

Studies on Seryl and Phenyl-alanyl Synthetases from Ascitic Tumor Cells Treated with 20-Methylcholanthrene (37668)

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It is well established that biological components required for the translation of mRNA (tRNA, aminoacyl-tRNA synthetases, ribosomes, and protein synthesis factors) exert a regulatory effect on protein synthesis. Because of the central role of tRNA in the translation of the genetic code it is possible that in higher organisms changes in the abundance of specific types of tRNA are associated with metabolic regulation, cellular differentiation, and neoplastic transformation.

In an extensive study of mammalian tissues, Taylor *et al.* (1) found that the column elution profiles of several tRNAs (phenylalanyl, seryl, glycyl, and tyrosyl) from Ehrlich tumor cells differed appreciably from those of the normal mouse organs. Yang and Novelli (2) have also described differences in the seryl-tRNA elution profiles between two plasma cell tumors. Goldman *et al.* (3) Muskinski *et al.* (4) and Gonano *et al.* (5) found differences in the tRNA profiles of the hepatoma cells as compared with normal cells.

The aminoacylation of tRNA is an important step in the regulation of protein synthesis. This is due to the high specificity of the reacting components of the systems, *e.g.*, tRNAs and the aminoacyl-tRNA synthetases. Therefore, it is not difficult to envisage that not only the tRNAs but also the aminoacyl-tRNA synthetases must have a regulatory function in the transformation of normal cells to neoplastic cells.

In our laboratory, the alteration of mammalian cells undergoing "spontaneous" malignant conversion has been studied *in vitro* (7-8). The modifications of the malignant properties of such cells which may be obtained

in vitro with various carcinogenic compounds are under investigation. In the present work our observations of changes in the aminoacyl-tRNA synthetases are reported.

Materials and Methods. Chemicals. 20-Methylcholanthrene (MCA) was purchased from Sigma. Uniformly labeled [I-¹⁴C]serine and phenylalanine were obtained from Radio Chemical Centre, Amersham, England, and had the following specific activities: 440 mCi/mmole, and 513 mCi/mmole or 492 mCi/mmole, respectively. Unlabeled amino acids, ATP, and Tris were obtained from Sigma. Double-distilled glycerol and glutathione were purchased from Merck and dithiothreitol from Calbiochem. The DEAE cellulose (DE 11) was obtained from Whatman, CM Sephadex from Pharmacia, and the Biogel HTP from Biorad.

tRNA. Isolation and purification of the total tRNA fraction in rat liver was performed as described by Yang and Novelli (6).

Cells. The C3H-L1a ascites tumor was established from a 67-month-old cultured C3H mouse cell line (C3H-L1) derived from normal adult C3H lung explants which apparently spontaneously converted into malignant cells after 6 mo of propagation *in vitro* (7, 8). These tumor cells grow equally well in culture and in the peritoneal cavity, but frequently regress after sc inoculation. Their isoimmunizing properties were demonstrated *in vitro* and *in vivo* in active protection experiments using inbred C3H mice preimmunized with subthreshold doses of viable tumor cells (8) injected both sc and ip. After treatment with MCA *in vitro* the cells were passed *in vivo* by weekly ip transplantations. Cells for experimental use were harvested in

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their exponential growth phase 3–4 days after transplantation.

Treatment with carcinogen. MCA was dissolved in acetone at a concentration of 1 mg/ml and added to 99 ml warm complete tissue culture medium (Fib. 14B) containing 20% fetal bovine serum and 80% of Eagle's minimum essential medium (9) modified as previously described (7, 8). This stock solution was prepared and kept in bottles wrapped in tinfoil and stored at 4° in darkness. After 1 week of storage, 1:10 dilutions were prepared in Fib. 14B, yielding a final concentration of 1 μ g carcinogen and 1 μ l acetone per ml Fib. 14B.

MCA containing Fib. 14B was added to monolayer cultures of C3H-L1a cells in their 57th *in vitro* passage. Control cultures received Fib. 14B with acetone alone. The media were renewed daily. After 1 wk all cultures were continued in normal Fib. 14B without carcinogen and acetone for a few more passages. During this period of rehabilitation all signs of cell damage disappeared. After complete recovery the cells were inoculated ip into 3-mo-old C3H mice, in which they produced ascites tumors at doses of 5×10^6 cells.

Experimental. The method of enzyme isolation used by us was based on the work of

LeMur (10, personal communication), but with slight modification. The cells were homogenized in 0.05 M KCl containing 1 mM glutathione, $(\text{NH}_4)_2\text{SO}_4$ was added to the 105,000g supernatant and the precipitate between 45% and 65% saturation (calculated at 20°) was obtained. The precipitate was dissolved in 0.01 M K- PO_4 buffer, pH 7.5, with 0.1 mM EDTA, 1 mM glutathione, 10% glycerol, and desalted on G 25 Sephadex column (1.7 \times 35 cm) equilibrated in the same buffer.

Chromatography on DEAE cellulose. The excluded protein peak from the Sephadex column was applied on DEAE cellulose (Whatman DE 11) column (3 \times 20 cm) equilibrated in a phosphate buffer as above with 0.1 mM dithiothreitol instead of glutathione. Elution was performed with a linear K- PO_4 gradient with phosphate concentration increasing from 0.02 to 0.25 M and pH decreasing from 7.5 to 6.5 (Fig. 1).

Phenylalanyl and seryl-tRNA synthetases were eluted from this column between 0.07–0.13 M K- PO_4 . The active enzyme fractions from the DEAE cellulose column were pooled and diluted to 0.02 M with respect to K- PO_4 .

Chromatography on CM Sephadex C-50. The pooled enzyme fractions from the DEAE column were applied on CM Sephadex C-50

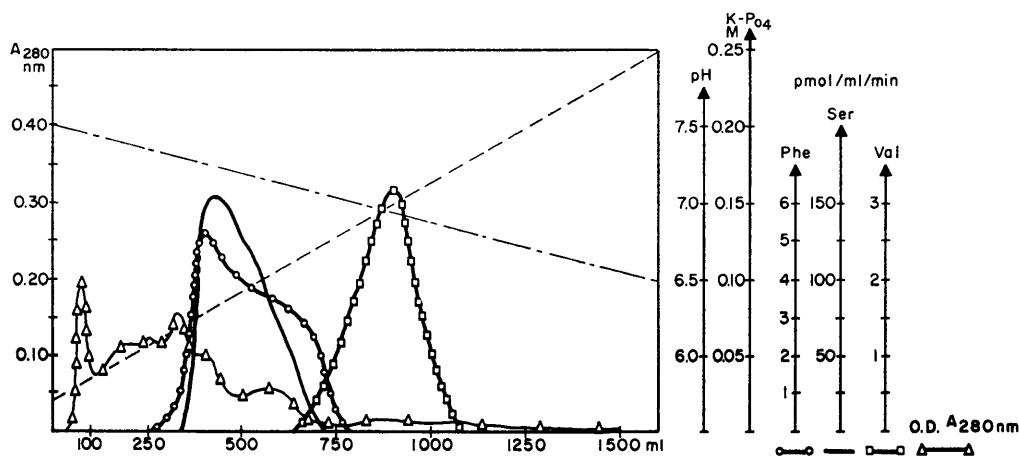


FIG. 1. Chromatography on DEAE cellulose (3 \times 20 cm) column of the ammonium sulphate (45–65%) fractionated extract from the MCA-treated tumor cells from C3H mice. Eluted with a linear K- PO_4 and pH gradient as shown in the figure, with a flow rate of 80 ml/hr. The buffer contained 0.1 mM EDTA, 0.1 mM dithiothreitol, and 10% glycerol.

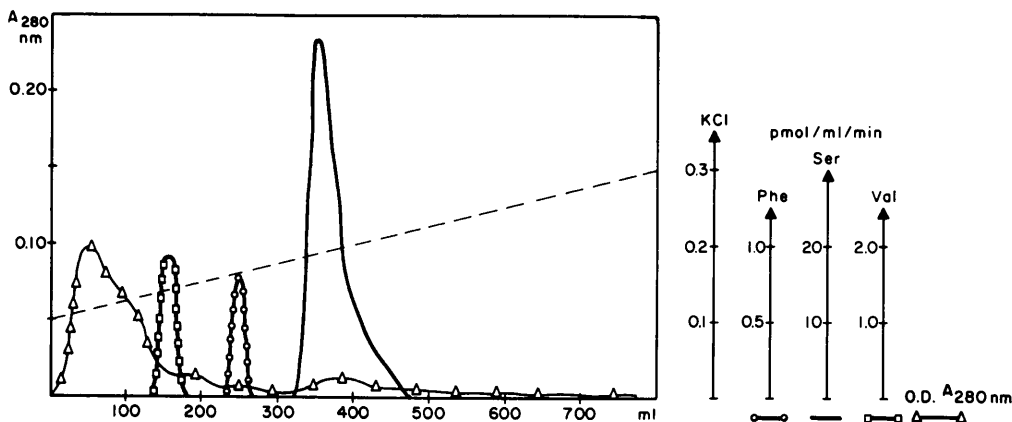


FIG. 2. Chromatography on CM Sephadex C-50 (3×30 cm) of the pooled active synthetase fraction from the DEAE cellulose column. The fraction was eluted with a linear KCl gradient as shown in the figure. The buffer contained $0.02 M$ $K-PO_4$ (pH 6.5), $0.1 mM$ EDTA, $0.2 mM$ dithiotheritol, and 10% glycerol. Flow rate was 40–60 ml/hr.

column equilibrated with $0.02 M$ $K-PO_4$ (pH 6.5) containing $0.1 mM$ EDTA, $0.2 mM$ dithiotheritol, and 10% glycerol. Elution was performed with a linear KCl gradient from 0.1 – $0.3 M$ in a $K-PO_4$ buffer of pH 6.5 (11) (Fig. 2).

The seryl- and phenyl-alanyl-tRNA synthetase peaks came out between 0.17 – 0.21 and 0.15 – $0.17 M$ KCl.

Chromatography on hydroxyapatite. The pooled fractions of each individual enzyme were applied on a small (1.5×5 – 7 cm) hydroxyapatite column. The column was

equilibrated with $0.02 M$ $K-PO_4$ at pH 6.5 containing $0.1 mM$ EDTA, $0.5 mM$ dithiotheritol, and 10% glycerol.

The seryl-tRNA synthetase was eluted with a double linear gradient with respect to $K-PO_4$ concentration (0.02 – $0.45 M$) and pH (6.5 – 7.5).

The seryl-tRNA synthetase was eluted from the hydroxyapatite column between 0.13 – $0.17 M$ $K-PO_4$ (Fig. 3).

Table I gives the different steps of the purification of the seryl-tRNA synthetase from the MCA-treated cells. The enzyme was

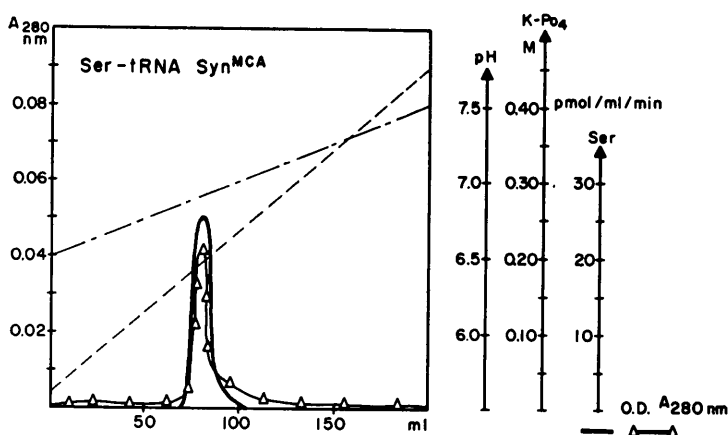


FIG. 3. Chromatography of seryl synthetase on hydroxyapatite (1.5×5 cm) after CM Sephadex C-50. The fraction was eluted with a double linear $K-PO_4$ gradient with respect to PO_4 concentration and pH. The buffer contained $0.1 mM$ EDTA, $0.5 mM$ dithiotheritol, and 10% glycerol.

TABLE I. Purification of Ser-tRNA Synthetase from MCA-Treated Ascites Tumor Cells/C3H Mice.

	Protein (mg)	Specific activity (μ^a /mg)	Total act. (μ^a)	Recovery (%)	R 280/260
Particle-free supernatant	2520	33.0	83550	100	0.76
	4260	28.5	121000	100	0.88
	2300	31.0	71200	100	0.89
AMS, ^b 45-65%	765	95.5	73050	85	0.81
	1075	89.5	96050	79	0.88
	570	92.5	52650	74	0.90
G-25	486	126.5	61550	74	0.94
	718	113.5	81650	64	0.94
	383	119.5	45810	64	0.95
DEAE-cellulose	9.6	2260	21700	26	1.48
	19.1	2165	43050	35	1.51
	6.1	2475	15100	21	1.50
Phosphocellulose	0.2	5690	1285	16	1.75
	0.4	4875	2060	17	1.75
CM Sephadex C50	0.2	5300	1060	14.8	1.74
Hydroxyapatite	0.05	11805	595	0.7	1.84
	0.06	11295	670	0.6	1.87
	0.04	11390	405	0.6	1.85

^a 1 μ = 1 pmole/min purified rat liver tRNA.

^b AMS = Ammonium sulphate saturation.

400-fold purified in terms of specific activity, and was stored in 50% glycerol at -20° .

Table II gives the different steps of the purification of seryl-tRNA synthetase from the acetone-treated cells. The enzyme was 500-560-fold purified.

The phenylalanyl-tRNA synthetase was eluted under the same conditions from the hydroxyapatite column between 0.23-0.27 *M* K_2PO_4 . A 550-600-fold purification of phenylalanyl-tRNA synthetase from the MCA-treated cells and 500-550 from the control cells was obtained.

Determination of the activity of seryl-tRNA synthetase: aminoacylation of tRNA. The activity of the enzymes of the various steps of purification was determined by measuring the amount of ^{14}C -labeled amino acid fixed to rat liver tRNA.

Each test was carried out in a volume of 0.25 ml of a solution containing 50 mM Tris (pH 7.5), 50 mM KCl, 4 mM Mg acetate, 4 mM ATP, 2 mM dithiothreitol, 20 μM ^{14}C -labeled amino acid (0.25 μCi /assay). All the

assays were carried out in the same medium with the exception of the concentration of Mg acetate which was sometimes 8 mM.

After incubating the reaction mixture at 30° for 30 min, 100 μ liter of the reaction mixture was applied on Whatman 3 mm paper discs. The discs were immersed in a solution of trichloroacetic acid 10% at 4° . After 15 min of precipitation the discs were washed twice in 5% trichloroacetic acid at 4° , and once in 95% cold ethanol. The discs were dried and the [^{14}C]aminoacyl-tRNA was counted in a Beckman liquid scintillation counter CPM 200 in the presence of a scintillation mixture composed of 4 g of PPO per liter of toluene.

The unit of activity was determined by the amount of enzyme that catalyzed the formation of 1 pmole of aminoacyl-tRNA per min at 30° .

Kinetic studies. Using 50 mM Tris buffer the optimal conditions for the determination of K_m values were found to be as follows: 1) K_m for seryl-tRNA synthetase from MCA-

TABLE II. Purification of Ser-tRNA Synthetase from Control (Acetone-Treated) Ascitic Tumor Cells.

	Protein (mg)	Specific activity (μ^a /mg)	Total act. (μ^a)	Recovery (%)	R 280/260
Partiele-free supernatant	2739	20.0	54000	100	0.80
	3520	21.0	73750	100	0.83
AMS, ^b 45-65%	550	69.5	38250	71	0.85
	668	70.0	46800	68	0.90
G-25	362	95	34050	63	1.12
	413	105	43625	59	1.20
DEAE-cellulose	7.5	2105	15775	29	1.60
	10	2170	21950	30	1.65
CM Sephadex C50	0.2	4515	8150	15.1	1.81
	0.2	1930	10390	14.1	1.84
Hydroxyapatite	0.02	9670	205	0.4	1.86
	0.02	10035	220	0.3	1.88

^a 1 μ = 1 pmole/min tRNA from yeast.

^b AMS = Ammonium sulphate saturation.

treated cells: pH 7.0, 4 mM Mg acetate, 4 mM ATP, 20 mM KCl, 80 μ M [¹⁴C]serine, 4.0 A₂₆₀ tRNA, and 2 mM dithiotheritol; 2) seryl-tRNA synthetase from control cells: as above with the exception of the concentration of Mg acetate which was 8 mM; 3) phenylalanyl-tRNA synthetase from MCA-treated cells: pH 8.0, 8 mM Mg acetate, 4 mM ATP, 40 mM KCl, 80 μ M [¹⁴C]phenylalanine, 4 A₂₆₀ tRNA, and 2 mM dithiotheritol; and 4) phenylalanyl-tRNA synthetase from control cells: treated as in the case of MCA-treated cells with the exception of the concentration of Mg acetate which was 4 mM. The results obtained are shown in Figs. 4-5.

Figure 4a gives the Lineweaver-Burk plot of amino acylation at 30° of purified total liver tRNA with purified seryl-tRNA from control ascitic tumor cells with different concentrations of Ser as substrate. Figure 4b gives the corresponding plot with the seryl synthetase from MCA-treated cells. Figures 5a and b give the Lineweaver-Burk plot for aminoacylation at 30° for different concentrations of ATP as variable substrate. Figure 6 gives the Lineweaver-Burk plot of aminoacylation at 30° of purified total liver tRNA with purified seryl synthetase from (a) control ascitic tumor cells, and (b) MCA-

treated cells with different concentrations of liver tRNA as variable substrate, at 2 different concentrations of ATP, 4 mM and 0.5 mM.

The K_m apparent and V_{max} values for (1) control cells are 24.5×10^{-7} M and 450 pmole/ml/min (4 mM ATP) and 75×10^{-8} M and 160 pmole/ml/min (0.5 mM ATP), and (2) MCA-treated cells, 11.9×10^{-8} M and 11 pmole/ml/min (4 mM ATP) and 8.4×10^{-8} M, and 9 pmole/ml/min (0.5 mM ATP).

The K_m values for Ser as substrate was determined at an ATP concentration of 4 mM which was markedly inhibitory in the case of control cells and slightly inhibitory in the case of MCA-treated cells.

Discussion. The isolation and the characteristics of the seryl-tRNA synthetase have been studied in the other systems by several authors: Katze and Koningsberg (12) from *E. coli*, Heider *et al.* (13) from yeast and LeMur (10) from hen's liver.

In the purification of the seryl-tRNA synthetase it seems the enzyme is isolated completely (hydroxyapatite column) and the purification fold was about 450 times. Marianne LeMur (10) obtained a purification of 950 times from hens' liver while Heider obtained a purification fold of 2000 times from

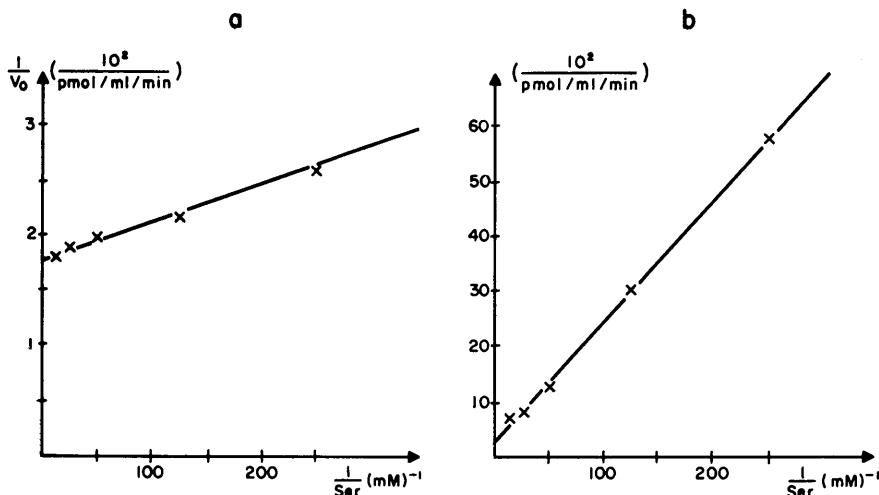


FIG. 4a, b. Lineweaver-Burk plot of aminoacylation at 30° of purified total tRNA with purified seryl synthetase from (a) control ascitic tumor cells, and (b) MCA-treated cells. Each reaction mixture (0.5 ml) contains per ml: 50 μ moles Tris (pH 7.0), 4 μ moles of Mg acetate, 4 μ moles of ATP, 4 A_{260} units tRNA, 2 μ moles dithioeritol, 20 μ moles of KCl, the concentration of the serine as shown and 100–200 mg of enzyme. Each point represents the mean of the initial velocities of 2 different reactions. After 3, 4, and 5 min, 0.1 ml of the reaction mixture was removed and applied on Whatman 3 mm filter discs and dried for 20 sec in a cold air stream and then precipitated in 10% cold trichloroacetic acid. K_m apparent for serine was 1.6 μ M for the control cells and 31 μ M for the MCA-treated cells: V_{max} was 56 pmole/ml/min for the control cells and 20 pmole/ml/min for the MCA-treated cells.

yeast.

From the V_{max} values (Table III) and the enzyme concentration the turnover number was calculated. We assumed that the enzyme seryl-tRNA synthetase was a pure protein and the mol wt of the enzyme was 120,000. The turnover numbers for serine and ATP were in the range of 150, *i.e.*, 150 molecules of substrate are reacting per min per mole of enzyme, assuming that all enzyme molecules are active. The turnover number of the seryl-tRNA synthetase from the control cells and the MCA-treated cells were the same for two of the substrates, ATP and serine.

When tRNA is the substrate there is a difference in turnover number by a factor 10 for the two seryl-tRNA synthetases at 4 mM ATP and a factor of 5 at 0.5 mM ATP. At those two concentrations of ATP there is a difference in K_m for tRNA as substrate by a factor of 20 and 10, respectively. This difference in the K_m values might be due to change in activity, but as there are differences in the affinity for serine and ATP, the differences in

K_m for tRNA are probably due to differences in affinity for tRNA.

The K_m values for tRNA as substrate are assumed to be true at 0.5 mM ATP because of inhibition at 4 mM ATP, and even if 0.5 mM ATP is at subsaturating concentration, the K_m values at 0.4 mM are supposed to be far from the limiting K_m values. At 0.05 mM ATP the K_m value for tRNA is about 20 times greater for the seryl-tRNA synthetase from control cells than from MCA-treated cells.

In the case of a rather strong binding to enzymes possessing low turnover numbers (14), K_m values should closely approximate the equilibrium dissociation constants (K_d values). In this case the K_m values (75 and 9.4×10^{-8} M) for tRNA as substrate indicates that the tRNA is bound more tightly to the synthetase from the MCA-treated cells than to the synthetase from control cells.

According to Bender *et al.* (15) differences in K_m values under these conditions will indicate differences in substrate affinity. The

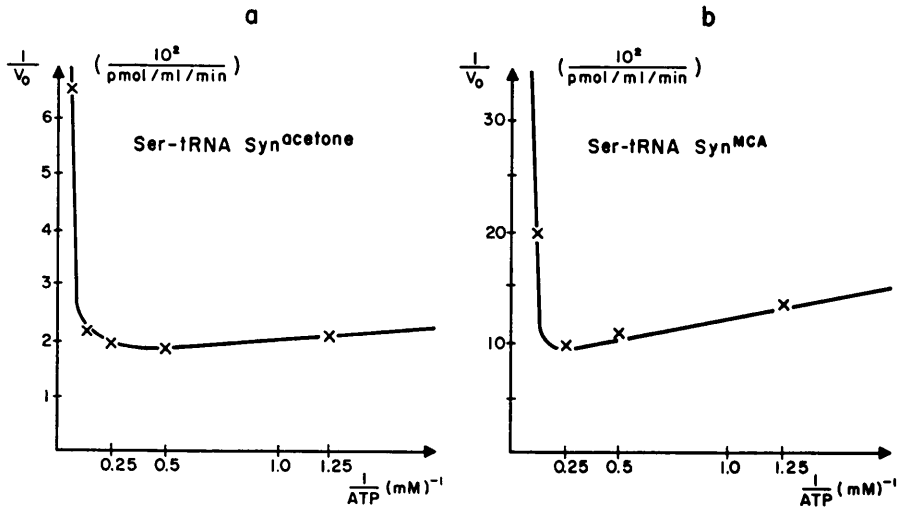


FIG. 5a, b. Lineweaver-Burk plot of aminoacylation at 30° of purified total liver tRNA with purified seryl synthetase from (a) control ascitic tumor cells and (b) MCA-treated cells, with different concentrations of ATP as variable substrate. The reaction conditions, except for the concentration of [¹⁴C]serine (80 nmoles) and the sampling procedure are exactly as those given in the text of Fig. 4. The K_m apparent and V_{max} values for control cells (a) and MCA-treated cells (b) are respectively 0.19 mM, 59 pmole/ml/min, and 0.72 mM, 14 pmole/ μ l/min.

present studies have shown that K_m for serine was about 20 times higher in MCA-treated cells than in the controls, while K_m for ATP was only increased 3–4 times.

These values indicate that the synthetases possibly have a regulatory role in the protein synthesis of the cancer cells with and without treatment with MCA. Similar differences

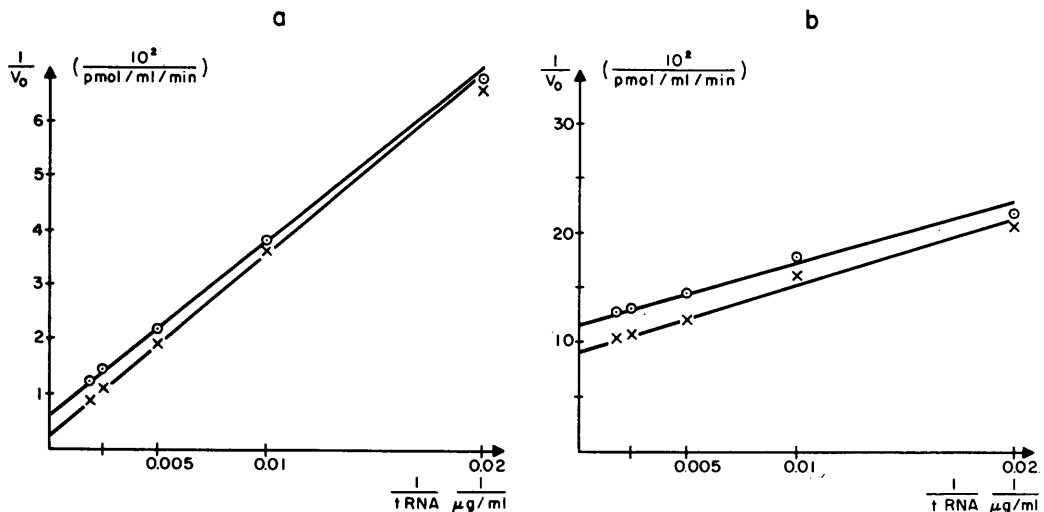


FIG. 6a, b. Lineweaver-Burk plot of aminoacylation at 30° of purified total liver tRNA with purified seryl synthetase from (a) control ascitic tumor cells, and (b) MCA-treated cells with different concentrations of tRNA as variable substrate. The reaction conditions and the sampling procedure are the same as described in the legend of Fig. 4 except for the concentration of [¹⁴C]serine (80 nmoles), ATP (4 mM) and (0.5 mM) and the different concentrations of tRNA.

TABLE III. K_m Apparent and V_{max} Values for the Seryl Synthetase of (1) Control Cells, and (2) MCA-Treated Cells, by Amino Acylation of Rat Liver tRNA.^a

K_m app		Seryl-synthetase (acetone)	Seryl-synthetase (MCA)
V_{max} app			
Serine	μM	1.6	31
	pmole/ml/min	56	20
ATP	mM	0.19	0.72
	pmole/ml/min	59	14
tRNA ^{ser} (4 mM ATP)	$\mu g/ml$	1400	68
	pmole/ml/min	—	—
	$10^{-8} M$	245	11.9
tRNA ^{ser} (0.5 mM ATP)	pmole/ml/min	450	11
	$\mu g/ml$	500	48
	pmole/ml/min	—	—
	$10^{-8} M$	75	8.4
	pmole/ml/min	160	9

^a The reaction is as follows: —50 mM Tris (pH 7.0), 2 mM dithiothreitol, 20 mM KCl, 4 mM Mg acetate (control cells), 8 mM Mg acetate (MCA-treated cells), 80 μM serine, 4 mM ATP or 0.5 mM ATP, and 4.0 A_{280} of tRNA per ml.

were not found in the case of phenylalanyl synthetase.

Summary. The aminoacylation of tRNA is an important step in the regulation of protein synthesis. The aminoacyl-tRNA synthetases may have a regulatory function in the transformation of normal cells to neoplastic cells.

Seryl and phenylalanyl synthetase have been isolated from ascitic tumor cells which were previously treated with 20-methylcholanthrene. The different steps of preparation are $(NH_4)_2SO_4$ precipitation of the 105,000g supernatant (between 50 and 65% saturation), chromatography on DEAE cellulose, CM Sephadex C-50 and hydroxyapatite.

The purification fold of the seryl-tRNA synthetase was 450 times with the molecular weight of 120,000. The present studies have shown that the K_m for serine was about 20 times higher in MCA-treated cells than in the controls, while the K_m for ATP was only increased 3–4 times. These values indicate that the synthetases possibly have a regulatory role in the protein synthesis of the cancer cells with and without treatment with MCA. Similar differences were not found in the case of phenylalanyl synthetase.

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