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The determination by radiochemical assay of argininosuccinase produced in an *Escherichia coli* system *in vitro*

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A highly sensitive radiochemical assay used to measure the synthesis and regulation of the product of the *argH* gene, argininosuccinase, in an *Escherichia coli* system *in vitro* is described. With L-[*guanidino*-^{14}C]argininosuccinic acid as a substrate, and in the presence of excess arginase and urease, $^{14}CO_2$ is collected in a simply designed micro-vessel. With this method less than 1 nmol of product can be measured in the presence of various concentrations of L-arginine.

The *argECBH* gene cluster in *Escherichia coli* is divergently transcribed from an internal control region between *argE* and *argC*. The major control of this cluster appears to be by a repressor protein that acts together with arginine or its derivative, although evidence with whole-cell studies suggests that a second site control might exist (Krzyzek & Rogers, 1976*b*). In our studies of the regulation of the right wing of the *argECBH* gene cluster in *E. coli* by using a transcription–translation system *in vitro*, we found the previous methods for assay of the *argH* enzyme, argininosuccinase, (L-argininosuccinate arginine-lyase, EC 4.3.2.1), to be inadequate.

Colorimetric assays for argininosuccinase are based on assay of the product, arginine, after its conversion by adding arginase into urea and formation of a chromophore with a high absorption coefficient (Hunninghake & Grisolia, 1966; Prescott & Jones, 1969; Geyer & Dabich, 1971). These assays cannot be used effectively in the situation that we have been studying because of the high and variable concentrations of free arginine in the cell extracts and the very low concentrations of enzyme to be assayed. Owing to those problems, we were unable to duplicate the data of Kelker *et al.* (1976), where cell-free synthesis of argininosuccinase was determined by a colorimetric method.

Three different types of radiochemical assays have been developed for or applied to the measurement of argininosuccinase activity. Schimke (1970) assayed enzyme activity with [*guanidino*-^{14}C]-argininosuccinate in the presence of excess arginase; after addition of urease in a second step, $^{14}CO_2$ was determined. Analogous assays for arginase (Righetti *et al.*, 1968; Klein & Morris, 1978) employed the collection of $^{14}CO_2$ and used [*guanidino*-^{14}C]arginine as a substrate. In a second type of method, *Kato et al.* (1976) added [^{14}C]fumarate and arginine. The product, [^{14}C]argininosuccinate, was separated from the [^{14}C]fumarate by adsorption and elution from a Dowex 50 column. In a third method, Ratner & Murakami-Murofushi (1980) assayed activity with [*arginino*-U-^{14}C]argininosuccinate and excess arginase, and measured both [U-^{14}C]ornithine and [^{14}C]urea as products that were eluted together and separated from the [^{14}C]argininosuccinate on a small Dowex 1 (acetate form) column. In an analogous assay for arginase, Ruegg & Russell (1980) removed [^{14}C]arginine in the second step with a sulphonate resin, and the [^{14}C]urea product was determined in the resin supernatant.

The radiochemical method described in the present paper is a simple microassay applicable to the micro-volumes and high sensitivity needed to determine the small amount of argininosuccinase produced under conditions of repression and derepression in the cell-free system. The assay is based on the method of Schimke (1970). [*guanidino*-^{14}C]Argininosuccinate, in the presence of excess added arginase, is converted into [^{14}C]urea, and after a second-stage incubation in a micro-vessel collected $^{14}CO_2$ is determined. This method allows quantitative determination of less than 1 nmol of arginine produced from radiolabelled argininosuccinate in the presence of various high concentrations of unlabelled arginine.

Materials and methods

Materials

L-[*guanidino*-^{14}C]Arginine (23 mCi/mmol) was obtained from New England Nuclear. The Dowex 50W X8 (200–400 mesh; H^+ form) resin was

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obtained from the Sigma Chemical Co. and was washed with 3 times its volume of water and with 2 times its volume of 10mM-HCl before use. Arginase (L-arginine amidinohydrolase, EC 3.5.3.1) was purchased from Worthington Biochemical Corp. and was suspended to 10mg/ml in 0.25M-potassium phosphate buffer, pH7.5. Urease (urea amidohydrolase, EC 3.5.1.5) (type VI) from jack beans was purchased from Sigma Chemical Co. All other chemicals were of the highest purity available.

Synthesis and purification of L-[*guanidino*-^{14}C]-*argininosuccinic acid*

[*guanidino*-^{14}C]Argininosuccinic acid was synthesized by a micro modification of methods previously reported by Ratner (1957) and Ratner & Kunkemueller (1966), with the use of [*guanidino*-^{14}C]arginine in a reversal of the argininosuccinase reaction. Commercial preparations of [*guanidino*-^{14}C]arginine are often contaminated with trace amounts of urea, which is later removed during the purification of [^{14}C]argininosuccinic acid. In a final volume of 0.2ml were added 250μCi of [*guanidino*-^{14}C]arginine (10μCi/μmol), 25μmol of potassium fumarate and 20 units (μmol of arginine/h) of a preparation of bovine liver argininosuccinase (150units/mg of protein), which was purified to the heat step at pH5.1 (Ratner, 1970). The reaction was allowed to reach equilibrium by incubation for 3h at 37°C. The reaction was stopped by addition of 0.05ml of cold 50% (w/v) trichloroacetic acid, and the protein was removed by low-speed centrifugation. The trichloroacetic acid was removed by four extractions with equal volumes of anhydrous diethyl ether. $BaCl_2$ (1.0M) was added to a final concentration of 0.25M, and the white $Ba_3(PO_4)_2$ precipitate was removed by centrifugation at 4°C. After adjustment to pH9 with saturated $Ba(OH)_2$, the [*guanidino*-^{14}C]argininosuccinic acid was precipitated by adding 2.5vol. of cold 95% (v/v) ethanol and leaving the mixture to stand for 1h at 4°C. The precipitate was collected by centrifugation and washed successively with 75%, 95% and 100% ethanol and diethyl ether at 4°C. The precipitate was dissolved in water and again precipitated as above without pH adjustment.

Contaminating fumarate and any traces of urea were removed on a 0.9cm × 4.0cm cation-exchange column of Dowex 50W X8 (200–400 mesh; H^+ form) (Kato *et al.*, 1976). The column was washed with 5.0ml of 10mM-HCl and 5.0ml of water, and argininosuccinate was eluted with 15ml of 1.0M-pyridine. [*guanidino*-^{14}C]Argininosuccinic acid was converted into the potassium salt by addition of K_2SO_4. The final preparation contained typically 100–120μCi and 10–12μmol of argininosuccinate with a final yield of approx. 40% of the [^{14}C]arginine originally added. The potassium [^{14}C]argininosuccinate was stored in 0.5ml of water adjusted to pH9.0 with KOH at −20°C, to minimize conversion into the anhydride I form (Ratner & Kunkemueller, 1966). Before use, the pH was adjusted to 7.5.

Cell-free synthesis of argininosuccinase

The cell-free transcription–translation system used for the synthesis of argininosuccinase was prepared by using an S-30 cell extract from *E. coli* and the necessary factors as described and modified by Zubay *et al.* (1970). Template DNA was derived from λ or ϕ80 bacteriophage carrying the *argECBH* gene cluster as described previously (Press *et al.*, 1971; Krzyzek & Rogers, 1976*a*). The incubation for enzyme synthesis *in vitro* was allowed to proceed at 37°C for 80min.

Assay of argininosuccinase activity in the cell-free transcription–translation system from Escherichia coli

After synthesis of argininosuccinase *in vitro*, a 40μl sample was added for enzyme assay to a 1.5ml plastic micro test tube. To this was added a 10μl solution containing 50μg of arginase (33units/mg) and 50nmol of potassium [*guanidino*-^{14}C]argininosuccinate to make a final volume of 50μl. The [^{14}C]argininosuccinate was adjusted to 0.5–5.0μCi/μmol with fresh unlabelled argininosuccinate, depending on the particular experiment. The plastic cover on the micro test tube was closed and the enzyme assay mixtures were incubated for various times at 37°C. For our standard assay the incubation time was for 18h, because of the small amount of enzyme present.

After the incubation, the assay mixture was transferred to a 1.5cm × 4.5cm disposable glass vial (Research Products International) sealed with a rubber sleeve stopper from which was suspended a straight-pin carrying a 2.0cm × 0.8cm piece of Whatman 3MM paper. The paper was wetted with 25μl of 5M-KOH, the system was sealed, and 50μl of urease (type VI) (2.0units/ml) was injected by hypodermic syringe into the assay mix through the rubber stopper. The urease reaction was allowed to go to completion at 37°C for 90min with moderate shaking. $^{14}CO_2$ release was completed by injecting 50μl of 1M-HCl followed by incubation with shaking at 37°C for at least 1h. Filter papers were then removed and air-dried. Similarly, the input radioactivity (c.p.m.) in the reaction was determined by spotting a 50nmol sample of [*guanidino*-^{14}C]-argininosuccinic acid on a folded filter paper wetted with 25μl of 5M-KOH and subsequently air-dried.

Measurement of radioactivity

Radioactivity was measured by placing the folded filter papers in 5ml of a dioxan/naphthalene scin-

tillation 'cocktail' and counting the radioactivity in glass vials in a Beckman LS-100 scintillation counter. The scintillation 'cocktail' contained: naphthalene, 375g; 2,5-diphenyloxazole, 23.5g; 1,4-bis-(*O*-methylstyryl)benzene, 0.48g; methanol, 200ml; dioxan, 3 litres.

Results and discussion

[^{14}C]Argininosuccinic acid was shown to be free of contaminating arginine by descending paper chromatography on Whatman 3MM paper with a phenol/water (6:1, w/v) solvent in the presence of NH_3 (Fig. 1). Arginine and argininosuccinic acid can be easily distinguished, with R_F values of 0.72 and 0.23 respectively. The chromatogram also shows the absence of significant anhydride I or II of argininosuccinate, since no radioactivity was found at R_F values 0.47 and 0.58 (Ratner *et al.*, 1953; Kato *et al.*, 1976). Enzymic assay showed that, after incubation of [^{14}C]argininosuccinate for 18h under our standard assay conditions in the absence of argininosuccinase, less than 2% of the substrate was converted into arginine and urea (Table 1).

A linear and quantitative recovery of $^{14}CO_2$ was obtained from 0.1nmol to 500nmol of added

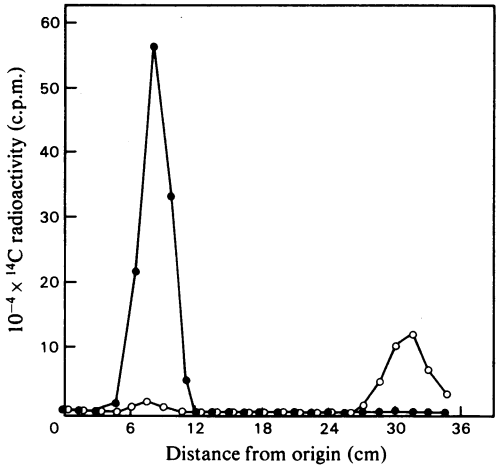

Fig. 1. *Paper chromatography of synthesized* [^{14}C]-*argininosuccinic acid*

Samples were spotted on Whatman 3MM chromatography paper and developed for 18h at 25°C by descending mode. The chromatogram was cut into 1.5cm sections and radioactivity was determined as described in the Materials and methods section. The solvent front was at 41cm. Symbols: ●, 1μl sample of purified barium [*guanidino*-^{14}C]-argininosuccinate (250μl total volume); ○, 10μl sample of pooled (7ml) ethanol supernatants containing mostly [^{14}C]arginine.

Table 1. *Micro assay for argininosuccinase*

The argininosuccinase produced by a cell-free extract of *E. coli* incubated for 18h with 400000c.p.m. of [*guanidino*-^{14}C]argininosuccinate at a final concentration of 1.0mM was assayed by using the system described in the Materials and methods section. $^{14}CO_2$ was collected and radioactivity determined as described in the text.

Incubation conditions	$^{14}CO_2$ produced (c.p.m.)
Complete system	132500
Plus 1.0mM-arginine	132300
Minus argininosuccinase	8000
Minus arginase	2000
Minus urease	500

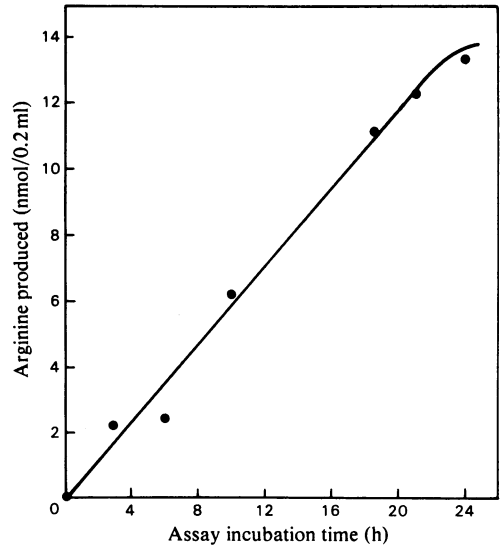

Fig. 2. *Time course of the assay of argininosuccinase produced in vitro*

Samples (40μl) of argininosuccinase synthesized *in vitro* in a crude *E. coli* extract were assayed under the conditions outlined in the Materials and methods section. At the times shown reactions were stopped by freezing at −70°C. After collection of the last sample, all were transferred to micro-vessels, and evolved $^{14}CO_2$ was collected and measured. The values shown (nmol of arginine/0.2ml) are calculated by assuming 1nmol of arginine per 1nmol of collected $^{14}CO_2$ and multiplied by 5, since 0.2ml was the size of the original enzyme-synthesis reaction.

[*guanidino*-^{14}C]argininosuccinic acid. We found that incubation in the micro-vessel with urease for 60min followed by an additional 60min with added HCl gave maximum release of $^{14}CO_2$. In our standard assay procedure incubation with urease was extended to 90min to ensure complete hydrolysis of urea.

Added L-arginine up to 1.0mM was found to have no effect on this assay for argininosuccinase activity (Table 1). This is due to the presence of excess arginase added to the reaction mixture, which converts all arginine into urea and ornithine.

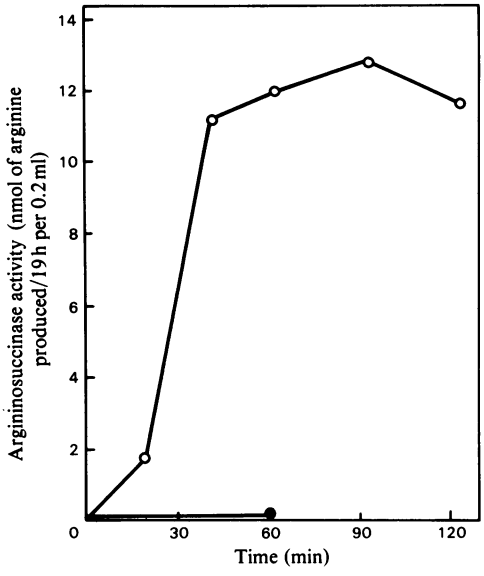

Fig. 3. *Progress of the synthesis of argininosuccinase in an E. coli system in vitro*

The synthesis mixture was incubated for 120min. At various times during synthesis samples (40μl) were removed and assayed for argininosuccinase activity (○). A reaction mixture was incubated without DNA as a control (●). Enzyme activity for argininosuccinase is plotted as nmol of arginine/19h per 0.2ml.

Argininosuccinase activity produced by the cell-free system of *E. coli* was found to remain constant for 24h at 37°C under these assay conditions (Fig. 2). A wide range of argininosuccinase activities can be detected owing to the high specific radioactivity of the [*guanidino*-^{14}C]argininosuccinate used and the sensitivity of the assay.

The synthesis of argininosuccinase in an *E. coli* system *in vitro* was monitored by the use of this radiochemical assay. With λ d*argECBH* DNA as a template, the time course of the synthesis of the *argH* gene product was monitored. Activity can be detected at low levels only 20min after the start of incubation (Fig. 3). Synthesis of the enzyme reaches a maximum after about 90min of incubation *in vitro*. Cell-free synthesis and repression of synthesis of argininosuccinase by arginine can be observed when S-30 cell extracts from *E. coli* containing the arginine repressor protein, the *argR* gene product, are used (Table 2). The problem of a high arginine background in the cell extract has been circumvented and the low enzyme activities observed under repressed conditions can be monitored. Indeed, we found that the presence of L-arginine (1mM) in the synthesis mixture affects the rate of synthesis of argininosuccinase by *argR*$^+$ extracts and not *argR*$^-$ extracts (Table 2), and this effect was not due to any influence of arginine on the rate of the argininosuccinase reaction, as was shown in Table 1.

Sensitive colorimetric assays (Hunninghake & Grisolia, 1966; Prescott & Jones, 1969; Geyer & Dabich, 1971) can effectively measure 10nmol of urea produced from arginine in the argininosuccinase–arginase couple. Ratner & Murakami-Murofushi (1980) evaluated their radiochemical assay for argininosuccinase as accurately measuring 5–10nmol of product formed during a 30min assay

Table 2. *Assay of argininosuccinase produced by cell-free synthesis*

Argininosuccinase was synthesized under various conditions in the *E. coli* S-30 system as described in the Materials and methods section. Enzyme activity was measured by the evolution of $^{14}CO_2$. Input of [*guanidino*-^{14}C]argininosuccinate per sample in Expt. A was 211000c.p.m. and in Expt. B 95000c.p.m. The values shown for $^{14}CO_2$ radioactivity (c.p.m.) are the averages of actual values of $^{14}CO_2$ obtained on paired samples.

		Enzyme activity per reaction mixture	
Cell extract	Incubation conditions	$^{14}CO_2$ (c.p.m.)	[^{14}C]Arginine (nmol)
Expt. A			
ArgR$^-$	+Arginine (0.5mM)	55700	15.2
ArgR$^-$	No arginine	45600	10.8
ArgR$^+$	+Arginine (0.5mM)	3400	0.8
ArgR$^+$	No arginine	106300	25
Expt. B			
ArgR$^-$	+ϕ80 d*argECBH* DNA	20500	10.8
ArgR$^-$	+ϕ80 DNA	1900	1.0
ArgR$^-$	No DNA	1500	0.8

period. These workers found a background interference by the anhydride I of [^{14}C]argininosuccinate, which was eluted from the columns together with the products, urea and ornithine. Under the standard conditions of the micro-assay reported in the present paper, 1.0nmol of arginine produced from argininosuccinic acid was easily measured as $^{14}CO_2$. By using [^{14}C]argininosuccinate without added carrier (23mCi/mmol), the sensitivity of this assay could theoretically be improved another 40-fold.

This present assay method measures the conversion of potassium L-[*guanidino*-^{14}C]argininosuccinate into $^{14}CO_2$ by using the coupling enzymes arginase and urease. Other assays employ the collection of $^{14}CO_2$ for the measurement of arginase (Righetti *et al.*, 1968; Mora *et al.*, 1972; Whitney & Magasanik, 1973; Klein & Morris, 1978) and argininosuccinase (Schimke, 1970) activity in eukaryotic cells. The assay described in the present paper measures bacterial argininosuccinase produced in a cell-free system in the presence of variable background arginine concentrations. With the use of micro-volumes and the high specific radioactivity of the substrate, we have described a convenient, sensitive and inexpensive method for studying the regulation of the *E. coli argH*-gene product.

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References

Geyer, J. W. & Dabich, D. (1971) *Anal. Biochem.* **39**, 412–417

Hunninghake, D. & Grisolia, S. (1966) *Anal. Biochem.* **16**, 200–205

Kato, H., Oyamada, I., Mizutani-Funahashi, M. & Nakagawa, H. (1976) *J. Biochem.* (*Tokyo*) **79**, 945–953

Kelker, N., Maas, W. K., Yang, H. L. & Zubay, G. (1976) *Mol. Gen. Genet.* **144**, 17–20

Klein, D. & Morris, D. R. (1978) *Biochem. Biophys. Res. Commun.* **81**, 199–204

Krzyzek, R. A. & Rogers, P. (1976*a*) *J. Bacteriol.* **126**, 348–364

Krzyzek, R. A. & Rogers, P. (1976*b*) *J. Bacteriol.* **126**, 365–376

Mora, J., Salceda, R. & Sanchez, S. (1972) *J. Bacteriol.* **110**, 870–877

Prescott, L. M. & Jones, M. E. (1969) *Anal. Biochem.* **32**, 408–419

Press, R., Glansdorff, N., Miner, P., DeVries, J., Kadner, R. & Maas, W. K. (1971) *Proc. Natl. Acad. Sci. U.S.A.* **68**, 795–798

Ratner, S. (1957) *Methods Enzymol.* **3**, 643–647

Ratner, S. (1970) *Methods Enzymol.* **17A**, 304–309

Ratner, S. & Kunkemueller, M. (1966) *Biochemistry* **5**, 1821–1832

Ratner, S. & Murakami-Murofushi, K. (1980) *Anal. Biochem.* **106**, 134–147

Ratner, S., Petrack, B. & Rochovansky, O. (1953) *J. Biol. Chem.* **204**, 95–113

Righetti, P., DeLuca, L. & Wolf, G. (1968) *Anal. Biochem.* **22**, 225–230

Ruegg, U. T. & Russell, A. S. (1980) *Anal. Biochem.* **102**, 206–212

Schimke, R. T. (1970) *Methods Enzymol.* **17A**, 324–329

Whitney, P. A. & Magasanik, B. (1973) *J. Biol. Chem.* **248**, 6197–6202

Zubay, G., Chambers, D. & Cheong, L. (1970) in *The Lactose Operon* (Beckwith, J. & Zipser, D., ed.), pp. 375–391, Cold Spring Harbor Laboratory, Cold Spring Harbor