Polyamines and Amino Acid Incorporation *in vitro* into Microsomes of Rat Cerebral Cortex

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1. Polyamines were found to be associated with microsomes of rat cerebral cortex, the amount of spermine being about four times that of spermidine. Cell sap contained more spermidine than spermine. 2. Both polyamines were able to stimulate the incorporation of [14 C]valine into microsomes *in vitro* with a maximum rate equal to 250% of the control. Polyamines stimulated at concentrations close to the amount of spermine and spermidine naturally present in the system. 3. Spermine (0.05 mM) was used to study the mechanism of action of polyamines. The increasing of microsome and cell-sap concentration facilitated the action of spermine, but the same process was inhibited by increasing pH 5-enzyme concentration. 4. Spermine did not affect the association of [14 C]valine with tRNA in cell sap, but increased the rate of aminoacyl-tRNA formation in pH 5 enzyme preparations. However, this process was not affected in any case when incorporating microsomes were present. 5. It is suggested that microsomes are the main site of action of polyamines.

The present concept of the biochemical function of the naturally occurring aliphatic polyamines spermine and spermidine is based on their ability to stabilize acid macromolecules, particularly nucleic acids (Tabor & Tabor, 1964; Agrell & Heby, 1968). In this way they may be involved in important cell functions, like DNA replication (Schwimmer, 1968), the synthesis of RNA (Abraham, 1968) and the synthesis of protein by ribosomes (Hershko, Amoz & Mager, 1961; Martin & Ames, 1962; Takeda, 1969). Although the mode of action of polyamines is far from being understood, it is generally accepted that they may play an important role in the regulation of nucleic acid and protein synthesis, and correlation has been claimed between polyamine concentration and growth pattern in various Vertebrate systems (Raina, 1963; Moruzzi, Barbiroli & Caldarera, 1968; Jänne, Raina & Siimes, 1968; Caldarera, Giorgi & Casti, 1969a; Pegg, Lockwood & Williams-Ashman, 1970).

As far as the mechanism of protein synthesis is concerned it has been established that polyamines, together with Mg^{2+} , are structural components of bacterial ribosomes, playing an important role in the association of the two ribosomal subunits (Cohen & Lichtenstein, 1960; Watson, 1964; Miyazawa, Dick & Tamaoki, 1968; Stevens & Morrison, 1968). The positive effect of polyamines on ribosome aggregation in eucaryotic organisms has

been demonstrated *in vitro* for guinea-pig pancreas (Siekevitz & Palade, 1962) and *in vivo* for chick embryo (Moruzzi *et al.* 1968). A study by Datta, Sen & Ghosh (1969) suggests that spermine and spermidine stabilize brain-cortex ribosomes *in vitro*.

A number of workers have briefly reported some isolated experiments on the effect of polyamines on various amino acid-incorporating systems *in vitro*. Tissières & Hopkins (1961) and Nakamoto & Hamel (1968) with *Escherichia coli* ribosomes, Bretthauer, Marcus, Chaloupka, Halvorson & Bock (1963) in yeast and Ochoa & Weinstein (1964) in mouse ascites leukaemia all obtained stimulation with polyamines; Allen & Schweet (1962) found that polyamines inhibit haemoglobin synthesis; Maden & Monro (1968) did not obtain any effect of spermidine on the ribosome-catalysed peptidyl transfer in *E. coli*. Few studies have been specifically dedicated to the problem and even less relate to mammalian tissue. Martin & Ames (1962) have studied the effect of polyamines on *Salmonella typhimurium* protein synthesis *in vitro* by using synthetic mRNA, and they obtained stimulations between six- and ten-fold. Tanner (1967) has reported the stimulation of aminoacyl-tRNA binding to yeast ribosomes by spermine and spermidine. Takeda (1969) has published a series of studies on various aspects of polyamine

stimulation of protein synthesis in *E. coli*. Two papers have dealt with mammalian systems: Hershko *et al.* (1961) with rat liver and Rosiek, Beer, Sablinski & Sawicki (1968) with rabbit reticulocytes have obtained the stimulation of amino acid incorporation into microsomes and ribosomes by polyamines.

As polyamines are natural components of mammalian ribosomes (Siekevitz & Palade, 1962; Raina & Telaranta, 1967; Datta *et al.* 1969), they may regulate the capacity of ribosomes to support protein synthesis. The rat brain is an appropriate system for the study of the regulation of protein synthesis, as some authors (Murthy & Rappoport, 1965; Adams & Lim, 1966; Yamagami & Mori, 1970) reported differences in synthetic activity between young and adult microsomal preparations, though the cause of this difference is still far from being known. Spermine and spermidine were studied in the whole brain of rat (Jänne, Raina & Siimes, 1964) and mouse (Shimizu, Kakimoto & Sano, 1965) and their concentrations were found to be much higher in young than adult animals. Calderara, Moruzzi, Rossoni & Barbiroli (1969b) have demonstrated the capacity of polyamines to enhance the aggregation of polyribosomes in developing chick embryo brain.

The present work reports the presence of polyamines in rat brain microsomes and cell-sap preparations and the capacity of spermine and spermidine to stimulate amino acid incorporation into rat brain microsomes *in vitro*, and offers an interpretation of the mode of action of polyamines.

MATERIALS AND METHODS

DL-[1-¹⁴C]Valine was obtained from The Radiochemical Centre (Amersham, Bucks., U.K.) and used undiluted (32.2 mCi/mmol). ATP (disodium salt), GTP (disodium salt), creatine phosphate, spermine, spermidine and putrescine free bases were obtained from Sigma Chemical Co. (St Louis, Mo., U.S.A.).

Subcellular fractionation. Adult Wistar albino rats (weighing about 200 g) bred in this Unit were killed by decapitation under light ether anaesthesia. The brain was removed and briefly cooled in ice; the cerebral cortex was separated and dropped into cold medium A (0.25 M-sucrose–4 mM-MgCl₂–25 mM-KCl–25 mM-tris-HCl, pH 7.6), rinsed and homogenized in 5 vol. of the same medium by means of 15 strokes in a glass homogenizer with a Teflon pestle. After centrifugation at 15000g for 10 min in an MSE 18 centrifuge at 0°C, the supernatant was collected and recentrifuged in the same way, to obtain the post-mitochondrial supernatant fraction. This was centrifuged in a Spinco 50 Ti rotor at 150000g for 70 min. Cell sap was obtained from the upper three-quarters of the supernatant. The pellet was rinsed and resuspended with medium A in an all-glass homogenizer. The microsome suspension was centrifuged for 5 min at 1500g to remove aggregated material.

Samples of cell sap and microsome preparations were collected for analytical determinations.

pH5 enzyme preparation. This fraction was prepared from cell sap as described by Zomzely, Roberts & Rappoport (1964). Cell sap was slowly adjusted to pH 5.2 with 1 M-acetic acid and centrifuged at 34000g for 15 min. The pellet was rinsed and resuspended in medium A with an all-glass homogenizer and the resuspension was centrifuged for 10 min at 5000g. The supernatant obtained in this way was adjusted to pH 7.6 with 5 M-KOH and stored at –20°C for periods of not longer than two weeks.

Cell-free incorporating system. The standard incubation mixture contained, in a total volume of 1 ml, 0.20 M-sucrose, 3 mM-MgCl₂, 20 mM-KCl, 1 mM-ATP, 0.5 mM-GTP, 20 mM-creatine phosphate, 20 mM-tris-HCl buffer, pH 7.6, 0.5 μ Ci of labelled valine and different concentrations of microsomes, cell sap or pH 5 enzymes. The same mixture composition was used when cell sap or pH 5 enzymes were incubated without microsomes. Polyamines were added as free bases diluted in water. The presence of 0.05 mM-spermine in the system caused an increase in pH of not more than 0.05 unit. The incubation was carried out in a water bath at 37°C and was stopped by chilling the tubes in ice and adding 1 ml of cold water and 2 ml of cold 20% (w/v) trichloroacetic acid containing 50 mM-valine. Non-incubated samples were used as blanks. No change in radioactivity was detected during the first minute of incubation; this was regarded as a warming-up time and added to all the incubation times (except for the experiment of Fig. 8).

Sample preparation and radioactivity measurement. After 1 h or more at 0–4°C the trichloroacetic acid-precipitated material was centrifuged and washed three times with cold 10% (w/v) trichloroacetic acid. RNA was digested with 5% (w/v) trichloroacetic acid at 80°C for 15 min and appropriate portions of the supernatant were used to measure the hot-acid-soluble radioactivity. The insoluble fraction was further washed twice with ethanol, once with ethanol-ether (1:1, v/v), once with ether, evaporated to dryness under vacuum and dissolved in a small volume of hot concentrated formic acid. The preparation of samples for aminoacyl-tRNA formation experiments was basically the same, except that the hot-acid digestion was omitted and the first ethanol treatment was carried out at 0°C for 1 h. About 95% of the radioactivity was solubilized if these samples were treated with hot acid.

The samples used for radioactivity determination were pipetted on to 32 mm stainless-steel planchets (Sigma Chemical Co.), dried under a stream of warm air and their radioactivities counted in a Nuclear-Chicago end-window gas-flow counter (30% efficiency). The lipid-solvent fractions did not show any appreciable radioactivity.

Extraction and determination of polyamines. Appropriate samples of the same microsome, cell-sap and pH 5 enzyme preparations used for the incubation experiments, were precipitated with 5% (w/v) (final concentration) HClO₄ and left overnight at 4°C. Polyamines were extracted and determined by a modification of the method of Raina (1963). Samples were centrifuged and the pellets were used for protein and RNA determinations. The supernatant was adjusted to pH 12–13 with 10 M-KOH; perchlorate salts were left to form a precipitate at 4°C, which was removed by centrifugation. The supernatant

Fig. 1. Identification of bands after separation of polyamines by paper electrophoresis. Known compounds were run together with samples obtained from microsome and cell-sap preparations. After staining with ninhydrin paper strips were cut into serial bands of 0.25 cm, extracted in 1 ml of eluent and the extinction was read at 505 nm. (a) Standard; (b) microsome sample; (c) cell-sap sample. Abbreviations: PT, putrescine; SPD, spermidine; SP, spermine. Bands a, b, and c are unidentified compounds.

was transferred to stoppered tubes, saturated with anhydrous Na_2SO_4 (0.3 g/ml) and an equal volume of butan-1-ol was added. After vigorous shaking for 20 min the two fractions were left overnight to separate. The butanol fraction was collected, acidified with conc. HCl (about 1%, v/v) and evaporated to dryness in small crucibles exposed to a gentle stream of warm air. The dry residue was dissolved carefully in appropriate small volumes of 0.1 M-HCl and stored at -20°C . Appropriate amounts (10–20 μl) of samples were applied on a 120 g/m² paper strip (40 mm $\times$ 410 mm) (LKB Produkter, Stockholm, Sweden) for electrophoresis separation at 300 V for 3 h in 0.1 M-citric acid–NaOH buffer, pH 3.7. If shorter times of electrophoresis and buffers of lower pH were used the unknown band 'a' did not separate from spermidine (Fig. 1). Paper strips were dried for 15 min at 100°C and stained with 1% ninhydrin solution (0.1 g of cadmium acetate, 10 ml of water, 5 ml of acetic acid and 1 g of

Table 1. Composition of subcellular fractions used in the incubation systems

Polyamines, protein and RNA determination was as described in the Materials and Methods section. Average values of three or more determinations are given.

	RNA/ protein ratio	Polyamines (nmol/mg of protein)	
		Spermine	Spermidine
Microsomes	0.103	4.08	1.21
Cell sap	0.012	1.95	4.59
pH 5 enzymes	0.026	—	—

ninhydrin dissolved in 85 ml of acetone). The colour was developed at 100°C for 45 min. The bands were cut into small pieces and extracted for 2 h in the dark with acetic acid–ethanol eluent (2 g of cadmium acetate, 10 ml of water, 40 ml of ethanol and 50 ml of acetic acid). Shorter times of elution are not satisfactory for a complete removal of the polyamine–ninhydrin complex and for constancy in duplicate samples. After centrifugation the extinction was read at 505 nm. Standard samples were used for identification of the bands and the determination of the extinction coefficients (E_{505} for 1 $\mu\text{g}/5\text{ ml}$ of eluent was 0.085 for spermidine and 0.070 for spermine). Blank bands were taken within 3 cm from the origin. Fig. 1 shows the identification of bands obtained from extraction of microsomes and cell-sap preparations. In this condition a good separation and determination of polyamines can be carried out when the quantity of material applied to the paper strip is between 0.5 and 4 μg of each polyamine.

Other determinations. Protein determination was carried out by the method of Lowry, Rosebrough, Farr & Randall (1951) with bovine serum albumin (Sigma Chemical Co.) as a standard. RNA was determined both by the orcinol reaction (Hurlbert, Schmidt, Brumm & Potter, 1954) and by the method of Fleck & Munro (1962).

RESULTS AND DISCUSSION

Polyamine content of subcellular fractions. The amount of polyamines associated with a particular fraction after subcellular preparation does not necessarily represent the natural distribution of polyamines because of ionic binding to macromolecules, which is influenced by the ionic strength of the medium (Tabor & Kellog, 1967; Stevens, 1969). Relative redistribution occurs during homogenization; in particular Mg^{2+} can replace polyamines and remove them partially from microsomes, although the concentration of Mg^{2+} used in the present experiments is believed to have a limited effect (Raina & Telaranta, 1967). Therefore the values in Table 1 represent the content of endogenous polyamines of the system *in vitro* under the present conditions of preparation.

As shown in Table 1 the pattern of polyamines associated with microsomes and cell sap is sensibly

different, spermine being present in a greater amount in microsomes whereas cell sap is richer in spermidine.

Little or nothing is known about the nature of the molecules that actually bind polyamines in the cell. It has been suggested that in ribosomes polyamines are associated with the phosphate groups of RNA (Watson, 1964; Choi & Carr, 1967). Cohen, Morgan & Streibel (1969) have shown that tRNA is associated with polyamines. The results in Table 1 make it unlikely that RNA is the only material with which polyamines are associated, since cell sap contains too small a quantity of RNA in comparison with the microsome fraction. However, it must be noted that about half of brain cell-sap polyamines are free, i.e. not associated with macromolecules (P. P. Giorgi, unpublished work). In theory polyamines might bind to the negative radicals of every macromolecule, e.g. acid proteins, phospholipids and acid polysaccharides (Agrell & Heby, 1968).

No polyamine was found in the pH5-enzyme fraction, presumably because they dissociate and are not precipitated with the pH5 enzymes.

Optimum concentration of polyamines as additive.

Both spermine and spermidine stimulated the incorporation of [14 C]valine into rat brain microsomes, the maximum rate being about twice that of the control (Fig. 2). The optimum concentration of spermine was tenfold lower than that of spermidine. However the optimum concentration of spermidine was able to reach a higher stimulation than that of spermine. The present results are consistent with those reported by Rosiek *et al.* (1968) with rabbit reticulocyte ribosomes.

Increasing polyamine concentration beyond the

Fig. 2. Effect of different concentrations of spermine and spermidine on amino acid incorporation in a microsome-cell sap incubation system containing 0.6mg of microsomes and 2.2mg of cell sap. Results are indicated as percentage values compared with control samples. The incorporation rate of controls was 450 c.p.m./mg of microsomal protein (1200 c.p.m./mg after correction for valine pool size). ●, Spermine-treated samples; ○, spermidine-treated samples.

optimum resulted in an inhibition of amino acid incorporation into microsomes (Fig. 2). This may explain the report that 1mM-spermine inhibited the synthesis *in vitro* of haemoglobin (Allen & Schweet, 1962).

It is of interest to consider the quantitative relations between endogenous polyamines (naturally associated with the subcellular fractions) and exogenous polyamines (introduced as additive in the cell-free system). From Table 1 it can be calculated that a microsome (0.6mg of protein)-cell sap (2.2mg of protein) system contains about 10nmol of spermidine and 5nmol of spermine as endogenous polyamines. These values are very near to the values of exogenous spermine and spermidine at which stimulation begins (Fig. 2), i.e. between 5 and 10nmol of spermine and between 50 and 100nmol of spermidine. This suggests that these two polyamines might act as regulators of protein synthesis in mammalian brain, where young animals contain higher concentrations of polyamines than the adults (Shimizu *et al.* 1965).

Effect of spermine on different cell-free preparations.

Three different cell-free systems were used to examine further the effect of polyamines on [14 C]-valine incorporation into rat cerebral microsomes. Spermine at its optimum concentration (0.05mM) was used.

No significant difference in spermine stimulation was found between the postmitochondrial supernatant system (Fig. 3b) and the microsome-cell sap system (Fig. 3a), indicating that the manipulations to which microsomes are subjected during their preparation neither prevent their response to polyamine stimulation nor modify this capacity. However, when microsomes were coupled with pH5 enzymes the same concentration of spermine produced no significant stimulation (Fig. 3c). The bottom graph of Fig. 3(a) shows the pattern of radioactivity associated with the hot-5%-trichloroacetic acid-soluble fraction. The radioactivity of this fraction may be used to study the concentration of labelled aminoacyl-tRNA complexes present in the system (Bretthauer *et al.* 1963). As far as this fraction is concerned there is no difference between control and spermine-treated samples.

Effect of microsome, cell-sap and pH5-enzyme concentration on spermine action. The results shown in Fig. 3 are not conclusive because they may represent the effect of a particular quantitative relationship among the components of the system. On the assumption that there is no great difference between a postmitochondrial supernatant system and a microsome-cell sap system, the difference between the latter and the microsome-pH5 enzyme system was studied. In all experiments 0.05mM-spermine was used.

The graphs of Fig. 4 show the effect of microsome

Fig. 3. Effect of spermine (0.05mM) on amino acid incorporation in different types of incubation systems. (a) Microsome (0.5mg)-cell sap (2.5mg) system; the top graph shows the specific radioactivity of microsomal protein; the bottom graph shows the radioactivity associated with the hot-acid-soluble material. (b) Post-mitochondrial supernatant system, containing 0.4mg of microsomes and 3.0mg of cell sap. (c) Microsome (0.5mg)-pH 5 enzyme (1.0mg) system. O, Control samples; ● spermine-treated samples.

and cell-sap concentration on the action of spermine. The radioactivity of control curves is decreasing with the increase of cell-sap concentration, but this does not mean that the system is working at inhibiting concentrations of cell sap. The pattern is due to isotope dilution caused by cell-sap endogenous valine pool, as demonstrated by Adams & Lim (1966); in fact if appropriate corrections are applied, the incorporation rate is found to be increasing until a concentration of about 3.0mg of cell sap. The approximate correction that has to be made for pool size is as follows: c.p.m. $\times 1.2 \times$ mg of cell-sap protein. Since this isotope dilution occurs to the same extent in both control and spermine-treated samples, no particular attention was given to the estimation of the valine pool and to the correction for isotope dilution.

With 0.3mg of microsomal protein (Fig. 4a) and low concentrations of cell sap, spermine inhibits [^{14}C]valine incorporation into microsomes. The degree of inhibition decreases with the increase of cell-sap concentration and at high concentrations of cell sap spermine has a stimulating effect. With 0.6mg of microsomal protein (Fig. 4b) the spermine-treated samples incorporate more actively than

the controls at any cell-sap concentration; again the extent of spermine stimulation is proportional to cell-sap concentration. The results shown in Fig. 4 suggest that both microsomes and cell sap are involved in the mechanism of action of spermine and that the effect of spermine does not depend simply on its concentration, but on its quantitative relationship with the two subcellular fractions. For example, 500nmol of spermine are needed to cause a 50% inhibition in a system with 0.6mg of microsomes and 2.2mg of cell sap (Fig. 2), whereas only 50nmol are sufficient to cause the same effect in a system with only 0.3mg of microsomes and 1.5mg of cell sap (Fig. 4). On the other hand the same concentration of spermine may be inhibiting or stimulating according to the quantity of microsomes and cell sap present in the system. The radioactivity of the hot-acid-soluble fraction was checked in all samples and the results were plotted under the corresponding values of protein specific radioactivity (Figs. 4a and 4b, bottom graphs). No difference was detectable in this fraction between control and spermine-treated samples.

Fig. 5 shows the effect of microsome and pH 5-enzyme concentration on the action of spermine.

Fig. 4. Effect of microsome and cell-sap concentration on the action of spermine (0.05 mM) added in a microsome-cell-sap system. (a) 0.3 mg and (b) 0.6 mg of microsomal protein were used with different cell-sap concentrations. Top graphs show the specific radioactivity of microsomal protein, where the thick line represents the pattern of percentage values of treated samples compared with controls. Bottom graphs show the radioactivity of the hot-acid-soluble fraction. The incubation time was 30 min. $\circ$, Control samples; $\bullet$, spermine-treated samples.

Fig. 5. Effect of microsome and pH5-enzyme concentration on the action of spermine (0.05 mM) added in a microsome-pH5-enzyme incubation system. (a) 0.6 mg and (b) 1.2 mg of microsomal protein were used with different pH5-enzyme concentrations. Top graphs show the specific radioactivity of microsomal protein, where the thick line represents the pattern of percentage values of treated samples compared with controls. Bottom graphs show the radioactivity of the hot-acid-soluble fraction. The incubation time was 30 min. $\circ$, Control samples; $\bullet$, spermine-treated samples.

The range of the pH 5 enzyme/microsome ratio is such that in the control systems the incorporation rate was increased by increasing the ratio; this was to avoid conditions where the system was limited. With 0.6mg of microsome protein (Fig. 5a) and very low concentrations of pH 5 enzymes, spermine scarcely stimulates the incorporation of [14 C]valine into microsomes. The increase of pH 5 enzymes corresponds to an increasing inhibition by spermine up to values of about 50% of the control. High extents of stimulation are reached with 1.2mg of microsomes (Fig. 5b) coupled with low concentrations of pH 5 enzymes; but the rate of stimulation is rapidly diminished by increasing pH 5 enzyme concentrations until it becomes an inhibition at high concentrations of this fraction. The results of Fig. 5 confirm the suggestion that microsomes are the site of action of spermine; but the pattern shown by pH 5-enzyme concentration is the opposite of that obtained with cell sap. Again no difference can be found in the concentration of labelled aminoacyl-tRNA complexes (Figs. 5a and 5b, bottom graphs).

The results of this group of experiments (Figs. 4 and 5) strongly suggest that microsomes are the subcellular components that respond to the stimulating action of polyamines. This is in agreement with the results obtained by other authors on the effect of polyamines on ribosome structure and polyribosome aggregation (Siekevitz & Palade, 1962; Watson, 1964; Caldarera *et al.* 1969b; Datta *et al.* 1969). Cell sap also seems to be involved in the mechanism of the stimulation of amino acid incorporation by spermine. The cell-sap component more likely to respond to polyamine action is tRNA. Therefore the possibility was considered that polyamines affect the process of aminoacyl-tRNA formation.

Effect of spermine on aminoacyl-tRNA formation. Hershko *et al.* (1961) claimed that spermine has no appreciable effect on amino acid incorporation into tRNA, without providing any evidence. Takeda & Igarashi (1969) have studied this problem in *E. coli*, reaching the conclusion that polyamines can substitute for Mg^{2+} in the activation of the enzyme aminoacyl-tRNA synthetase. Experiments were carried out in both types of soluble fractions, cell sap and pH 5 enzymes, which showed in the previous experiments (Figs. 4 and 5) to have a different behaviour when coupled with microsomes.

Fig. 6 shows the effect of spermine on the incorporation of [14 C]valine into the cold-acid-insoluble fraction of cell sap. If cell sap was prepared in the standard way, by centrifuging the postmitochondrial supernatant for 70min at 150000g, a certain degree of stimulation was obtained with spermine. On the other hand if cell sap was further centrifuged for 3h with the same force absolutely

Fig. 6. Effect of spermine (0.05mM) on the association of [14 C]valine with tRNA in a cell-sap incubation system. Δ and $\blacktriangle$, 3.0mg of cell-sap protein obtained by 70min centrifugation of the postmitochondrial supernatant; Δ , control samples; $\blacktriangle$, spermine-treated samples. $\circ$ and $\bullet$, 1.5mg of cell-sap protein obtained by 3h extra centrifugation at 150000g of standard cell sap; $\circ$, control samples; $\bullet$, spermine-treated samples.

Fig. 7. Effect of spermine (0.05mM) on the association of [14 C]valine with tRNA in a pH 5-enzyme incubation system. The amount of pH 5-enzyme protein was 0.85mg. $\circ$, Control samples; $\bullet$, spermine-treated samples.

no effect was detectable from the addition of spermine in the system. In fact, the second step of cell-sap preparation led to a small pellet with an RNA/protein ratio of 0.022 and a quantity of protein equal to the 7% of microsomal protein obtained after the first centrifugation step. Probably the stimulation caused by spermine with standard cell sap is due to its effect on a small quantity of ribosomes still present in the soluble fraction. The same experiment carried out with

Fig. 8. Comparison between amino acid incorporation into protein and amino acid association with tRNA in a microsome (0.5 mg)-pH 5-enzyme (1.5 mg) incubation system treated with spermine (0.05 mM). The first minute of incubation corresponds to the warming up of samples, as explained in the Materials and Methods section. (a) Specific radioactivity of the hot-acid-insoluble material; (b) radioactivity associated with the hot-acid-soluble fraction. ○, Control samples; ●, spermine-treated samples.

purified cell sap shows no effect of spermine on the formation of [^{14}C]valyl-tRNA. Failure to detect any effect of spermine on the cell-sap incubation system (Fig. 6) could suggest that polyamines are not involved in the process of aminoacyl-tRNA formation. Since the present results are not in agreement with those obtained with a bacterial system (Takeda & Igarashi, 1969), further investigations are needed.

Ribosome-free cell sap after 3 h centrifugation at 150 000 g was used to prepare pH 5 enzymes and the same experiment of Fig. 6 was carried out with this fraction. Fig. 7 shows that the effect of spermine on the incorporation of [^{14}C]valine into the cold-acid-insoluble fraction of pH 5 enzymes is quite different from that obtained with cell sap. During the first minutes of incubation the radioactivity of samples treated with spermine was clearly higher than the control; no difference was found after 18–20 min incubation. This means that spermine

increases the reaction rate so that tRNA becomes saturated in a shorter time.

It must be pointed out that in the experiments with the microsome-pH 5-enzyme system (Fig. 5) spermine did not cause any difference in the radioactivity associated with tRNA. But the information provided by this result is relative because, as shown in the experiment of Fig. 7, the effect of spermine on this fraction must be studied with short incubation times, whereas the experiment of Fig. 5 was carried out with 30 min incubation. Therefore a microsome-pH 5-enzyme system was set up and the consequence of spermine treatment was studied in both hot-acid-soluble and -insoluble fractions at the very beginning of the incubation. In spite of the clear inhibiting effect of spermine on amino acid incorporation into microsomes (Fig. 8a), no difference was detectable in the radioactivity of the hot-acid-soluble fraction (Fig. 8b). This means that in the presence of incorporating microsomes it is not possible to detect the change in aminoacyl-tRNA formation caused by spermine in pH 5 enzyme when this fraction is incubated alone (Fig. 7).

The different effect of spermine on cell sap and pH 5 enzymes is difficult to explain, but it seems likely that this is due to the lack of endogenous polyamines in pH 5 enzymes and to the nature of the chemical bonds between exogenous spermine and pH 5-enzyme macromolecules.

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