCTION

The phenomena of unbalanced growth and thymineless death were discovered in *E. coli*, strain $15T^-$, a thymine requiring mutant, some of whose properties have been described in considerable detail[1-4]. The lethal effects of thymine deficiency are expressed under conditions of continuing metabolism and biosynthesis in the absence of the synthesis of deoxyribose nucleic acid (DNA). In the absence of exogenous thymine, numerous synthetic processes such as the synthesis of protein and ribose nucleic acid (RNA), may continue despite the prevention of DNA synthesis and cell division.

As one approach to the nature of these phenomena, mutants of the thymineless

* This work was done while this author (D. K.) was on leave from the Boris Kidric Institute in Belgrade, Yugoslavia.

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organism have been isolated which lack additional synthetic capacities. A number of these polyauxotrophs have been described; these include strains requiring a single amino acid[5] or the pyrimidine, uracil[6], and such organisms have also been found to have unusual properties. The latter, for example, was found to be able to synthesize protein in the absence of exogenous uracil, a property which appeared to depend on the turnover of a fraction of the cellular ribose nucleic acid (RNA).

During the isolation of these polyauxotrophs[6], a mutant of *E. coli* strain $15T^-$ was obtained which required exogenous arginine and uracil for continuing growth and multiplication. This mutant of *E. coli* strain $15T^-A^-U^-$, hereinafter to be designated as strain TAU, appeared potentially useful for the problem of dissecting further the interrelationships of DNA, RNA, and protein synthesis, as well as for the further analysis of the apparent mutagenicity of thymine deficiency[7] which we had begun to explore in our thymineless uracilless strain[6].

This paper presents some data on physiological and genetic properties of strain TAU. The strain has been found to be a "leaky" mutant, *i.e.* capable of a small degree of synthesis of arginine and uracil.

Microbial strains and methods

The properties of the parent bacterial strain, strain $15T^-$, and the isolation of the mutant, strain TAU, have been described[5,6] as have been the composition of the media used[8], the estimation of viable cells, and the application of turbidimetry to the study of bacterial growth.

The starvation of deficient strains and the procedure used to recover the revertants have been reported[6]. The deficient media in which the appearance of revertants was studied contained mineral salts[8] + glucose + uracil + arginine. Uracil- or arginine-independent mutants were discovered by plating starved cells on minimal Difco agar containing mineral medium, 1 mg of glucose, 2 μg of thymine, and 10 μg of uracil or 20 μg of arginine per ml of agar. To test thymine dependence and uracil or arginine independence, the mutants which appeared on the plates were replicated to two of each of the following minimal agar plates containing: (1) glucose, to score possible thymine-independent cells, (2) glucose + thymine to reveal U^+A^+ revertants, (3) glucose + thymine + uracil, to recover arginine-independent mutants, and (4) glucose + thymine + arginine to identify uracil-independent mutants.

Chemical materials and methods

Carbamyl phosphate was synthesized by the method of JONES *et al.*[9]. Citrulline and arginosuccinic acid were kindly supplied by DR. SARAH RATNER of the Public Health Research Institute of the City of New York. Ureidosuccinic acid and dihydroorotic acid were gifts from DR. D. WRIGHT WILSON of this Department. Other nucleic acid derivatives and amino acids were obtained from the California Foundation for Biochemical Research, Los Angeles.

The techniques used for the extraction of protein and nucleic acids from the bacteria, and methods for their estimation have been described[6], as have been the chromatographic techniques for the isolation of RNA derivatives[6]. RNA nucleotides were dephosphorylated by purified alkaline intestinal phosphatase[1]. The enzymic

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transfer of ribose from a labeled nucleoside to an unlabeled base has been described[6,11]. The isolation of alanine and arginine will be described below.

Uniformly labeled [^{14}C]glucose was obtained from the Nuclear Instrument and Chemical Corporation, Chicago, Illinois. Radioactivity was measured in a gas-flow counter using samples plated on stainless steel planchets under conditions in which self-absorption was negligible. The specific activity of isolated substances has been expressed as activity per μmole of carbon. The specific activity of the glucose contained in the medium was $3.33 \cdot 10^3$ counts/min per μmole of carbon.

Enzyme assays

Three enzymic reactions involving carbamyl phosphate (CP) have been studied with bacterial extracts. These are: (1) The transphosphorylation to adenosine diphosphate (ADP) to form ATP. (2) The transcarbamylation to aspartate to form ureidosuccinate. (3) The transcarbamylation to ornithine to form citrulline. Bacterial extracts were prepared as follows: Parental and mutant strains were grown with aeration in a minimal medium containing glucose as the sole energy source and supplemented with suitable essential nutrients. The cells derived from cultures in the exponential phase were harvested by centrifugation. This and subsequent steps were carried out at 4°. The moist pellet was ground with 2.5 times its wet weight of Alumina A-301 and the paste was extracted with three portions (2.5 ml) of water. After centrifugation for 10 min at 5000 rev./min the supernatant fluid was used for enzyme assays.

The systems used for the study of the CP-ADP transphosphorylation reaction (carbamate phosphokinase) had the following composition in 1 ml: 50 μmoles tris buffer pH 7.4, 10 μmoles $MgSO_4$, 50 μmoles glucose, 0.1 μmole ADP, 0.25 μmole TPN: 100 μg glucose-6-phosphate dehydrogenase (Sigma), 200 μg hexokinase (Pabst), 10 μmoles carbamyl phosphate, bacterial extract. Components were added to a cuvette in the order given. After mixing, the increase in optical density at 340 mμ was determined in a Beckman spectrophotometer. Controls run simultaneously omitted carbamyl phosphate in one instance and bacterial extract in a second. This system made use of the following reactions:

$$\text{CP} + \text{ADP} \rightleftharpoons \text{Carbamate} + \text{ATP} \quad (1)$$

$$\text{ATP} + \text{glucose} \longrightarrow \text{glucose-6-phosphate} + \text{ADP} \quad (2)$$

$$\text{Glucose-6-phosphate} + \text{TPN}^+ \longrightarrow \text{TPNH} + \text{6-phosphogluconolactone} + \text{H}^+ \quad (3)$$

The increase in TPNH, measured at 340 mμ, corrected for changes in the control systems, was a measure of the extent of transphosphorylation from CP to ADP to form ATP. Rates were recorded after the first minute after mixing. A unit of enzyme was defined as the amount of enzyme giving a change of optical density at 340 mμ of 0.01 per min.

The systems used for the study of carbamyl transfer reactions had the following composition in 0.5 ml: 50 μmoles tris buffer pH 7.4, 12.5 μmoles carbamyl phosphate (lithium salt), 100 μmoles DL-aspartate or 10 μmoles DL-ornithine, and bacterial extract. Blanks were prepared by omitting the acceptor, aspartate or ornithine. The mixture was incubated at 37° for 15 min and then placed in an icebath. An aliquot of 0.1 ml was removed and added to 4.7 ml of ammonium acetate at pH 4.2. Inorganic phosphate was estimated by the LOWRY AND LOPEZ method[12], the amount of inorganic phosphate liberated being a measure of carbamyl transfer.

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RESULTS

Nutritional requirements of strain TAU and the site of the metabolic lesion

The mutant was seeded on agar plates containing glucose, thymine, and one of the known intermediates in arginine synthesis. To such a seeded plate was added a paper disc containing one of the intermediates in uracil biosynthesis. The growth responses of the strain are recorded in Table I. It can be seen that ureidosuccinate but not aspartate will replace uracil and that citrulline but not ornithine will replace arginine. These results would appear to suggest a block in the carbamylation of aspartate and of ornithine, a result apparently consistent with the origin of the double requirement in a single genetic change. This had indeed been suggested by the method of isolation of the mutant from strain $15T^-$ in a single step[6]. In addition, it will be seen subsequently that reversions to uracil and arginine independence occur simultaneously indicating the existence of a single metabolic lesion controlling both biosynthetic pathways.

TABLE I

GROWTH RESPONSES OF STRAIN TAU

Minimal medium supplemented with*	*Growth responses with additional supplements of*				
	Aspartic acid	*Ureidosuccinic acid*	*Dihydro-orotic acid*	*Orotic acid*	*Uracil*
Arginine	o	+	++	++	++
Citrulline	o	+	++	++	++
Arginosuccinic acid	o	o	o	o	o
Ornithine	o	o	o	o	o

* Difco agar containing mineral medium, glucose, and thymine.

Since carbamyl phosphate is known to be a common intermediate for uracil and arginine and other mutants of comparable nutritional requirements had been inferred to lack the ability to form this common precursor, we undertook to test strain TAU for the enzyme, carbamate phosphokinase. This enzyme is presumed to control the formation of carbamyl phosphate in a reversible reaction, *i.e.* carbamate + ATP $\rightleftharpoons$ CP + ADP. It may be noted that the formation of carbamate is not considered to be enzymic[13]. As recorded in Table IIA, no differences were observed in carbamate phosphokinase activity in extracts of *E. coli* strain B, the parent strain *E. coli* $15T^-$, and strain TAU. Thus, the metabolic lesion did not appear to exist in the amount of the enzyme governing the formation of the single essential precursor, carbamyl phosphate.

The presence of the carbamyl transferases was estimated as described earlier. As can be seen in Table IIB, strain TAU grown in arginine and uracil has a lower content (approx. 30 %) of the enzyme for transfer to aspartate than did the parent, strain $15T^-$, and only 15 % as much of the enzyme for transfer to ornithine. Nevertheless, these transferases are present in this mutant strain, in contrast to strain $15T^-U^-$ which appears to possess a normal complement of ornithine transcarbamylase and is devoid of the aspartate transcarbamylase. This finding also supports the view that two distinct enzymes are involved in carbamyl transfer. Of interest is the finding that a one step revertant, strain $15T^-A^+U^+$, has supranormal amounts of both enzymes.

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TABLE IIA

THE CONTENTS OF CARBAMATE PHOSPHOKINASE IN BACTERIAL EXTRACTS

Bacterial strains	*Units per mg*
E. coli strain B	15.8
E. coli strain 15_{T^-}	13.9
E. coli strain $15_{T^-A^-C}$	13.6

TABLE IIB

THE CONTENTS OF CARBAMYL TRANSFERASES IN EXTRACTS OF VARIOUS MUTANT STRAINS

Bacterial strains	*Decrease in carbamyl phosphate (μmoles per 15 min)*	
	Aspartate	*Ornithine*
Strain 15_{T^-} (Parent)	1.20	7.46
Strain $15_{T^-U^-}$ (Mutant)	0	7.62
Strain TAU (Mutant)	0.37	1.33
Strain $15_{T^-A^+U^+}$ (TAU revertant*)	5.44	12.96

* All extracts were regularly found to have similar contents of protein; however, a protein estimation was not run for this extract.

Adaptation to D-*xylose*

The ability of the mutant to adapt to D-xylose was studied under different experimental conditions. As demonstrated earlier[4,6], the metabolism of D-xylose requires an induced biosynthesis of a xylose isomerase. In the experiments described below, the adaptation was followed by estimating the disappearance of the substrate, D-xylose. An exponentially growing culture of TAU was chilled when the bacterial concentration had reached 3 to $4 \cdot 10^8$ cells per ml; the culture was sedimented and washed twice in mineral medium. Aliquots were resuspended in media containing D-xylose in place of glucose and lacking either pyrimidines or arginine. The compositions of the various media are given in the legend to Fig. 1. The figure presents data on the adaptation of TAU to D-xylose, *i.e.* the growth and viability of the organism and its metabolism of the pentose.

It can be seen that in the presence of all growth requirements the exposure to xylose produces a lag of about 60 min before growth occurs. No increment in turbidity has been observed in the absence of arginine, although increases in this function occur in the absence of thymine and uracil. It has been inferred therefore that protein synthesis leading to a detectable increment in turbidity cannot take place in the absence of exogenous arginine.

After this lag, the cells either grew or died as metabolism and syntheses proceeded, *i.e.* either cell multiplication or sterilization accompanied substrate utilization depending on the presence or absence of thymine. As can be seen, the maximal killing effect produced by thymine deficiency was observed in cultures resuspended in minimal medium supplemented either with arginine plus uracil or with arginine alone. In the absence of thymine, the omission of arginine reduced death rate and

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the increase in turbidity. When both arginine and uracil were lacking, the killing rate was very low.

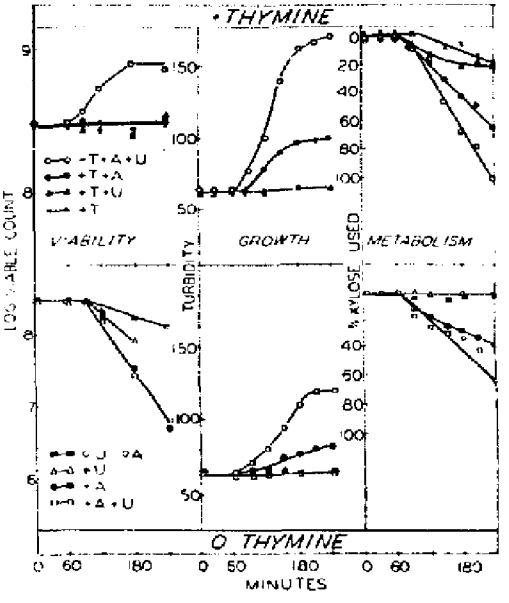

Fig. 1. The viability, growth, and metabolism of D-xylose of strain $15_{T^-A^-U^-}$. The bacteria were grown on glucose, thymine, arginine and uracil, washed and resuspended at turbidity = 60 in the following media at 37°: ○–○–○, minimal medium + thymine + arginine + uracil; ●–●–●, minimal medium + thymine + arginine; ▲–▲–▲, minimal medium + thymine + uracil; ×–×–×, minimal medium + thymine; ■–■–■, minimal medium; △–△–△, minimal medium + uracil; ⊗–⊗–⊗, minimal medium + arginine; □–□–□, minimal medium + arginine + uracil. Those media contained 1 mg of D-xylose per ml.

A complete utilization of substrate was observed at 240 min in minimal media supplemented with all three nutritional requirements. In this interval 65% of D-xylose disappeared if cells were resuspended in minimal medium supplemented either with thymine plus arginine (omitting uracil) or with uracil plus arginine (omitting thymine). Furthermore, as demonstrated in Fig. 1, about 45% of the substrate was used when the cells were resuspended in the minimal medium supplemented with arginine alone. These results showed that this auxotrophic mutant retains the power to adapt to D-xylose utilization in the absence of exogenous thymine and uracil. This phenomenon has already been discovered and described by COHEN AND BARNER for strain 15_{T^-} in the absence of thymine[4] and strain $15_{T^-U^-}$ in the absence of thymine and uracil[6].

A very low utilization of substrate was observed when arginine was omitted and cells were resuspended in minimal medium supplemented with thymine or with thymine plus uracil. In the absence of thymine, the omission of arginine completely repressed adaptation to xylose.

These experiments then show that TAU is capable of adaptation and production of a specific enzyme in the absence of thymine and uracil if the arginine requirement is satisfied.

It was thought that the occurrence of adaptation despite the absence of net

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RNA synthesis may arise in this strain in a manner similar to that for strain 15T–U– in which a turnover of RNA occurs when the synthesis of new specific protein takes place in the absence of exogenous pyrimidine[6]. A test of RNA turnover was therefore undertaken with TAU, with the additional aim of determining the effect of arginine deficiency on such possible turnover. This was accomplished by following the incorporation of isotope from uniformly labeled [^{14}C]glucose into RNA in the absence and presence of exogenous arginine, and in the absence of thymine and uracil in the medium.

Effect of arginine on polymer synthesis in absence of exogenous pyrimidines

It has been demonstrated in many biological systems that synthesis of protein is tied to a concomitant synthesis of RNA. In addition, it is known that RNA synthesis and to a smaller extent DNA synthesis are inhibited in *E. coli* under conditions of amino acid deficiency (PARDEE AND PRESTIDGE[15], GROS AND GROS[14], (BARNER AND COHEN[5]). We wished to study the metabolism of RNA in the absence of uracil and the effect of arginine deficiency on this metabolism.

Cells growing exponentially in synthetic (minimal) medium containing glucose, thymine, arginine, and uracil were chilled, centrifuged, and washed. One aliquot was resuspended in minimal medium containing 0.48 mg [^{14}C]glucose per ml and a second aliquot was resuspended in same medium as above, but supplemented with 20 μg arginine per ml. After 45 and 90 min of incubation, an aliquot of 25 ml was removed for analysis of viability, RNA, DNA, and protein and a second aliquot of 600 ml was removed for the study of incorporation of ^{14}C into RNA under those conditions. The data on the effect of exogenous arginine on polymer synthesis and cell viability in absence of pyrimidines are presented in Table III. From this Table,

TABLE III

EFFECT OF ARGININE ON POLYMER SYNTHESIS IN THE ABSENCE OF PYRIMIDINES

Time of Incubation min	*Turbidity*				*Viability*			
	*Minimal medium**		*+ Arginine*		*Minimal medium*		*+ Arginine*	
	O.D.	*% increase*	*O.D.*	*% increase*	*No. of cells/ml*	*% decrease*	*No. of cells*	*% decrease*
0	75	0	75	0	$2.4 \cdot 10^8$	0	$2.92 \cdot 10^8$	0
45	75	0	89	19	$1.0 \cdot 10^8$	58	$1.0 \cdot 10^8$	66
90	75	0	95	27	$4.7 \cdot 10^7$	80	$4.2 \cdot 10^7$	86

Protein				*Nucleic acids*							
				RNA				*DNA*			
Min. medium		*+ Arginine*		*Min. medium*		*+ Arginine*		*Min. medium*		*+ Arginine*	
mg/10 ml	*% increase*	*mg/10 ml*	*% increase*	*µg/10 ml*	*% increase*	*µg/10 ml*	*% increase*	*µg/10 ml*	*% increase*	*µg/10 ml*	*% increase*
1.83	0	1.9	0	275	0	264	0	49	0	49	0
2.62	44	3.52	86	315	15	300	14	49	0	49	0
2.70	47	3.80	100	310	13	340	29	53	8	53	8

* Minimal medium contains salts supplemented with glucose.
Minimal medium + arginine = contains salts, glucose as above plus 20 μg/ml of arginine.

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it can be seen that an increase in turbidity was observed only if cells were resuspended in minimal medium supplemented with arginine. This increase was about 19% at the 45 min interval and 27% at 90 min. At 90 min the content of protein had doubled in the cells resuspended in the presence of exogenous arginine. However, in the absence of arginine an increase of biuret-reactive material was also observed (+47%) although in this time no increase in turbidity was revealed. A comparable surprising result separating protein synthesis from the increase of turbidity, had been seen with *E. coli* strain $15T^{-}U^{-6}$. With TAU in the absence of arginine the increment of biuret-reactive material seems to be complete after 45 min. Nevertheless, it will recalled that TAU did not adapt to xylose under these conditions.

This increase of biuret-reactive material in the absence of arginine might suggest a leakage, *i.e.* slow synthesis of arginine or the formation of arginine-free peptides, perhaps in cell wall, or both. The idea of a leakage in arginine synthesis is supported also by the fact that transfer of carbamyl group from carbamyl phosphate to ornithine, although depressed under the experimental conditions mentioned above, is not completely prevented. However, the possibility of the existence of some endogenous source of arginine for protein synthesis, which might be exhausted after 45 min of incubation, is not completely excluded. In any case the inability to adapt in the absence of exogenous arginine implies that the leakage in arginine synthesis or endogenous reserves of the amino acid must be very low.

A small increase of RNA content was observed under conditions with or without arginine although uracil had been omitted from the medium. In cells resuspended without arginine this increase ranges between 12 to 14% and this small difference might conceivably arise as an error in the analysis; however, if real, it might also indicate leakage, *i.e.*, a slow synthesis of uracil, as already suggested by results on transfer of the carbamyl group from carbamyl-phosphate to aspartic acid. If the increase is real, it seems that without exogenous arginine a plateau level is reached after 45 min. As recorded above the same result was observed for biuret-reactive material in those cells.

In the absence of both pyrimidines, but in the presence of arginine, a continuing increase of RNA or acid precipitable Bial-reactive pentose was observed. However, it should be noted that in the absence of thymine no significant synthesis of DNA was observed even if synthesis of protein was allowed to proceed.

Metabolism of RNA and protein in uracil-deficient media

Continuing the above experiment, aliquots were removed at 45 and 90 min intervals from the 600-ml batches of the cells resuspended in minimal medium supplemented with [^{14}C]glucose plus arginine. The cells were sedimented, washed, and resuspended in mineral medium, from which the cells were precipitated with 5% cold trichloroacetic acid (TCA). After precipitation, the cells were washed three times each with ethanol, ether, and finally with boiling ethanol-ether (1:1). A modified SCHMIDT-THANNHAUSER procedure[5] was used for extraction of RNA since digests of DNA derived from TAU do not reprecipitate on addition of acid. Dried, defatted bacterial pellets were extracted overnight with 5 ml NaOH at 37°. The clear solution obtained by this treatment was neutralized, and chilled. The addition of 3 vol. of ethanol precipitated DNA and protein. The supernatant fluid containing RNA nucleotides was neutralized, concentrated, and tested for DNA by the diphenyl-

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amine reaction. The RNA nucleotides contained less than 2% of DNA. The RNA nucleotides were precipitated in the cold as silver salts by adding 0.5 ml of 2 *M* $AgNO_3$ at pH 7.6. The silver salts were washed once with cold water, resuspended in water, and the free nucleotides were regenerated by passing H_2S through this suspension. After centrifugation and concentration of the supernatant fluid, the nucleotides were chromatographed in solvent systems containing isobutyric acid–0.5 *N* NH_4OH (10:6) at pH 3.7. This solvent separates adenylic acid from cytidylic acid. The combined uridylic and guanylic acids, not separated in this solvent, were treated with intestinal alkaline phosphatase and the resulting nucleosides were separated readily in isopropanol–water–concentrated NH_4OH (28% NH_3) (85:15:1.3). Adenylic and cytidylic acids were also treated with phosphatase and the corresponding nucleosides were separated in *n*-butanol saturated with 5% NH_4OH.

Further purification steps involved enzymic transfer of ribose to an unlabeled base. The ribose moiety of adenylic and guanylic acid was transferred from corresponding nucleosides to unlabeled uracil and the activity of the resulting uridine was determined. The activity of the ribose moiety of the isolated uridylic acid was determined after exchange of the labeled uridine with unlabeled hypoxanthine.

Labeled adenine, guanine, and uracil were also derived from their corresponding nucleoside after their liberation in the exchange reactions. Cytosine was obtained by perchloric digestion of cytidine.

The protein precipitated by 5% trichloroacetic acid was hydrolyzed with 6 *N* HCl by refluxing overnight. After the hydrolysate was taken to dryness repeatedly to remove hydrochloric acid, the residue was dissolved in water and applied to Whatman No. 3 paper for chromatography in butanol–acetic acid[16]. The arginine band was located by spraying a narrow strip with α-naphthol reagent[17]. Further purification of the arginine involved the use of the phenyl thiohydantoin (PTH) derivative prepared on a micro scale as described by SJÖQUIST[18,19]. The PTH derivatives of arginine from the chromatogram and a known sample of arginine were run consecutively through the following steps of purification: heptane–formic acid–butanol chromatography, electrophoresis in 0.05 *M* ammonium formate at pH 3.5, and chromatography in ethyl acetate saturated with H_2O. The R_F of arginine PTH in the first solvent is sufficiently different from that of other amino acid PTH derivatives to effect complete separation.

Alanine, separated in the butanol–acetic acid chromatogram, was rerun in butanol–methyl ethyl ketone–NH_4OH. Complete isolation of alanine could be expected from this combination of solvents but one further purification was carried out. This consisted of either chromatography with phenol at pH 12.0[20] or preparation and chromatography of the PTH derivative.

It was found that considerable ^{14}C was incorporated into purine and pyrimidine derivatives of RNA when the cells were resuspended in the minimal medium. This indicates that a metabolism of RNA occurred in those cells even in the absence of exogenous uracil.

Data on the isotope contents of isolated compounds and derivatives are presented in Table IV. These data confirm in general the analytical evidence presented in Table III. Thus, isotope is found in alanine, indicating amino acid synthesis and incorporation in acid insoluble cell residues in the absence of exogenous uracil. The isotope content of alanine indicates a synthesis of this amino acid to the extent

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TABLE IV

RATIOS OF ACTIVITY OF CARBON OF BACTERIAL DERIVATIVES TO ACTIVITY OF GLUCOSE CARBON*

Compound	*Minimal medium*		*Minimal medium + Arginine*	
	45 min	*90 min*	*45 min*	*90 min*
RNA				
Adenosine-ribose	0.139	0.109	0.207	0.184
Adenine	0.091	0.069	0.139	0.124
Guanosine-ribose	0.125	0.097	0.177	0.181
Guanine	—	0.110	—	0.133
Uridine	0.091	0.104	0.139	0.167
Ribose	0.164	0.175	0.235	0.280
Uracil	0.014	0.015	0.022	0.025
Cytidine	0.096	0.079	0.124	0.146
Ribose	0.147	0.126	0.182	0.235
Cytosine	0.033	0.021	0.052	0.029
Amino acids				
Alanine	0.204	0.197	0.376	0.346
Arginine	—	0.053	—	0.015

* Specific activity of [^{14}C]glucose 3330 counts/min/μ mole carbon.

of 25 % of the alanine originally present when arginine was absent from the medium. In the presence of arginine, about twice this incorporation was obtained. These biosynthetic functions were substantially completed in both systems at 45 min. An approximately two-fold discrepancy between protein increment measured by the biuret reaction and that determined by isotope incorporation into amino acids has been reported in our earlier paper on strain 15T$^-$U$^-$[6].

Significant radioactivity (5.3 % of that of the glucose fed) was found in the arginine of cell protein after extensive purification of this amino acid, indicating the existence of an incomplete block of this synthesis. The biosynthesis of arginine was further repressed in the presence of exogenous arginine, *i.e.* now only 1.5 % of the activity of the glucose was incorporated. Radioactivity of this low order was also found in all samples[8] of uracil and cytosine isolated from RNA formed in the absence of exogenous uracil. Thus, an incomplete block also existed in uracil synthesis.

In the absence of exogenous arginine, maximal isotope was found in the RNA derivatives at 45 min. In the presence of exogenous arginine, significantly greater amounts of isotope were obtained in these derivatives at 45 min; in addition, in this case the isotope contents of the ribose of the pyrimidine nucleotides continued to increase after 45 min in this system.

In the absence of exogenous arginine the isotope content of the purine ribose was slightly less than that of pyrimidine ribose. In addition, with one exception the isotope content of purine carbon was less than that of its ribose. The latter point is particularly striking in the medium containing arginine. These data indicate a greater de novo synthesis of purine ribose than of purine and suggest either the presence of stored purine or a turnover of RNA in which a significant percentage of the purine is conserved (one-third of the total).

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Apparent mutagenicity of thymine starvation

Strain TAU was grown to $3 \cdot 10^8$ bacteria per ml, washed, and subjected to thymine starvation with aeration at 30° in a mineral medium supplemented with glucose, arginine, and uracil. Plating methods for the demonstration of revertants have been presented earlier. Experimental results are recorded in Table V.

TABLE V

THE APPARENT MUTAGENICITY OF THYMINE STARVATION*

Time of starvation min	No. of viable cells per ml	% killing	Number of revertants					
			per 10^7 viable cells			per ml of culture		
			A+	U+	A+U+	A+	U+	A+U+
0	$1.88 \cdot 10^8$	0	17.4	17	17	285	295	285
15	$1.9 \cdot 10^8$	0	79	80	81	1510	1420	1450
30	$1.65 \cdot 10^8$	12	150	158	154	1330	1400	1460
60	$2.2 \cdot 10^7$	88.2	200	265	220	555	750	615
120	$1.0 \cdot 10^6$	99.5	250	216	256	13.5	15.8	16
240	$3.75 \cdot 10^5$	99.8	365	370	372	10.3	10.3	10.4

* Starvation in minimal medium supplemented with glucose, arginine, and uracil.

It can be seen in Table V that the revertants to arginine independence, to uracil independence, and to both together are equivalent in number at the beginning of the experiment and appear at the same rate. Thus the reversion to independence with respect to both requirements appeared to occur in a single genetic change.

Thymine starvation of TAU produced a five fold increase in the appearance of revertants before the cells began to die. This effect was recorded earlier by the senior author (D.K.) for strain 15T–U– (see [6]). After this period, the total numbers of mutants decreased in general paralleling the loss of viability, as can be seen in Fig. 2. Although a slow increase occurred in the proportion of revertants to total viable cells, the latter increase may only reflect the relative resistance of a proportion of the back mutants to thymineless death.

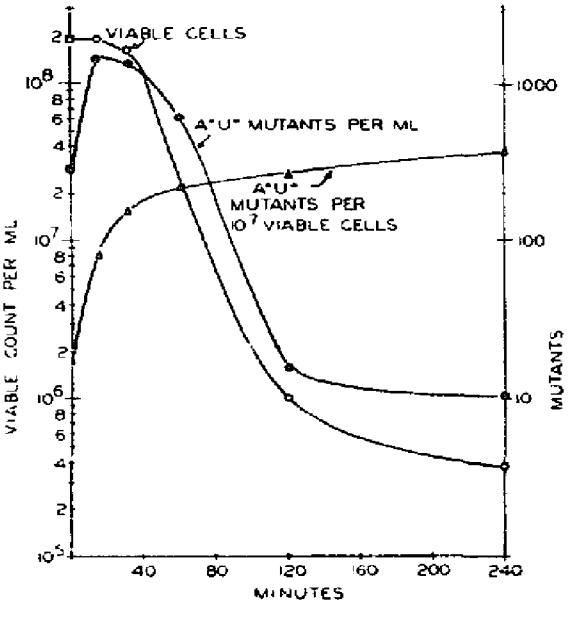

Fig. 2. A comparison of the survival of strain TAU and the appearance of T–A+U+ revertants under conditions of thymine starvation. The appearance of mutants is expressed both for a given volume of culture and for a given number of viable cells.

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DISCUSSION

Although the initial purposes of these experiments were somewhat frustrated by the complexities of the mutant; a number of interesting results have been obtained. Thus, the mutant TAU is able to synthesize up to 5 % of its arginine requirement and about 2 % of its uracil requirement; nevertheless, the exogenous precursors citrulline and ureidosuccinate are essential for growth of the organisms. The position of the metabolic block and its origin or elimination in a single genetic step implicate an error in carbamylation. However, the three known enzymes catalysing reactions of the carbamyl moiety are present in the mutant although the contents of the two enzymes catalysing carbamyl transfer to form citrulline and ureidosuccinate are somewhat depressed. The amounts of these enzymes are depressed only 70 to 80 %, as compared to the amounts in the parent strain $15T^-$, and even this might be ascribed to a negative feed-back mechanism arising from growth in the presence of arginine and uracil. It may be imagined that the very significant levels of these transferases even under these conditions would permit a greater synthesis of arginine and uracil than that actually observed.

It may be suggested then that in this mutant there is not only an inhibition of enzyme synthesis but more important an inhibition of the activity of these lowered levels of enzymes produced. RAVEL *et al.*[21] have recently demonstrated the inhibitory activity of carbamyl phosphate towards the growth of *Streptococcus* and *Lactobacillus arabinosus*, an inhibition overcome by citrulline, purines, and uracil. These authors have also shown that cultivation of *S. lactis* in the presence of carbamyl phosphate ($2 \cdot 10^{-3}$ *M*) reduces the ornithine carbamyltransferase in the organism to less than 10 % of the normal value. At high concentrations of carbamyl phosphate, the activity of the enzyme is inhibited as well. Since the enzyme for synthesis of carbamyl phosphate is present in normal amounts, it appears possible that this compound may be accumulated in amounts sufficent to be seriously inhibitory to both enzyme production and activity in strain TAU. Nevertheless, this explanation of the behavior of the mutant is most inadequate since strain $15T^-U^-$ which lacks the aspartic carbamyl transferase alone and therefore also probably produces more carbamyl phosphate than it can use, is not inhibited in the production of the ornithine carbamyl transferase nor in its activity. It is possible that further study of these transferases in these mutants and their revertants may reveal differences in their sensitivity to inhibition by carbamyl phosphate. Such an analysis may also indicate whether particular revertants arise by a true back mutation or by development of a suppressor mechanism.

As described in Fig. 1, in the presence of exogenous arginine the mutant behaved in many respects like strain $15T^-U^-$[6]. Exogenous arginine permitted the production of xylose isomerase in the absence of uracil and markedly facilitated the death of strain TAU in the absence of thymine. An increase of cell numbers and of turbidity occurred only in the presence of the amino acid. As with strain T^-U^-, both thymine and uracil were also essential to the increase in cell number.

In the absence of an exogenous supply of amino acid, the production of xylose isomerase did not occur although an increase of biuret reactive substance and the synthesis of acid-insoluble bound alanine was demonstrated. The nature of this material is not clear; the possibility that some cell wall material or other acid in-

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soluble polypeptides, deficient in arginine, has been laid down, is not excluded.

Of particular interest is the problem of the apparent RNA synthesis in deficient organisms. A very small net synthesis (14 %) was observed for the first 45 min in the absence of arginine and uracil. The extent of isotope incorporation in RNA ribose in this system tends to confirm that for the most part this small apparent increment was indeed net synthesis (see Table V). It seems possible that this amount of incorporation may be accounted for by the utilization of ingested uracil present in the low molecular pool.

However, even in this system a slight but significant discrepancy is seen between incorporation into uridine ribose and purine ribose and between incorporation into adenine ribose and adenine, suggesting that the sources of the purine ribotides may be somewhat more complicated than that of a simple de novo synthesis. Indeed in the presence of exogenous arginine these discrepancies become much more marked, and although a de novo synthesis of purine ribose stops at 45 min, the incorporation of isotope into pyrimidine ribose continues from 45 to 90 min. It should be noted that this RNA metabolism continues for a period at least five times as long as that necessary to exhaust an amount of exogenous uracil sufficient to permit a 28 % increase in RNA under conditions of normal growth. These results in this system seem most reasonably explained as arising from a turnover of RNA with a conservation of pyrimidines to which newly formed ribose is added, even as in strain $15T^{-}U^{-}$[6]. With strain TAU however, the observed turnover appears in considerable measure dependent on the presence of amino acids, more particularly the required exogenous arginine.

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