Protein Synthesis Group, Shanghai Institute of Biochemistry (1975) *Sci. Sin. 18*, 745.
Schröder, E. (1967) in *Peptides* (Beyerman, H. C., van de Linde, A., & Maassen van den Brink, N., Eds.) pp 245–257, North-Holland Publishing Co., Amsterdam.
Shaltiel, S. (1967) *Biochem. Biophys. Res. Commun. 29*, 178.
Sieber, P., & Iselin, B. (1968) *Helv. Chim. Acta 57*, 622.
Staub, A., Sinn, L., & Behrens, O. K. (1955) *J. Biol. Chem. 214*, 619.
Stewart, J. M., Knight, M., Paiva, A. C. M., & Paiva, T. (1972) *Prog. Pept. Res.*, [*Proc. Am. Pept. Symp.*] *2nd, 1970 2*, 59–64.
Unger, R. H., & Orci, L. (1975) *Lancet 1*, 14.
Wang, S. S. (1973) *J. Am. Chem. Soc. 95*, 1328.
Wieland, H. (1912) *Ber. Dtsch. Chem. Ges. 45*, 484.
Windridge, G. C., & Jorgensen, E. C. (1971) *J. Am. Chem. Soc. 93*, 6318.
Wünsch, E. (1966) presented at the 8th European Peptide Symposium, Noordwijke, Sept 1966.
Wünsch, E., & Wendelberger, G. (1968) *Chem. Ber. 101*, 3659.
Wünsch, E., Jaeger, E., Kisfaludy, L., & Löw, M. (1977) *Angew. Chem., Int. Ed. Engl. 16*, 317.
Yang, C. C., & Merrifield, R. B. (1976) *J. Org. Chem. 41*, 1032.

Increased Translatable Messenger Ribonucleic Acid for Argininosuccinate Synthetase in Canavanine-Resistant Human Cells†

Tsung-Sheng Su,* Arthur L. Beaudet, and William E. O'Brien

ABSTRACT: The level of argininosuccinate synthetase activity in the human tissue culture cell line RPMI 2650 was 6-fold higher when citrulline was substituted for arginine in the culture medium. Canavanine-resistant (Can^r) variants were isolated and had enzyme activity up to 25 nmol min^{-1} (mg of protein)$^{-1}$ or 180-fold higher than that of the wild-type cells grown in arginine. The differences in enzyme activity were paralleled by differences in the amount of enzyme determined immunologically. The micrograms of enzyme per milligrams of protein, determined by complement fixation, were 0.03 for wild-type cells grown in arginine, 0.29 for wild-type cells grown in citrulline, and 6.73 for a Can^r variant. In vivo labeling studies suggested increased synthesis of argininosuccinate synthetase in Can^r cells, and in vitro translation of poly(adenylic acid) [poly(A)] messenger ribonucleic acid (mRNA) from wild-type and Can^r cells confirmed a quantitatively compatible increase in translatable poly(A) mRNA for the enzyme in Can^r cells. No precursor for the enzyme was recognized by using in vitro translation, and the poly(A) mRNA for the enzyme had a sedimentation value of 16 S by sucrose-gradient analysis. The levels of argininosuccinate synthetase activity in the Can^r cells were similar to those found in normal liver.

Argininosuccinate synthetase (EC 6.3.4.5) and argininosuccinate lyase (EC 4.3.2.1) are important enzymes in the urea cycle, and genetic deficiencies can cause hyperammonemia, mental retardation, and death. Through the reactions shown in eq 1 and 2, these enzymes can synthesize arginine from citrulline + aspartate +

$$\text{ATP} \underset{\text{Mg}^+}{\overset{\text{synthetase}}{\rightleftharpoons}} \text{argininosuccinate} + \text{AMP} + \text{PP}_i \quad (1)$$

$$\text{argininosuccinate} \overset{\text{lyase}}{\rightleftharpoons} \text{fumarate} + \text{arginine} \quad (2)$$

citrulline, a function which may be important in nonhepatic tissues and in cultured cell experiments. The activity of the urea-cycle enzymes in mammalian liver is increased with increased dietary protein (Schimke, 1962; Nuzum & Snodgrass, 1971). Argininosuccinate synthetase and lyase are subject to metabolite control in cultured mammalian cells. Schimke (1964) observed that argininosuccinate synthetase and argininosuccinate lyase activities were repressed coordinately by the presence of arginine in the growth medium of HeLa, KB, and L cells. Conversely, when arginine was removed and replaced by citrulline in the growth medium, the argininosuccinate synthetase and argininosuccinate lyase activities increased 4–20-fold in these cell lines. Irr & Jacoby (1978) found that argininosuccinate synthetase in cultured human lymphoblasts was increased when citrulline replaced arginine in the medium, but that argininosuccinate lyase activity remained constant.

Variation in argininosuccinate synthetase also is observed in certain cell variants. Jacoby (1978) described the isolation of human lymphoblast cell lines which were resistant to the arginine analogue canavanine. Canavanine is not a specific inhibitor of argininosuccinate synthetase but rather is toxic when incorporated into protein in place of arginine. The canavanine-resistant cells had increased argininosuccinate synthetase activity, and presumably were resistant to canavanine because of an increased conversion of citrulline to arginine. The canavanine-resistant lymphoblasts isolated by Jacoby (1978) had lost the ability to respond to changes in arginine concentration.

We have isolated canavanine-resistant variants of the human cell line RPMI 2650. The amount of argininosuccinate synthetase protein and its messenger ribonucleic acid (mRNA) were increased in the variant cell lines.

† From the Howard Hughes Medical Institute and the Departments of Pediatrics and Cell Biology, Baylor College of Medicine, Houston, Texas 77030. *Received September 16, 1980.* This work was supported in part by National Institutes of Health Grant GM 07526.

* Correspondence should be addressed to this author at the Department of Pediatrics, Baylor College of Medicine, Houston, TX 77030.

0006-2960/81/0420-2956$01.25/0

Materials and Methods

Materials. Purified argininosuccinate synthetase from human liver and rabbit antiserum to the purified enzyme were prepared as described previously (O'Brien, 1979). [^{14}C]Aspartate, [^{14}C]fumarate, and [^{35}S]methionine were obtained from Amersham.

Tissue Culture Conditions. RPMI 2650 was obtained from the American Type Culture Collection (Hay et al., 1979) where it is designated CCL 30. This human cell line has a near diploid karyotype (Moorhead, 1965) and is not a derivative of HeLa (Hay et al., 1979). Cells were cultured in minimal essential medium supplemented with nonessential amino acids (Gibco), with 10% fetal calf serum (Rehatuin) and with 0.6 mM arginine or citrulline as indicated. Cultures were examined regularly for mycoplasma by using culture (Hayflick, 1965) and fluorescent staining (Del Giudice & Hopps, 1978) techniques.

Canavanine-resistant cell lines were obtained without mutagenesis by culturing cells in medium with arginine replaced by 0.6 mM citrulline and an initial concentration of canavanine of 10 μM. The vast majority of cells initially placed in this medium died, and clonal growth was detected after 10–14 days. Dependent upon the rate of growth of subcultures, the concentration of canavanine was increased to 20 μM and then 40 μM over a 10-week period. Two separate clones were further purified by single-cell isolation and were designated as Can^{r}1 and Can^{r}2.[1]

Cell Harvest, Enzyme Assays, and Complement Fixation. Cells were harvested from 150- or 100-mm plates by removing the medium, washing 3 times with 0.9% NaCl, and scraping the cells with a Teflon policeman into 0.6 mL of 50 mM Tris-HCl, pH 7.5, containing 1 mM citrulline. This cell suspension was frozen and thawed 3 times and centrifuged at 12000g for 20 min. The supernatant fluid was recovered as the cell extract.

Argininosuccinate synthetase activity was determined by using a method based on the conversion of [^{14}C]aspartate to [^{14}C]argininosuccinate (O'Brien, 1979), but modifications were necessary to avoid interference from asparagine synthetase activity. Reactions were stopped by the addition of 0.70 mL of cold H_2O, and the mixture was applied to 0.4 × 4 cm columns of Dowex 1-X8 acetate equilibrated with 10 mM Tris–acetate, pH 7.4. Two 1-mL volumes of the same buffer were used to rinse the reaction tubes and also were applied to the column. The effluent was discarded, and argininosuccinate was eluted with 4 mL of 0.05 M HOAc. A 1-mL aliquot of the effluent was added to 6 mL of Aquasol (New England Nuclear) for scintillation counting.

Argininosuccinate lyase activity was determined by measuring the conversion of [^{14}C]fumarate into [^{14}C]argininosuccinate. Reactions contained 100 mM potassium phosphate at pH 6.8, 12 mM [2,3-$^{14}C_2$]fumarate (500 dpm/nmol), 50 mM arginine, and enzyme, in a total volume of 0.1 mL. Reactions were incubated at 37 °C and were terminated by the addition of 0.05 mL of 1 M HOAc. Reactions then were heated for 30 min at 90 °C to convert argininosuccinic acid to its anhydrides (Ratner & Kunkemueller, 1966). After being heated, 0.85 mL of H_2O was added, and the entire 1 mL was applied to a 0.5 × 4.0 cm column of Dowex 1-X8 acetate prevously equilibrated in 50 mM HOAc. Two washes of 1 mL of 50 mM HOAc were used to rinse the tube and were applied to the column. The anhydrides of argininosuccinate acid were totally eluted in the 3-mL volume, and 1 mL was removed and placed into 6 mL of Aquasol.

The microcomplement fixation procedures for quantifying the amount of total synthetase protein in the cell extracts were performed as described by Levine (1978). Purified human liver argininosuccinate synthetase was used as the standard.

Incorporation of [^{35}S]Methionine into Intact Cells. For radioactive labeling, cells in 150-mm plates were incubated for 5 h in 12 mL of serum-free medium containing as the sole methionine source 1 mCi of [^{35}S]methionine (specific activity 1075 Ci/mmol), and extracts were prepared as described for the enzyme assay.

Messenger RNA Isolation. Poly(adenylic acid) [poly(A)] RNA from cultured cells was isolated as described by Wahl et al. (1979) with a modification to include proteinase K treatment. The RNA was further purified by two sequential fractionations by using oligo(dT)–cellulose chromatography as described by Aviv & Leder (1972), and the poly(A) mRNA was stored at −70 °C.

Sedimentation Analysis of RNA in a Sucrose Gradient. An aliquot of the oligo(dT)–cellulose-purified RNA was dissolved in 10 mM Tris-HCl at pH 7.4 with 1 mM EDTA, heated at 68 °C for 10 min, and then cooled rapidly to 4 °C. $NaDodSO_4$ was added to 0.5% (w/v), and the sample was loaded onto a 10–30% (w/v) linear sucrose-density gradient containing 10 mM Tris-HCl at pH 7.4, 1 mM EDTA, and 0.5% (w/v) $NaDodSO_4$. The gradient was sedimented in a Beckman SW 41 rotor at 35000 rpm (147500g) at 20 °C for 14 h. Fractions of 0.75 mL were collected, concentrated by ethanol precipitation, and translated in vitro.

In Vitro Protein Synthesis. Rabbit reticulocyte lysate was prepared according to the method of Allen & Schweet (1962), and the mRNA-dependent protein-synthesizing system was prepared essentially as described by Pelham & Jackson (1976). The incorporation from [^{35}S]methionine in reactions of 250 μL was determined by using 2-μL aliquots, and portions of the remainder of the in vitro products were analyzed by immunoprecipitation and gel electrophoresis.

Immunoadsorption and Gel Electrophoresis. Immunoadsorption was conducted with formalin-fixed *Staphylococcus aureus* as described by Kessler (1975) with modifications. Volumes of 100–150 μL of [^{35}S]methionine-labeled cell extract or in vitro synthesized protein were preabsorbed by addition to a conical centrifuge tube containing 10 μL of packed IgGsorb (The Enzyme Center). After the solution was mixed and incubated for 10 min at 4 °C, the cells were removed by centrifugation, and the step was repeated with the supernatant fluid. The final supernatant fluid was removed and mixed with either immune serum or preimmune serum and incubated for 15 min at 4 °C. To this solution was added 100 μL of a 10% suspension of IgGsorb, and a 10-min incubation was allowed. IgGsorb was removed by centrifugation, and the resulting pellet was washed as described by Kessler (1975). The final pellet was suspended in 75 μL of 4% $NaDodSO_4$ containing 0.7 M β-mercaptoethanol and heated at 95 °C for 6 min. A 50-μL aliquot of the supernatant was applied for electrophoresis in 15% acrylamide gels as described by Laemmli (1970), and autoradiography or fluorography (Bonner & Laskey, 1974) of the dried gel was carried out by using Kodak SB-5 film.

Results

Enzyme Activity. As shown in Table I, the argininosuccinate synthetase activity of the RPMI 2650 cells was increased approximately 6-fold when cells were grown in

[1] Abbreviations used: HOAc, acetic acid; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; Can^{r}1 and Can^{r}2, the RPMI 2650 cell lines selected for resistance to the arginine analogue, canavanine.

Table I: Enzyme Activity in Wild-Type and Variant RPMI 2650 Cell Extracts

cell line	culture condition	activity [nmol min^{-1} (mg of protein)$^{-1}$][a] arginino-succinate synthetase	arginino-succinate lyase
RPMI 2650	arginine	0.14	1.27
RPMI 2650	citrulline	0.86	0.94
Can^{r}1	arginine	25.1	1.38
Can^{r}1	citrulline	25.0	1.66
Can^{r}2	arginine	8.5	1.23
Can^{r}2	citrulline	4.7	1.24

[a] These data are the average of two determinations made on each of two cultures.

medium with 0.6 mM citrulline compared to cells grown in medium with 0.6 mM arginine. The argininosuccinate lyase activity was not significantly different in the two conditions. The increase in synthetase activity upon transfer to medium containing citrulline occurred over a 96-h period. Analysis of the canavanine-resistant variants indicated that the activity of argininosuccinate synthetase in Can^{r}1 cells was increased to about 180-fold; activity for Can^{r}2 cells was increased about 50-fold. Argininosuccinate lyase activity of the Can^r cells was not altered. Unlike the wild-type cells, the level of argininosuccinate synthetase activity in the Can^r cells was not influenced significantly by the substitution of citrulline for arginine in the culture medium. The difference in the activity in the Can^{r}2 cell line was not consistently observed and was not thought to be significant. The data from multiple experiments showed similar relative activities to those shown in Table I although the absolute activities varied slightly between experiments, probably related to culture conditions and cell density (Hudson et al., 1980). The Can^{r}1 cell line consistently yielded argininosuccinate synthetase activity in the range of 20–30 nmol min^{-1} (mg of protein)$^{-1}$. This is in comparison to a level of 10–20 nmol min^{-1} (mg of protein)$^{-1}$ for analogous extracts of human autopsy liver and 4200 nmol min^{-1} (mg of protein)$^{-1}$ for the purified enzyme from human liver (O'Brien, 1979). If one assumes that argininosuccinate synthetase in the Can^{r}1 cells has a specific activity identical with that of the liver enzyme, it can be calculated [(25/4200) × 100] that argininosuccinate synthetase was approximately 0.6% of the soluble protein in the extracts from Can^{r}1 cells.

The activity of argininosuccinate synthetase in the variant cell lines was very stable. After maintenance for almost 1 year in two culture conditions, (1) normal medium with arginine and (2) medium without arginine but containing citrulline and 40 μM canavanine, there was no significant change in argininosuccinate synthetase activity. In addition, argininosuccinate synthetase activity did not change when variant cell lines were grown in concentrations of canavanine up to 150 μM.

Immunologic Quantitation of Argininosuccinate Synthetase. In order to investigate various possible mechanisms for increased enzyme activity, we used an antibody to the human argininosuccinate synthetase (O'Brien, 1979). The antibody had indistinguishable affinity for immunoadsorption of enzyme from human liver or from the Can^r cell lines (data not shown). Analysis by microcomplement fixation indicated a 10-fold increase in antigenic material in wild-type cells grown in citrulline as compared to wild-type cells grown in arginine (Table II). In addition, there was a greater than 200-fold increase in antigenic material in Can^{r}1 cell extracts. Thus, between 0.6 and 0.7% of the protein in the extract from Can^{r}1 cells

Table II: Determination of Immunoreactive Argininosuccinate Synthetase in Cell Extracts by Microcomplement Fixation

cell line	culture condition	μg of antigen/mg of protein[a]	relative amount of antigen
RPMI 2650	arginine	0.03	1
RPMI 2650	citrulline	0.29	10
Can^{r}1	arginine	6.73	225

[a] The data are the average of independent determinations on two culture plates.

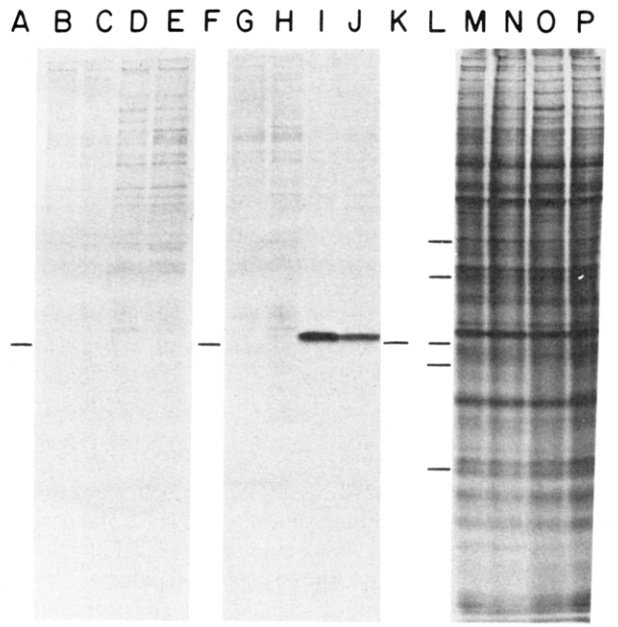

FIGURE 1: Autoradiograph of NaDodSO$_4$ gel analysis of immunoprecipitates of [^{35}S]methionine-labeled cell extracts. Cell extracts were diluted to give 150000 cpm/μL. Lanes B, G, and M are extracts from RPMI 2650 cells grown in arginine; lanes C, H, and N are from the same cells grown in citrulline; lanes D, I, and O are from Can^{r}1 cells, and lanes E, J, and P are from Can^{r}2 cells. A 50-μL sample of extract was reacted with preimmune serum for lanes B–E or with immune serum for lanes G–J. Lanes M–P represent 1 μL of extract directly applied for gel analysis. Lanes A, F, and K are marked to indicate the location of the purified human liver argininosuccinate synthetase, and lane L is marked to indicate the migration of molecular weight markers of 68000, 60000, 45000, 39500, and 23300 as determined by Coomassie Blue staining of the gel prior to autoradiography. The gel was exposed 174 h for autoradiography.

was argininosuccinate synthetase. This provided good agreement regarding the relative and absolute amounts of argininosuccinate synthetase in the various extracts, whether determined immunologically or enzymatically. The data were consistent with the view that variable amounts of normal enzyme rather than alteration in the kinetic properties of the enzyme accounted for the differences in enzyme activity.

Gel Electrophoretic Analysis of Immunoprecipitable Argininosuccinate Synthetase Synthesized in Vivo. As a first step in examining the synthesis and degradation of the synthetase enzyme, the cultured cells were pulse labeled with [^{35}S]methionine and the newly synthesized proteins analyzed by immunoprecipitation followed by NaDodSO$_4$ gel electrophoresis. An autoradiograph of such a gel is shown in Figure 1. With the two Can^r cell extracts, a major radioactive component was precipitated by immune (lanes I and J) but not by preimmune serum (lanes D and E). This component migrated in a position similar to but possibly larger than purified human liver argininosuccinate synthetase (lanes A, F, and K). This radioactive band was very intense for the two

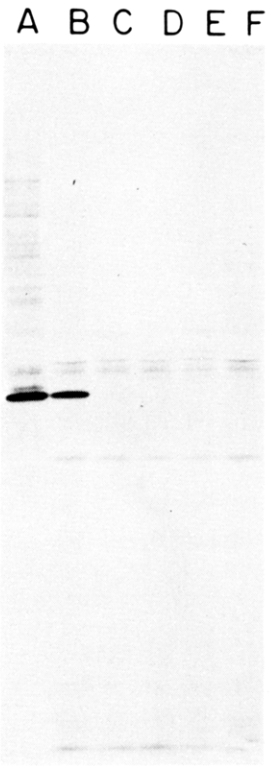

FIGURE 2: Gel electrophoresis of products of in vitro translation of mRNA from cultured cells. Translation reactions contained 0.2 A_{260} unit/mL poly(A) mRNA from Can^r cells (lanes B and C) or wild-type cells (lanes D and E). The products of the in vitro synthesis were reacted with immune serum (lanes B and D) or preimmune serum (lanes C and E). Lane F is identical with lane B except 22 μg of purified liver argininosuccinate synthetase was added to the extract prior to immunoprecipitation. Lane A was from an immunoprecipitation of in vivo labeled extract from Can^r cells for comparison. The gel was exposed for fluorography for 6 h. Incorporation of [^{35}S]-methionine was 4.08×10^4 cpm/μL for wild-type mRNA and 3.39×10^4 cpm/μL for Can^{r}1 mRNA, and 4.5×10^6 cpm of each reaction product was immunoprecipitated for each lane shown.

Can^r cell lines (lanes I and J), was detectable but faint for the wild-type cells grown in citrulline (lane H), and was not detected in the wild-type cells grown in arginine (lane G). Scintillation counting of the excised bands from the NaDodSO$_4$ gel analysis indicated that 0.07% of the radioactivity in the soluble extract from Can^{r}1 cells was in argininosuccinate synthetase. This value was not corrected for any losses and cannot be used to calculate the percent of cell protein which is argininosuccinate synthetase, since we do not know the methionine content of the enzyme. Comparison of lanes M, N, O, and P in Figure 1 suggested no definite change in the region where argininosuccinate synthetase would migrate in the analysis of crude extracts. If the half-life of the enzyme was similar in the different cell lines and conditions, and if the half-life was long compared to the 5-h pulse duration, the differences in the amounts of immunoprecipitated radioactive enzyme would indicate major differences in the rates of synthesis of the enzyme.

Translatable Argininosuccinate Synthetase mRNA Content in the Cultured Cells. While analysis of in vivo synthesized enzyme as shown in Figure 1 suggested that differences in synthesis were important, more direct evidence was sought by analysis of cellular mRNA. Poly(A) mRNA from both wild-type and Can^{r}1 cells was prepared, and the products of the in vitro translation were analyzed by immunoprecipitation and NaDodSO$_4$ gel electrophoresis as shown in Figure 2. When similar amounts of poly(A) mRNA from wild-type cells and Can^{r}1 cells were added to synthesis reactions, equivalent amounts of [^{35}S]methionine were incorporated into proteins. An intense band of immunoprecipitable product was found with the mRNA from Can^{r}1 cells but not with the mRNA from wild-type cells (land B vs. lane D, Figure 2). The immunoprecipitated products from the in vitro translation (lane B) migrated identically with the products from the in vivo synthesis (lane A). The similar migrations of the in vitro and in vivo synthesized enzymes provided no evidence for a higher molecular weight precursor of the enzyme in this cell line. The specificity of the result was demonstrated in lane F (Figure 2), where purified human liver argininosuccinate synthetase was used to compete with the radiolabeled enzyme and displace it from the immunoprecipitate. The radioactivity in the region of the gel corresponding to argininosuccinate synthetase was quantified by scintillation counting. The immunoprecipitable radioactivity with poly(A) mRNA from Can^{r}1 cells was greater than 3000 cpm (3760 cpm in lane B minus 328 cpm in lane C) while that with poly(A) mRNA from wild-type cells was not measurable (185 cpm in lane D minus 198 cpm in lane E). The immunoprecipitable radioactivity represented 0.1% of the total with Can^{r}1 poly(A) mRNA, and this proportion was similar to that for in vivo incorporation with Can^{r}1 cells. In an experiment not shown, an equal mixture of poly(A) mRNA from wild-type cells and Can^{r}1 cells gave an intermediate level of synthesis of the enzyme, indicating that neither inhibition nor nuclease activity explained the absence of synthesis with the mRNA from wild-type cells. These data demonstrated that the Can^{r}1 cell line contained a major increase in translatable poly(A) mRNA for argininosuccinate synthetase and that the increase was quantitatively compatible with the differences in enzyme activity.

Sucrose-Gradient Analysis of Argininosuccinate Synthetase mRNA. The poly(A) mRNA fraction from the canavanine-resistant cells was further fractionated on a 10–30% sucrose gradient containing 0.5% NaDodSO$_4$. The bulk of the argininosuccinate synthetase mRNA was found in fraction 8 (Figure 3) when the products of the in vitro translation were immunoprecipitated and analyzed. This location in the gradient indicated that the mRNA for argininosuccinate synthetase has a sedimentation value of approximately 16 S.

Discussion

The experiments presented here are in good agreement with previous work. The substitution of citrulline for arginine in the culture medium for growth of RPMI 2650 cells caused an increase in argininosuccinate synthetase but not in argininosuccinate lyase, similar to previous observations with human lymphoblasts (Irr & Jacoby, 1978). These results differ from previous observations with HeLa and KB cells, where both the synthetase and lyase enzymes increased in activity when cells were grown in citrulline. Canavanine-resistant variants of RPMI 2650 were isolated readily, as occurred with human lymphoblasts (Irr & Jacoby, 1978). In both lymphoblasts and RPMI 2650, the resistance to canavanine was associated with a marked increase in argininosuccinate synthetase activity and with a loss of metabolite regulation. The data presented here provide new information about the underlying mechanism of alterations in argininosuccinate synthetase activity. Immunological determinations revealed steady-state increases of argininosuccinate synthetase both in wild-type cells grown in citrulline and in Can^r cells. Immunoprecipitation of [^{35}S]methionine-labeled cells suggested an increase in newly synthesized enzyme in the variant cells. This was confirmed by demonstration of increased translatable poly(A) mRNA for argininosuccinate synthetase in Can^r cells. All of these observations support the conclusion

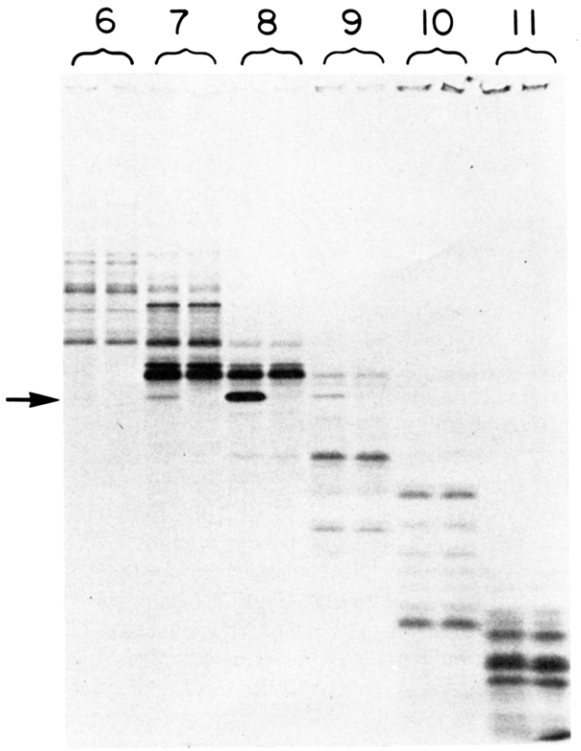

FIGURE 3: Sucrose-gradient fractionation of poly(A) mRNA from Can^{r}1 cells. Products of in vitro translation were prepared as described under Materials and Methods, and 3×10^6 cpm of precipitable material was applied to each lane for gel analysis. The numbered pairs of lanes correspond to the gradient fraction reacted with either immune serum (left) or preimmune serum (right). The arrow indicates the position of in vivo synthesized argininosuccinate synthetase. Fluorography was for 14 h. Fractions 6–11 include that portion of the gradient containing molecules with a sedimentation coefficient of 20–10 S.

that canavanine resistance is associated with increased synthesis of argininosuccinate synthetase.

Three of the urea-cycle enzymes from mammalian sources now have been synthesized in vitro. We could find no evidence for a significantly larger precursor for argininosuccinate synthetase as was observed for carbamoyl-phosphate synthetase (Raymond & Shore, 1979) and for ornithine transcarbamylase (Conboy et al., 1979). Thus, we do not find a precursor for a known cytosolic enzyme, while the two enzymes for which precursors were found are located in the mitochondria.

The exact mechanism of enzyme overproduction by canavanine-resistant cells requires further study. Gene amplification as a mechanism of enzyme overproduction and drug resistance has been observed for dihydrofolate reductase (Schimke et al., 1978) and for the early steps in the pyrimidine biosynthetic pathway (Wahl et al., 1979). Gene amplification is known to occcur physiologically for ribosomal RNA in amphibians (Brown & Dawid, 1968; Gall, 1968) and for chorion proteins during oogenesis in *Drosophila* (Spradling & Mahowald, 1980). It should be noted from our data that the level of enzyme activity found in the Can^r cells was within the range of activity found for normal liver. At this time, it is not known if the Can^r cells achieve their level of overproduction by a similar or different mechanism than that which accounts for high levels of enzyme activity in liver. It is not known if gene amplification is involved, but data on gene dosage in the various cells should answer that question definitively.

Acknowledgments

We thank Aesta Manschreck, Ray Barr, and Joe Gatto for technical assistance and Dr. Georg Bock, who provided help to modify the argininosuccinate synthetase assay. We also thank Drs. Jeffery M. Rosen and Robert J. Schwartz for helpful discussions and review of the manuscript.

References

Allen, E. H., & Schweet, R. S. (1962) *J. Biol. Chem. 237*, 760–767.
Aviv, H., & Leder, P. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 1408–1412.
Bonner, W. M., & Laskey, R. A. (1974) *Eur. J. Biochem. 46*, 83–88.
Brown, D. D., & Dawid, I. B. (1968) *Science (Washington, D.C.) 160*, 272–280.
Conboy, J. G., Kalousek, F., & Rosenberg, L. E. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 5724–5727.
Del Giudice, R. A., & Hopps, H. E. (1978) in *Mycoplasma Infection of Cell Cultures* (McGarrity, G. J., Murphy, D. G., & Nichols, W. W., Eds.) pp 57–69, Plenum Press, New York.
Gall, J. G. (1968) *Proc. Natl. Acad. Sci. U.S.A. 60*, 553–560.
Hay, R., Lavappa, K. S., Macy, M., Shannon, J., & Williams, C. (1979) in *Catalogue of Strains II*, 2nd ed., p 28, American Type Culture Collection, Rockville, MD.
Hayflick, L. (1965) *Tex. Rep. Biol. Med. 23*, 285–303.
Hudson, L. D., Erbe, R. W., & Jacoby, L. B. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 4234–4238.
Irr, J. D., & Jacoby, L. B. (1978) *Somatic Cell Genet. 4*, 111–124.
Jacoby, L. B. (1978) *Somatic Cell Genet. 4*, 221–231.
Kessler, S. W. (1975) *J. Immunol. 115*, 1617–1624.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Levine, L. (1978) *Handb. Exp. Immunol. (3rd Ed.)*, 5.B.1.
Moorhead, P. S. (1965) *Exp. Cell Res. 39*, 190–196.
Nuzum, C. T., & Snodgrass, P. J. (1971) *Science (Washington, D.C.) 172*, 1042–1043.
O'Brien, W. E. (1979) *Biochemistry 18*, 5353–5356.
Pelham, H. R. B., & Jackson, R. J. (1976) *Eur. J. Biochem. 67*, 247–256.
Ratner, S., & Kunkemueller, M. (1966) *Biochemistry 5*, 1821–1832.
Raymond, Y., & Shore, G. C. (1979) *J. Biol. Chem. 254*, 9335–9338.
Schimke, R. T. (1962) *J. Biol. Chem. 237*, 459–468.
Schimke, R. T. (1964) *J. Biol. Chem. 239*, 136–145.
Schimke, R. T., Kaufman, R. J., Alt, F. W., & Kellems, R. F. (1978) *Science (Washington, D.C.) 202*, 1051–1055.
Spradling, A. C., & Mahowald, A. P. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 1096–1100.
Wahl, G. M., Padgett, R. A., & Stark, G. R. (1979) *J. Biol. Chem. 254*, 8679–8689.