CYTOCHEMICAL STUDY ON THE PANCREAS OF THE GUINEA PIG

VII. Effects of Spermine on Ribosomes

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ABSTRACT

Pancreatic ribosomes (guinea pig) aggregate and lose upon treatment with polyamines, particularly spermine, their bound secretory enzymes. Spermine, at 0.5 mM, for example, causes the release of about 85 per cent of the chymotrypsinogen and RNase, and from 85 to 100 per cent of the ribosomal amylase. At the same time, the particles lose about 10 per cent of their RNA, 7 to 24 per cent of their total protein, and from 75 to 100 per cent of their Mg^{++}. Observations with the electron microscope confirm the heavy agglutinating of the ribosomes but otherwise show little change in the structure of the particles. Using radioactive spermine it was found that, concomitant with the loss of bound enzymes and Mg^{++} from the ribosomes, spermine became bound to the particle. The extent of binding ranged from 0.29 to 1.49 μmoles per 10 μmoles RNA-P. The bound radioactive spermine can be removed by subsequent treatment of the ribosomes with GTP, ATP, or P-P, which treatment also removes most of the RNA of the particles, leaving behind ribosomes with a much lower RNA/protein ratio. From this evidence it was inferred that spermine, in releasing the Mg^{++} of the particle, becomes salt-linked to the free phosphate hydroxyl groups of the RNA. Freshly isolated pancreatic and hepatic ribosomes contain very little spermine, about 0.1 to 0.2 μmoles polyamine/10 μmoles RNA-P. The results are discussed in terms of the linkages between the structural protein, the bound secretory enzymes, and the RNA of the ribosomes.

INTRODUCTION

We have been interested in pancreatic RNP[1] particles, or ribosomes, for the past few years (1–7). In particular, we have obtained relatively pure preparations of particles which have a diameter of ~150 A (1, 6, 7), an RNA content of from 35 to 45 per cent (1, 7), a sedimentation coefficient of ~85 S (7), and a molar amount of Mg^{++} equivalent to about $\frac{1}{10}$ that of the phosphate groups in the RNA (7). We have shown that ribosomes, as isolated from guinea pig pancreas, have bound amylase, RNase, and TAPase activities (6, 7). Moreover, we have found that, shortly (1 to 3 minutes) after an intravenous injection of radioactive leucine, the α-chymotrypsinogen isolated from these particles has a higher specific radioactivity than the same zymo-

[1] Abbreviations used are: RNA, ribonucleic acid; RNA-P, ribonucleic acid phosphate; RNP, ribonucleoprotein; P-P, inorganic pyrophosphate; ATP and GTP, adenosine and guanosine triphosphates; tris, tris (hydroxymethyl) aminomethane; RNase, ribonuclease; TAPase, trypsin-activatable proteolytic activity; TCA, trichloracetic acid; DOC, deoxycholate.

gen isolated from other subcellular units (6). This finding indicates that the RNP particles synthesize α-chymotrypsinogen, and suggests that they may be involved in the synthesis of other pancreatic digestive enzymes.

When ribosomal Mg^{++} is removed by chelating agents such as versene, ATP, GTP, and P-P, all the above-named enzymes are released together with ~80 per cent of the RNA and ~25 per cent of the total protein of the particles (7), with a large part of the protein removed being made up by the released enzymes. Ribosomes treated by chelating agents are still recognizable as particles, but they are agglutinated and their diameter is increased about twofold (7).

As a result of these findings, we assumed that *in vivo* the newly synthesized proteins remain on the particles for a finite period and have to be removed therefrom to complete the process of soluble protein synthesis. In addition, we postulated that in the intact cell the synthesized enzymes are released without concomitant loss of RNA, for we have shown that the turnover rate of the total ribosomal RNA is very low *in vivo* (5). For these reasons we were interested in finding conditions under which only the enzymes are released from the particles, while the RNA is retained. We now report experiments which show that the polyamines, putrescine, cadaverine, and particularly spermine, effect a release of ribosomal enzymes without materially affecting ribosomal RNA. In addition, we demonstrate that spermine replaces the Mg^{++} of the particles, thereby rendering them stable in the absence of Mg^{++}, and preventing or minimizing RNA losses. The implications of these findings on the type of bonds which link the RNA, structural protein, and enzymes of the particles are discussed.

METHODS

Biochemistry

GENERAL PROCEDURES: RNP particles were isolated from guinea pig pancreatic microsomes by deoxycholate treatment, as already described (6, 7). Their RNA was determined by the orcinol method, their protein by Nesslerization after Kjeldahl digestion, and their Mg^{++} by a colorimetric method; details of these procedures have already been given (7). RNA-P was calculated as being 9 per cent of the RNA, and protein as being 6.25 times protein-N. The assays for determining amylase, RNase, and TAPase activities, the conditions for measuring the release of these activities from the particles, and the recoveries obtained were the same as in (7). Chymotrypsinogen was measured by specifically assaying for chymotrypsin activity by the method of Cohen and Erlanger (8) after trypsin activation.

INCUBATION WITH POLYAMINES AND OTHER COMPOUNDS: The incubation conditions were as follows: The surface of ribosomal pellets was carefully washed, and the particles resuspended by homogenization in 0.88 M sucrose. Ribosomes obtained from 250 mg fresh pancreas were incubated for 30 minutes at either 35°C or 4°C in a total volume of 2.0 ml, containing the various compounds to be tested in a final sucrose concentration of 0.44 M. After incubation, the contents of two tubes (500 mg tissue equivalents) were pooled, cold distilled water was added to make the final sucrose concentration 0.15 M (final volume 12 ml), and the suspension spun for 90 minutes at 105,000 *g* in a Spinco model L centrifuge.[2] Enzymatic activities were tested before incubation in the original particle suspension, and after incubation and resedimentation in the supernatant and sometimes in the companion pellet. The recovery of enzymatic activity was the same as given previously ((7), *cf.* Table I). Because of the small yields of ribosomes obtained, the RNA, protein, and Mg^{++} contents of the original particle suspension and of the resedimented particles were measured in a separate series of experiments. In some cases in which duplicates were particularly desirable, as in Mg^{++} determinations, more incubated particles were pooled for sedimentation.

EXPERIMENTS WITH RADIOACTIVE SPERMINE: When radioactive spermine was used, the surface of the pellet, sedimented after incubation, was washed several times with cold distilled water, to remove possible contamination by unbound spermine and then the ribosomes were resuspended in water. In these experiments, one-half of the suspension was used for RNA, protein, and Mg^{++} determinations, while the other half was dried down on aluminum planchets, kept in a desiccator, and counted with a Nuclear-Chicago gas-flow counter, with a counting error of less than 1 per cent. From the ~10,000 CPM spermine used in each experiment, 250 CPM to 1000 CPM were recovered in the corresponding ribosomal pellets (the recovery figure varied from one experiment to another). This is much more than would be expected from contamination by soluble spermine, since the pellet accounts for less than 1 per cent of the original suspension volume, and since its surface

[2] Direct sedimentation from the incubation medium (0.44 M sucrose) by using 4 ml tubes, a swinging bucket rotor and a centrifugal field of 100,000 *g* × 90 minutes, gave similar results in enzyme distribution between the ensuing pellets and supernatants.

was carefully washed after centrifugation. Moreover, upon resuspension and resedimentation, the loss of radioactivity was not greater than the loss of RNA (*cf.* Table IV).

In the experiments in which the release of radioactive spermine from particles was investigated, the following conditions were used: after incubation with radioactive spermine, as indicated above, the ribosomes were sedimented, the surface of the ensuing pellets washed repeatedly, and the pellets finally resuspended by homogenization in cold distilled water. An aliquot of this suspension was removed for immediate chemical analysis and radioactivity counting, while another aliquot was incubated at 35°C for 30 minutes with various additions in a total volume of 1.0 ml. After incubation, water was added, the suspension centrifuged at 105,000 *g* for 90 minutes, and the ensuing pellet resuspended in water and assayed, as above.

SPERMINE IDENTIFICATION: For the tentative identification of spermine in ribosomes, the methods of Zillig *et al.* were used (9). The particles were resuspended in a small volume of a mixture of 2.0 M acetic acid–0.6 M formic acid to extract the polyamines. The precipitated residues were separated by centrifugation, and the supernatant was subjected to paper electrophoresis, using Whatman #3 MM paper, a current of 1000 volts running for 2 to 2.5 hours, and 0.05 M acetate buffer, pH 3.2, as the liquid phase. Paper chromatography of the same extract was carried out on Whatman #1 paper, with isopropyl alcohol/water (65/35, v/v) as the liquid phase, running for 24 hours. After drying, the papers were sprayed with ninhydrin reagent. Commercial samples of the various amines or amino acids were used as markers.

Materials

The radioactive spermine had a specific activity of approximately 0.1 $\mu c/\mu mole$, was labeled with C^{14} in the 1 and 4 positions of the butane part of the molecule (N,N^1-bis(3-aminopropyl)-1,4-butanediamine-1,4-C^{14}) and was obtained through the generosity of Drs. H. Waelsch and D. Clarke. The non-radioactive polyamines were obtained from the California Corporation for Biochemical Research, Los Angeles, and were chromatographically pure, as tested by us. The solutions of all compounds were brought to pH 7.0 before use in these experiments.

Electron Microscopy

Pellets of pancreatic ribosomes, incubated in (*a*) 0.44 M sucrose (control), (*b*) 0.1 mM spermine in 0.44 M sucrose, (*c*) 0.1 mM spermine and 1 mM Mg^{++} in 0.44 M sucrose, (*d*) 0.3 mM tris buffer, pH 7.0, in 0.44 M sucrose, and (*e*) 0.1 mM spermine in 0.44 M sucrose followed by 0.5 mM GTP, were fixed *in situ*, *i.e.*, in the centrifuge tubes, in 2 per cent OsO_4 in 0.88 M sucrose. Only in the case of (*a*), (*d*), and (*e*) had the pellets the usual appearance and location at the bottom of the tubes. For (*b*) and (*c*) the sediments appeared as extremely thin deposits on the centrifugal side of the tubes, in continuity with a slightly thicker bottom pellet.

After fixation, carried out overnight at ~0°C, the preparations were dehydrated *in situ* with ethanol in concentrations increasing from 70 to 100 per cent. While in 100 per cent ethanol, the pellets were removed and trimmed into orientable strips, or, in some cases (*b* and *c*), the sides and bottoms of the tubes bearing the sediments were cut in strips. The tube wall completely dissolved during subsequent impregnation in butyl methacrylate, leaving behind strips of the fine ribosomal deposits. All strips were subsequently embedded in a 2:8 mixture of methyl and butyl methacrylate and finally cut parallel to the direction of sedimentation. The sections were stained with lead hydroxide and covered with a film of carbon before examination in an RCA EMU-2b or a Siemens Elmiskop I microscope.

A few preparations of liver ribosomes or pancreatic microsomes incubated in spermine were also processed and examined, as indicated above.

RESULTS

Release of Enzymes from Particles

In a previous paper (7), we reported that P-P, ATP, or GTP, at a concentration of 0.5 mM, released from 80 to 100 per cent of the bound amylase, RNase, and TAPase activities of the RNP particles, during an incubation of 30 minutes at 35°C. Repetition of this type of experiment, but using 0.5 mM spermine instead,[3] gives almost identical results: from 80 to 90 per cent of the amylase, RNase and chymotrypsinogen activities of the particles is released (Table I). The recovery of enzymatic activity in all cases was satisfactory, indicating that the added spermine did neither inhibit nor activate the enzymes to any great extent. Supplementary experiments, in which crystalline RNase and chymotrypsinogen were assayed in spermine concentrations equal to those used in the assay media in our release experiments, gave about 10 per cent activation of the RNase and 10 per cent inhibition of the chymotrypsin activities. In parallel experiments

[3] Immediately after adding spermine, the almost clear suspension of particles becomes clouded. This cloudiness leads to aggregation of the particles, either with time or with heat.

with ribosome-bound RNase, spermine gave variable results, from a 10 per cent inhibition to a 10 per cent activation. Furthermore, keeping the particles in spermine (sample 2, Table I) did not interfere with their enzymatic activities, except perhaps in the case of RNase. It appears, therefore, that the enzymatic activity found in the supernatant of particles resedimented after spermine treatment is a measure of the release of enzymes from these particles.

TABLE I

Effect of Spermine on Ribosomal Enzymatic Activities

The guinea pigs were fasted 24 hours; the ribosomes were obtained as described and resuspended in 0.88 M sucrose.

Particle aliquots equivalent to 0.25 gm tissue were incubated in 2 ml 0.44 M sucrose with or without 0.5 mM spermine. Samples 1 and 2 were kept at 4°C for 120 min., then assayed. Samples 3 and 4 were kept at 4°C for 30 min. and then spun at 100,000 *g* for 90 min. at 4°C, to separate the particles (pellet) from the incubating medium (supernatant). The particles from samples 3 and 4 were resuspended in 0.44 M sucrose prior to assay. Diluted aliquots of all samples were assayed for amylase, RNase, and chymotrypsin activities. The maximum concentration of spermine in the assay medium (for sample 2) was calculated to be 0.2 mM for amylase, 0.025 mM for RNase, and 0.04 mM for chymotrypsin.

Amylase activity is given in mg maltose released from a starch digest by 1 gm tissue equivalent, whereas RNase and chymotrypsin activities are given as micrograms of RNase or chymotrypsinogen per gm tissue equivalent, as calculated from curves obtained with crystalline bovine RNase and chymotrypsinogen.

		Amylase (Activity/gm)			RNase (Activity/gm)			Chymotrypsin (Activity/gm)		
	Incubation conditions	Pellet	Supernatant	Total	Pellet	Supernatant	Total	Pellet	Supernatant	Total
1	120 min. in 0.44 M sucrose	—	—	48.1*	—	—	6.24*	—	—	4.29*
2	120 min. in 0.44 M sucrose containing 0.5 mM spermine	—	—	49.8*	—	—	5.80*			4.26*
3	30 min. in 0.44 M sucrose; then centrifuged	40.8	9.4	50.2‡	5.10	1.02	6.12‡	3.39	0.67	4.06‡
4	30 min. in 0.44 M sucrose containing 0.5 mM spermine; then centrifuged	4.5	42.8	47.3‡	1.14	5.44	6.58‡	0.69	3.45	4.14‡

* These figures represent direct measurements.

‡ These figures represent additive totals.

Table II gives results of experiments with various amines and other compounds, including, for comparison, further experiments with P-P. In experiments not shown in the Table, spermine at 0.1 mM was equivalent to P-P at 0.5 mM in releasing also ribosomal RNase and TAPase activities.[4] Putrescine and cadaverine, although active, were markedly less effective than spermine, suggesting that releasing ability is related to the number of amine groups and hence the charge on the molecule. Puromycin, an aminonucleoside known to inhibit protein synthesis (11–13), also released amylase activity less effectively than spermine. Histamine, lysine, and

[4] Spermine (1 mM), however, was found (10) to inhibit the release of radioactive proteins from ribosomes in an *in vitro* system in which reticulocyte ribosomes were synthesizing soluble hemoglobin; we have no explanation for the discrepancy between these findings and our results.

ornithine caused some release and even tris buffer had an effect, which became marked only at 5 mM. It is noteworthy that pilocarpine, carbamylcholine, and acetylcholine have very little, if any, effect in amylase release, as compared to incubation in 0.44 M sucrose.

Effect of Spermine on Ribosomal RNA, Protein and Mg^{++}

The P-P mediated discharge of ribosomal enzymatic activities is probably a consequence of the concomitant chelation of all the Mg^{++} and release of most of the RNA of the particles (7). Table III shows the effects of various amine compounds on ribosomal protein, RNA and Mg^{++}; the result of a further experiment with P-P is included for comparison.

Unlike P-P, spermine solubilized, in most cases, only a small amount (up to 10 per cent) of ribosomal RNA, the loss reaching 26 per cent only in some cases (Exps. 4 and 7). Since the loss of ribosomal protein was similar to or higher (up to 24 per cent) than that of RNA, the RNA/protein ratio of spermine-treated particles was, except for one instance (Exp. 7, Table III), equal to or higher than that of particles incubated in 0.44 M sucrose. In contradistinction, P-P–treated particles, because of the greater RNA losses, have an RNA/protein ratio one-half (Table III), or even lower than one-half (7), that of control particles. The relatively large variations in the percentage of protein and RNA losses in Table III are assumed to reflect variations in the extent of damage incurred by the particles during preparation. Putrescine, at 1.0 mM, behaves like spermine at 0.5 mM, but at 2.0 mM it apparently begins to disrupt the particles, as indicated by a much greater release of ribosomal RNA, leading to lower RNA/protein ratios than that of control particles.

As in the case of the P-P–treated particles (7), we assume that the proteins released by spermine are mostly secretory enzymes, since it is known that pancreatic ribosomes have bound amylase, RNase, and chymotrypsin activities (the latter after activation by trypsin), and since the present experiments show that 80 to 100 per cent of these activities are released by spermine.

TABLE II

Release of Amylase Activity from Ribosomes by Spermine, other Amines, and Various other Compounds

Incubation at 35°C under conditions given in the text. Per cent release is the percentage of amylase activity which remained in the supernatant after centrifuging the incubated particles for 90 min. at 105,000 *g*. Each figure represents one experiment.

Compound added	Conc.	Per cent release	Compound added	Conc.	Per cent release
	mM			mM	
Spermine	0.05	83, 88	L-Ornithine	1.0	32
"	0.1	82, 86, 90, 93	L-Arginine	1.0	10
"	0.5	87, 90, 92, 96, 97, 99, 100, 100*	Tris, pH 7.0	0.5	33
			"	1.0	41‡, 63
"	5.0	100§	"	5.0	46, 71, 86
Putrescine	1.0	68‖	P-P	0.5	80, 81, 91, 99, 100
"	10.0	72			
Cadaverine	1.0	66	Serotonin	0.5	29
Puromycin	0.6	43	Carbamylcholine	0.5	23
"	1.2	71	Pilocarpine	0.5	19
"	2.5	80, 83	Acetylcholine	0.5	15
Histamine	1.0	48	Liver glycogen	1.0 mg	8
L-Lysine	1.0	30	Water	—	0, 0, 8, 9, 15, 18, 18, 21

* Incubation of the same preparation at 0°C for 30 min. released 83 per cent of the amylase activity.
‡ Incubation of same preparation at 0°C for 30 min. released 32 per cent of amylase activity.
§ Plus 1 mM Mg^{++} = 96.
‖ Plus 1 mM Mg^{++} = 77.

On the basis of specific enzymatic activities, it has been calculated that RNase and chymotrypsinogen alone could account for 3 to 5 per cent of the total ribosomal proteins in the case of the guinea pig pancreas. The percentage of all secretory proteins bound to RNP particles has been estimated at 10 to 20 per cent. Accordingly, what remains behind in these experiments seems to be the structural protein(s) of the ribosomes. Hence, spermine, like P-P, appears to release, preferentially, proteins produced for secretion.

In addition to the enzymatic activities mentioned, spermine, like P-P, releases all, or almost all, ribosomal Mg^{++} (Table III). It was previously assumed that the pyrophosphate compounds act by effectively competing with the phosphate groups of the RNA for ribosomal Mg^{++}, for the addition of Mg^{++} to the incubation medium along with the pyrophosphates prevented the enzyme-releasing effect of these compounds (7). When Mg^{++} was added together with spermine to the incubation medium, it did not counteract, however, the effect of spermine on amylase release (*cf.* footnote, Table II). In fact, in 4 ex-

TABLE III

Effect of Various Amines, P-P, and Mg^{++} on Release of RNA, Protein, and Mg^{++} from RNP Particles

The particles were incubated at 35°C as given in the text. Different amounts of ribosomes were used in the various experiments and this explains the large variations in total amounts of RNA and protein.

Exp. No.	Compounds added	Recovered in pellet after centrifuging incubated RNP particles for 90 min. at 105,000 *g*			
		RNA	Protein	RNA/Protein	Mg^{++}
		(μg)	(μg)		(μmoles)
1	None*	362	728	0.50	—
	P-P (0.5 mM)	140	515	0.27	—
	Spermine (0.1 mM)	355	555	0.64	—
	Tris, pH 7.0 (1 mM)	353	555	0.63	—
2	None	142	350	0.41	0.04
	Spermine (0.1 mM)	143	287	0.50	0.00
	Putrescine (1 mM)	111	281	0.40	0.01
3	None	112	256	0.44	0.03
	Spermine (0.1 mM)	102	237	0.43	0.00
	Putrescine (2 mM)	51	237	0.22	0.00
4	None	351	550	0.64	0.08
	Spermine (0.5 mM)	260	414	0.62	0.00
	Putrescine (5 mM)	179	506	0.35	0.00
5	None	289	—	—	0.08
	Spermine (0.1 mM)	272	—	—	0.02
	Spermine (0.1 mM) plus Mg^{++} (1 mM)	321	—	—	0.10
6	None	229	341	0.67	—
	Spermine (0.1 mM)	209	278	0.75	—
	Spermine (0.1 mM) plus Mg^{++} (1 mM)	273	319	0.85	—
7	None	211	315	0.67	—
	Spermine (0.1 mM)	154	282	0.55	—
	Spermine (0.1 mM) plus Mg^{++} (1 mM)	176	287	0.62	—

* None represents particles incubated in 0.44 M sucrose. By comparison with freshly isolated particles, ribosomes incubated in sucrose alone showed losses of RNA and protein of from 10 to 15 per cent (*cf.* page 223).

periments (of which only 2 are shown in Table III) it was found that the RNA/protein ratios of the particles, recovered after incubation in both Mg^{++} and spermine, were higher than those of particles treated with spermine alone. This finding might be related to the observation that, in the presence of Mg^{++} in the medium, spermine did not release the Mg^{++} of the particles (Table III, Exp. 5). In this respect, it should be mentioned that particles incubated in 0.44 M sucrose lose from 10 to 15 per cent of their RNA and protein, as compared to particles not treated at all. These lost materials might come from partly damaged particles, and the combined effect of spermine and Mg^{++} could be to stabilize such particles and thereby insure their sedimentability.

The results of these experiments suggest that the various amines, particularly spermine, replace the Mg^{++} complexed to the phosphate groups of the ribosomal RNA by forming a strong salt bond between their charged amine groups and the charged phosphate groups of the RNA. It has previously been found by Felsenfeld and Huang (14) that spermine displaces Mg^{++} from polynucleotides. Since the spermine apparently replaces Mg^{++} in the particles, and since Mg^{++} seems to be one of the principal factors in stabilizing these particles (*cf.* reference 7), it follows that spermine probably helps to bind the RNA to some of the ribosomal proteins, presumably the structural proteins. This assumption is supported by the fact that the major part of the RNA and protein are retained in spermine-treated ribosomes, while most of the enzymes are lost. The retention of RNA in the particles cannot be attributed to RNase inhibition, since spermine inhibits neither the free nor the ribosome-bound enzyme.

Binding of Spermine to Particles

To test this assumption, the ability of RNP particles to bind radioactive spermine was investigated. The results showed that spermine is bound to the particles, and that by far the greatest part of the ribosome-bound spermine cannot be washed off by further incubation in water. Previously (7), we had found that the ratio μmoles Mg^{++}/10 μmoles RNA-P of isolated RNP particles was uniformly approximately one. The corresponding ratio for bound radioactive spermine varied from a low of 0.29 to a high of 1.49 in a series of experiments. Put in another way, the ribosomes adsorbed from 0.6 to 3.0 μmoles primary amine/10 μmoles RNA-P, or from 1.2 to 6.0 μmoles total amine group/10 μmoles RNA-P. These variations from particle to particle preparation were not due to inaccuracies in the experimental procedure, for duplicate binding experiments on the same particles yielded results within 10 per cent of each other. The effect of adding Mg^{++} (1.0 mM) to the medium containing radioactive spermine was variable. It was noticed, however, that in those cases in which low binding of spermine occurred, Mg^{++} increased it, sometimes fourfold, while in the instances in which high binding was present, the addition of Mg^{++} decreased it, again sometimes fourfold. The reason for these variations in spermine binding and Mg^{++} effects thereon are not known, but they could reflect variations from one particle preparation to another in the ease of access of spermine to the phosphate groups of the RNA.

That the binding of the added spermine is primarily due to the RNA of the particles can be inferred from the following data. First, washing the particles with 5 per cent TCA released in two experiments, 80 and 86 per cent of the counts added as spermine, indicating a noncovalent bond; second, treatment with either GTP or ATP removes a variable (from 43 to 88 per cent), but usually large, amount of the radioactive ribosome-bound spermine (Table IV and Fig. 1). This treatment also removes most of their RNA but one-half or less of their total protein.[5] Fig. 1 shows that as the bound spermine is removed from the particles, RNA is proportionally released, whereas in this particular experiment little protein is lost. Hence, the ratio, RNA/protein, mirrors the removal of the spermine and of the RNA, as the concentration of GTP is increased. Table IV also indicates that when Mg^{++} is added to the incubation mixture along with the GTP or ATP, the loss of radioactive spermine is reduced to 20 to 25 per cent, indicating that the

[5] Previously (7) it had been found that GTP and ATP at this concentration removed nearly all the RNase, amylase, and TAPase activity of the ribosomes, together with only 10 or 15 per cent of their total protein. The substantially greater losses in total protein found in the experiment (Table IV) can be attributed to the fact that in this type of experiment the ribosomes went through two centrifugation cycles.

added Mg^{++} forms a complex with the GTP or ATP and thus interferes with their action in removing the spermine and the RNA. In this Table it can also be seen that under various conditions of spermine removal or retention the ratio of C^{14}-spermine bound to RNA-P is approximately the same. Accordingly it can be inferred that the amine groups of the bound spermine were salt-linked to the phosphate groups of the RNA, and that the added pyrophosphate compounds, GTP or ATP, effectively competed with the phosphate groups of the RNA for the bound spermine. When Mg^{++} was present, the pyrophosphate groups were complexed with it, and hence had little effect.

Occurrence of Spermine in the RNP Particles

The ability of ribosomes to bind exogenous spermine, together with the fact that Zillig *et al.* (9) found polyamines in *E. coli* and liver ribosomes, prompted us to inquire whether polyamines, primarily spermine, normally occur in pancreatic RNP particles.

TABLE IV

Effect of GTP and of Mg^{++} on the Release of RNA, Radioactive Spermine, and Protein from Pancreatic Ribosomes

The ribosomes were labeled with radioactive spermine, as described in the text, then resuspended and re-incubated at 35°C for 30 min. with the additions given below. After re-incubation, the particles were isolated by centrifugation at 105,000 *g* for 90 min., and their radioactivity, protein, and RNA determined as described in the text.

Exp. No.	Conditions	Sedimentable after 90 min. at 105,000 *g*				
		Protein	RNA (μmoles RNA-P)	RNA/Protein	C^{14}-Spermine (μmoles)	μmoles C^{14}-Spermine per 10 μmoles RNA-P
		(μg)				
1	Re-incubation without additions	—	—	—	0.018	—
	" with Mg^{++} (10 mM)	—	—	—	0.018	—
2	No re-incubation	250	0.33	0.47	0.049	1.49
	Re-incubation without additions	150	0.25	0.58	0.028	1.12
	" with Mg^{++} (10 mM)	—	—	—	0.023	—
	" " GTP (0.5 mM)	—	—	—	0.016	—
	" " GTP (0.5 mM) plus Mg^{++} (3 mM)	—	—	—	0.022	—
3	No re-incubation	282	0.44	0.55	0.050	1.12
	Re-incubation without additions	259	0.37	0.49	0.034	0.92
	" with GTP (2 mM)	122	0.04	0.12	0.004	1.00
	" " GTP (2 mM) plus Mg^{++} (3 mM)	188	0.23	0.44	0.026	1.13
4	No re-incubation	275	0.54	0.67	0.069	1.28
	Re-incubation without additions	231	0.37	0.56	0.045	1.22
	" with GTP (2 mM)	187	0.14	0.27	0.014	0.97
	" " GTP (2 mM) plus Mg^{++} (3 mM)	253	0.32	0.43	0.036	1.12
	" " ATP (2 mM)	228	0.18	0.28	0.019	1.08
	" " ATP (2 mM) plus Mg^{++} (3 mM)	250	0.27	0.38	0.034	1.26

In the paper chromatography system used (*cf.* reference), the following Rf values were found: spermine, 0.17 to 0.18; spermidine, 0.26 to 0.28; putrescine, 0.38 to 0.42; cadaverine, 0.43 to 0.46; histamine, 0.34 to 0.36; lysine, 0.36; and valine, 0.76. Chromatographed extracts of guinea pig pancreatic and hepatic ribosomes and of rat hepatic ribosomes yielded regularly a small

ninhydrin-positive spot with an Rf of 0.16 to 0.19, probably spermine, usually accompanied by a spot with an Rf of 0.27 to 0.30, possibly spermidine, and another with an Rf of 0.22 to 0.24 of unknown identity. Ninhydrin-positive streaks also appeared between Rf 0.42 and Rf 0.62.

Paper electrophoresis showed the following sequence in the rate of movement to the anode: arginine < lysine < spermine < spermidine < cadaverine < putrescine, with the following values for a typical 2-hour run: arginine, 17 cm; lysine, 18 cm; spermine, 27 cm; spermidine, 29 cm; cadaverine, 31 cm; and putrescine, 33.5 cm. Extracts of pancreatic (guinea pig) and hepatic (guinea pig and rat) ribosomes regularly yielded a small ninhydrin-reactive spot migrating with the rate of spermine (26.5 to 27.5 cm) frequently accompanied by a single small additional spot with a migration rate intermediary (28 cm) between those of spermine and spermidine. It appears, therefore, that isolated pancreatic and hepatic ribosomes contain spermine and possibly another polyamine, but it should be pointed out that the amount of material behaving as polyamine in the electrophoretic and chromatographic systems is very small and, as such, accurate quantitative data could not be obtained. For example, when ribosomes obtained by the deoxycholate treatment of guinea pig pancreas microsomes were extracted, and the polyamine content semi-quantitatively determined by comparing the ninhydrin color developed on paper chromatograms with known amounts of spermine (dissolved in the same solution with which the extract was made and identically chromatographed), it was found that an extract which contained 540 μg RNA gave a spermine spot which contained less than 2 μg. In order to obtain better data, larger amounts of particles were needed, necessitating the use of calf pancreas for the preparation of ribosomes (the pancreas from one adult guinea pig weighs about one gram). Particles were, therefore, prepared in large quantities from both calf pancreas and guinea pig liver. They were extracted with cold 5 per cent TCA, the TCA removed with ether, and the extracts spotted on Whatman #3 MM paper for electrophoresis. After electrophoresis, the paper was sprayed with ninhydrin, and all the stained areas corresponding to compounds behaving in the system like polyamines (*i.e.*, moving faster than lysine) were cut out. The ninhydrin-positive color was quantitatively removed from the paper by successive soakings of the paper in 100 per cent methanol, and the absorption at 545 mμ (its maximum) was recorded. A known amount of spermine placed on the paper and carried through the entire process was used as a standard. It was found that in an extract of calf pancreatic ribosomes containing 1910 μg RNA there were 13.2 μg spermine equivalents of polyamine (0.45

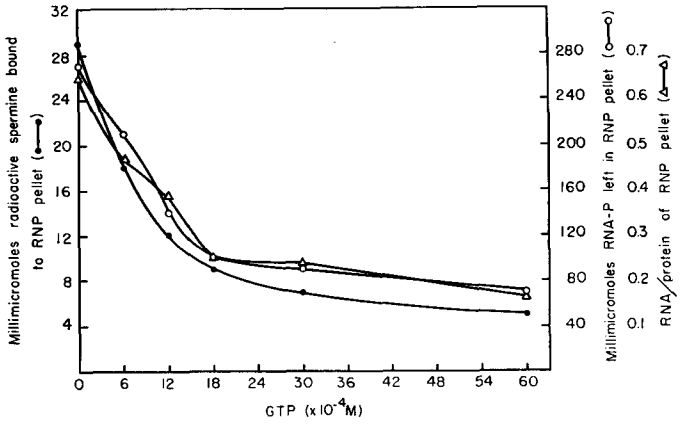

FIGURE 1

Effect of GTP concentration on the RNA, RNA/protein ratio and bound radioactive spermine of pancreatic ribosomes. Methods are given in the text.

μmoles total amine group, calculated as spermine, per 10 μmoles RNA-P), while an extract of guinea pig hepatic ribosomes containing 957 μg RNA had 5.6 μg spermine equivalents (0.39 μmols total amine group, calculated as spermine, per 10 μmoles RNA-P).[6]

Since it is possible that the DOC used to detach the ribosomes from the microsomal membranes may have removed polyamines associated with them *in situ*, free pancreatic RNP particles were obtained and examined. Such particles are prepared without the use of DOC (1). Extracts of free ribosomes, like those of attached particles, gave relatively small proportions of compounds reacting with ninhydrin and moving like polyamines in the chromatographic system; for example, an extract containing 780 μg RNA gave a spermine spot on paper which was equivalent to about 3 μg polyamine. Furthermore, very little of the radioactive spermine experimentally bound to DOC-prepared hepatic ribosomes

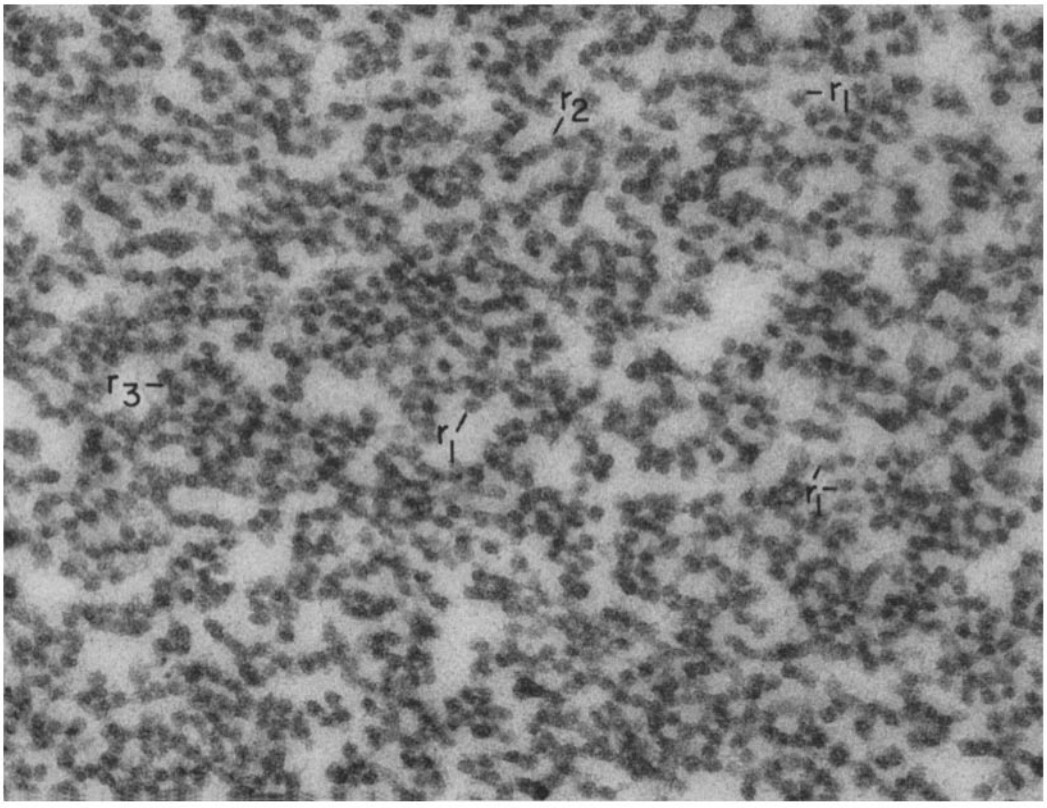

FIGURE 2

All micrographs represent sections of pancreatic ribosomal pellets (guinea pig) fixed *in toto* in 2 per cent OsO_4 in 0.88 M sucrose and embedded in a mixture of methyl and butyl methacrylate. All sections were stained with lead hydroxide before electron micrography.

Pancreatic ribosomes fixed immediately upon preparation. They appear as dense particles of ~150 A diameter, either isolated (r_1) or aggregated in chains (r_2) and clusters (r_3). × 120,000.

[6] It is instructive to compare these values with data in the literature (45) concerning the amount of spermine and spermidine in whole tissue. Guinea pig pancreas contains 280 μg spermine and 312 μg spermidine per gm tissue (45) or, as based on RNA values per gm tissue (1), per 5370 μg RNA. This value comes out to 6.20 μmoles total amine group per 10 μmoles RNA-P. Guinea pig liver contains 80 μg spermine and 32 μg spermidine per gm tissue (45) or per 9250 μg RNA. This comes out to 0.75 μmoles total amine group per 10 μmoles RNA-P.

could be removed by a further DOC treatment of the particles. For instance, when ribosomes having 0.16 μmoles C^{14}-spermine bound per 10 μmoles RNA-P as a result of the treatment mentioned were resuspended, incubated for 30 minutes at 0°C with water or with DOC, and then resedimented, their C^{14}-spermine/10 μmoles RNA-P ratio did not decrease: it was 0.20, 0.19 and 0.16 for particles incubated in water, 0.3 per cent DOC, and 0.6 per cent DOC, respectively. Since only 0.3 per cent DOC was used to prepare the particles, it is assumed that treatment of the microsomes with DOC removed very little of the polyamine which might have been bound to ribosomes *in situ*.

Effects of Spermine on Other RNP Particles

Hepatic RNP particles, obtained from DOC-treated microsomes (guinea pig), reacted like their pancreatic counterparts when incubated in 0.1 mM radioactive spermine: they bound the radioactive polyamine (0.80 and 1.57 μmoles/10 μmoles RNA-P in two experiments), lost 85 and 90 per cent of their Mg^{++} and ~10 per cent of their protein and RNA. In the absence of known enzymatic activities, the nature of the lost protein could not be ascertained. Mg^{++} loss could be prevented by including Mg^{++} (1 mM) in the incubation medium. The RNA/protein ratio of the spermine- and Mg^{++}-treated ribosomes was higher (1.08) than that of the sper-

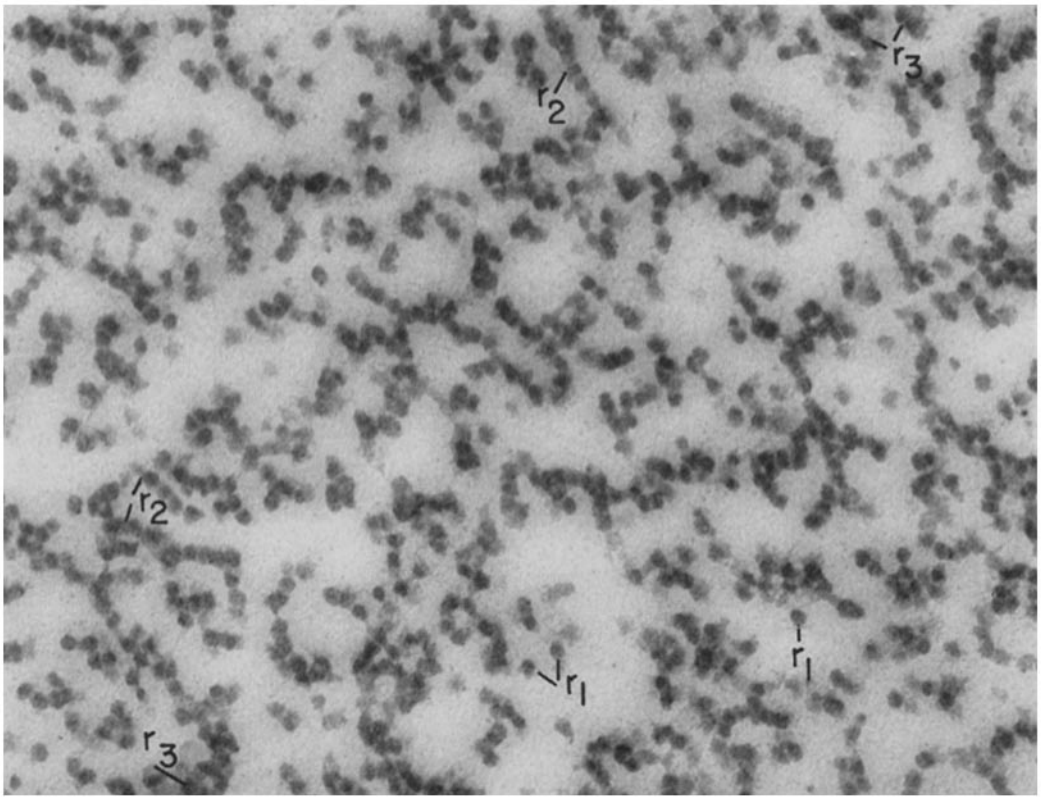

FIGURE 3

Pancreatic ribosomes incubated for 30 minutes at 35° in 0.44 M sucrose before fixation. The particles are comparable in size, density and degree of aggregation to the ribosomes fixed without previous incubation, the pellet is, however, less packed than that in Fig. 2; r_1, r_2 and r_3 designate similar degrees of ribosome aggregation. × 120,000.

mine-treated particles (0.84) and of the controls incubated in 0.44 M sucrose (0.91). In sectioned pellets, spermine-treated hepatic ribosomes were more variable in form and density than unincubated controls. They were also extensively agglutinated, but in chains, not in compact clusters like their pancreatic counterparts.

sedimentable. The second incubation caused, however, a protein loss proportionally higher than in the case of pancreatic ribosomes.

Since we found that spermine forms an insoluble salt with DOC at certain concentrations, and since traces of the bile acid might still persist in ribosomal pellets prepared from DOC-treated

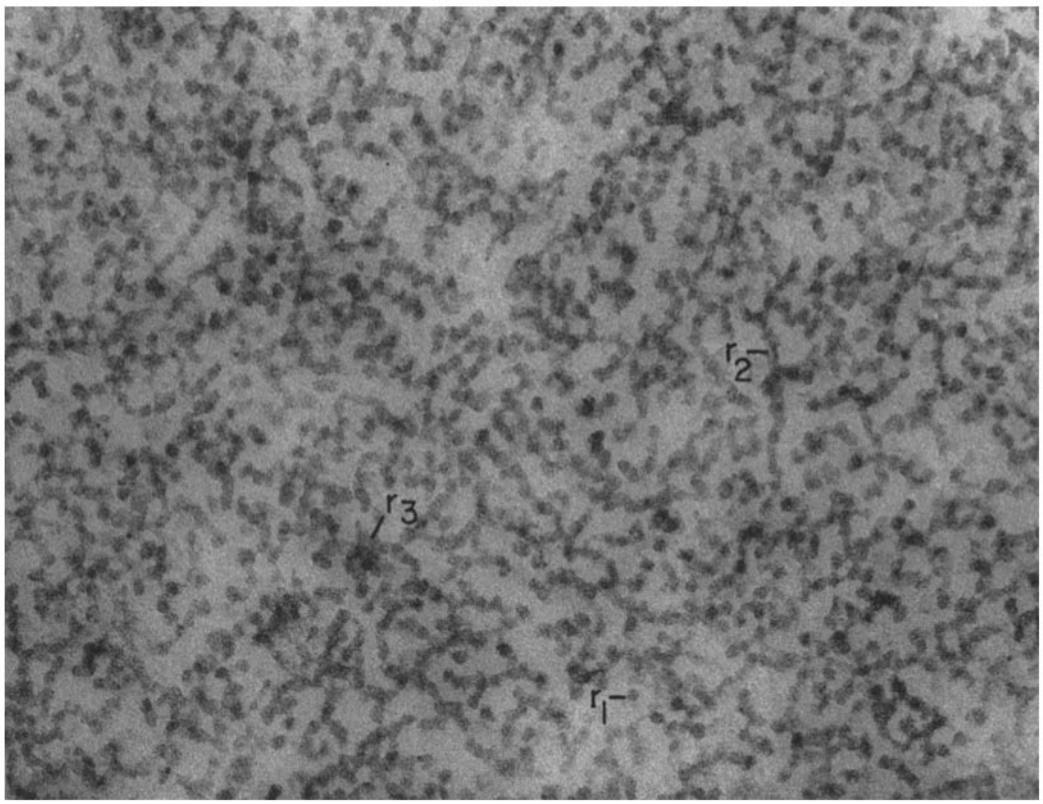

FIGURE 4

Pancreatic ribosomes fixed after incubation for 30 minutes at 35° in 0.44 M sucrose containing 0.3 mM tris buffer, pH 7.0. The aggregation of the particles in chains (r_2) and clusters (r_3) is as extensive as in Fig. 2, but the pellet is slightly less packed. Isolated particles (r_1) are rare. There is no considerable change in the density and dimensions of the ribosomes. × 120,000.

Upon reincubation in increasing concentrations of inorganic pyrophosphate, hepatic ribosomes treated with radioactive spermine lost in parallel their radioactivity and their RNA. The results were comparable to those obtained by treating pancreatic ribosomes with GTP as illustrated in Fig. 1, except that the release curves for spermine and RNA flattened only at 3 mM pyrophosphate, at which point ~70 per cent of both radioactivity and RNA were no longer

microsomes and presumably influence our results, we decided to investigate spermine effects on free pancreatic ribosomes. As already mentioned, such particles are prepared without the use of DOC. Incubation of free ribosomes in 0.1 mM radioactive spermine resulted in: *a*) binding of the radioactive polyamine to the particles (0.51 μmoles/10 μmoles RNA-P); *b*) complete loss of ribosomal Mg^{++}; *c*) loss of ~20 per cent of the original protein and RNA content of the prepa-

rations; *d*) extensive loss of amylase activity (~60 per cent as compared to ~30 per cent lost by controls incubated in 0.44 M sucrose). It appears, therefore, that free and attached ribosomes react basically alike to spermine treatment

outlined and more varied in shape than unincubated ribosomes (Fig. 2) but in general the differences were small, most particles appearing normal. Ribosomes incubated in spermine or in spermine and Mg^{++} were all agglutinated in

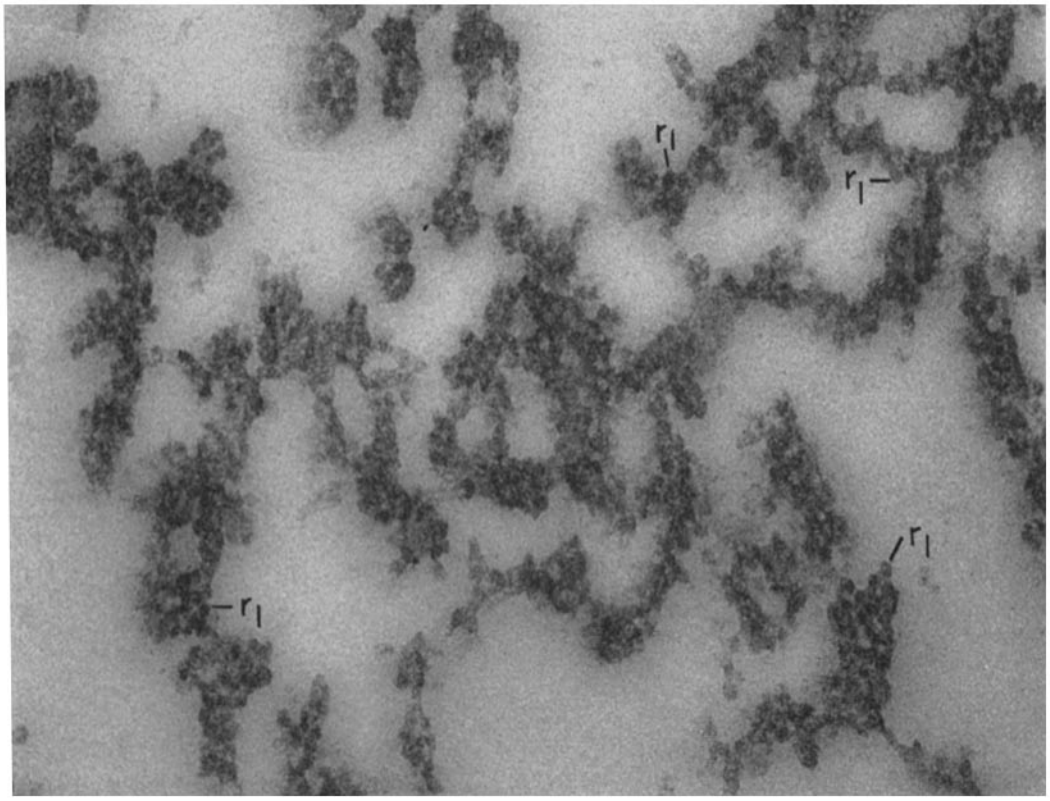

FIGURE 5

Pancreatic ribosomes fixed after incubation for 30 minutes at 35° in 0.44 M sucrose containing 0.1 mM spermine and 1 mM Mg^{++}. There is severe aggregation of the particles in tight clumps within which, however, individual ribosomes are still visible in many places (r_1). Where recognizable, the density and dimensions of the particles appear unaltered. The assumption advanced in our preliminary communication (44), that the ribosomes swell when incubated in spermine, was erroneous: it was due to the difficulty of resolving individual particles within the tight aggregates formed in the presence of the polyamine. × 120,000.

and it can be assumed that the influence of DOC in our previous results is negligible.

Electron Microscopy

Ribosomes incubated in 0.44 M sucrose, with or without tris buffer pH 7.0 (Fig. 3, Fig. 4), appeared as small dense particles 150 to 200 A in diameter, usually disposed in chains or small clusters. Some of these particles were less sharply compact clusters or strands 3 to 6 particles across. Within these aggregates, individual particles measuring 150 to 200 A could still be recognized, although the intervening spaces were small. A certain amount of particle damage and merging into more or less homogeneous masses were encountered, especially in spermine-treated preparations. The particles incubated in spermine and Mg^{++} were in general better preserved (Fig. 5)

than those incubated in spermine alone. In both cases, no difference was found between the particles of the bottom pellet and those of the thin deposit along the centrifugal side of the tubes. Ribosomes incubated in spermine followed by GTP were swollen up to 200 A diameter and aggregated in larger but less compact masses than spermine-incubated particles; as such they resembled particles incubated in GTP or ATP alone (7).

DISCUSSION

Although generally similar to ribosomes isolated from other sources, the pancreatic RNP particles appear to differ in certain details. Their sedimentation coefficient is 85 S (7) as compared to 80 S for yeast (15), pea seedling (16), and liver ribosomes (17), and to 70 S for bacterial RNP particles (18–21). Moreover, there is no evidence that they are comprised, like other ribosomes, of 30 to 40 S and 50 to 60 S subunits (17–21) which dissociate at low, and copolymerize at high, Mg^{++} concentrations. Admittedly, however, this aspect has not been systematically investigated in the case of pancreatic RNP particles. Their RNA content does not exceed 40 per cent, whereas that of bacterial ribosomes reaches 60 per cent (18–21). Under our conditions of isolation, their Mg^{++} content is low: 1 mole Mg^{++} for 10 moles of RNA-P; by comparison, that of pea seedling particles reaches 1 mole Mg^{++} for 4 moles of RNP-P (16). The Mg^{++} requirement of the *E. coli* particles also seems to be very high (20). Finally, their polyamine: RNA ratio is low, about one-tenth that reported for *E. coli* ribosomes (9).

There are at least three distinct components to be considered in these RNP particles: the RNA, the structural proteins and the proteins produced for secretion. Some of the latter are newly synthesized molecules which, for a finite period, seem to remain attached to their supposed RNA template (7). The RNA itself could be of more than one type; at least not all of it is released by Mg^{++} chelators or, in these experiments, by agents removing spermine.[7] The types of bonds which probably link these various ribosomal components, *i.e.* hydrogen bonds (22, 23), salt linkages (24) and Mg^{++} complexing (*cf.* literature cited in reference 7) have already been discussed (7). The experiments here reported point out again the importance of Mg^{++} complexing for the integrity of the particles; indicate that the cation can be replaced by polyamines, especially by spermine; and show clearly that upon such replacement the particles loose their enzymatic activities which, at least in part, seem to be due to newly synthesized proteins (*cf.* 6).

The information available permits a tentative inquiry into the role played by Mg^{++} in pancreatic ribosomes and into the mechanism by which spermine succeeds in partially replacing the cation.[8] The simplest assumption is that Mg^{++} links together ribosomal RNA subunits through their phosphate groups and possibly also through the amino groups of their purines (26), and that this linkage stabilizes the hydrogen and electrostatic bonds between the RNA and the structural protein, thereby insuring the existence and individuality of the particle. Alternatively it could be assumed that Mg^{++} forms complexes not only with the phosphate groups of the RNA but also with the carboxyl and amino groups of the structural as well as secretory proteins. Although the amount of ribosomal Mg^{++} is small, such a possibility is not excluded, for Mg^{++} has a coordination number of 4 and possibly 6 (27). Upon the displacement of Mg^{++}, the amine groups of spermine and other polyamines could form electrostatic bonds with the phosphate groups of the RNA (*cf.* 28), and these linkages in turn could stabilize the other bonds between RNA and structural protein. Since spermine releases ribosomal enzymes, it must be postulated that it cannot stabilize the assumed hydrogen and electrostatic bonds between RNA and those secretory enzymes, or that it cannot replace the hypothetic coordination bonds between the latter

[7] However, the base ratios of the RNA removed from spermine-treated particles by GTP and of the RNA remaining attached to the protein of the particle (*cf.* Fig. 1) are very similar to each other. Also, after massive *in vivo* injections of inorganic phosphate-P^{32}, both these RNA fractions acquire equally little label in their phosphate groups. For example, the phosphate in the phospholipid of the microsomal membranes had a specific activity of 20,000 CPM/μM phosphate, while the phosphates in the nucleotides isolated after alkaline hydrolysis from both these kinds of RNA had a specific activity of 5–10 CPM/μM phosphate.

[8] That spermine (25) and spermidine (12) can somewhat replace Mg^{++} in a radioactive amino acid—incorporating system has been shown with liver (25) and *E. coli* (12) ribosomes.

and Mg^{++}. The second alternative implies that the secretory proteins are held onto the ribosomes by bonds involving some basic groups on the protein for which spermine cannot compete.

The presence of endogenous spermine and possibly of another polyamine in pancreatic RNP particles may reflect a general ribosomal feature: various polyamines have already been found in bacterial and hepatic ribosomes (9). However, the amount of polyamines in pancreatic ribosomal extracts is much less than that found in similarly prepared extracts of *E. coli* ribosomes (9). In fact, the range of the amount of basic amine groups (1.2 to 6.0 μmoles/10 μmoles RNA-P) which can be bound to pancreatic particles, in the form of added spermine, brackets the amount of polyamines (3.1 μmoles basic amine group/10 μmoles RNA-P) shown to be already bound to freshly prepared *E. coli* ribosomes (9). In other words, as isolated, *E. coli* ribosomes already contain as much polyamine, mostly cadaverine and putrescine (9), as pancreatic ribosomes can bind *in vitro* in the form of spermine. When isolated, pancreatic RNP particles have only about one-tenth as much polyamine. It is not known whether this constitutes a real difference between bacterial and mammalian ribosomes, for the artifactitious adsorption of polyamine to bacterial or mammalian ribosomes during isolation is not excluded. It should be noted, however, that Cohen and Lichtenstein (29) found that *E. coli* ribosomes isolated in the presence of radioactive polyamines did not exchange the radioactive amines for the polyamines already bound to them. It would appear that the *E. coli* ribosome RNA is already saturated with polyamines which are not easily replaceable by amines in the medium. A comparable, apparently saturating concentration can be attained in the case of pancreatic ribosomes by mixing these particles with spermine.

Published evidence suggests that the polyamines may stabilize or otherwise affect the structure and chemistry of the particles (*cf.* 30). For instance, ribosomes isolated from *E. coli* grown in the presence of spermine consistently have higher sedimentation coefficients (*i.e.* 70 S or 100 S) than particles prepared from cells grown in the absence of spermine (31). *E. coli* ribosomes showed a greater preservation of the 65 S component in the presence of spermidine and Mg^{++} than in the presence of either alone (30). Finally, nucleotide incorporation into hepatic, presumably ribosomal RNA, is stimulated by the simultaneous addition of spermine and Mg^{++} (32). Polyamines were shown to act as stabilizing agents in many other cases. Spermine, for instance, can preserve nucleic acid structure (33, 34), maintain the integrity of bacterial cells (35, 36) and of bacterial protoplasts (35, 37), and confer stability to isolated mammalian mitochondria (37) and nuclei (38). Polyamines also bind strongly to polynucleotides (39), particularly to poly(adenylic + uridylic) acid, to form a complex which is precipitable at neutral pH's (40). They also combine with and neutralize phage DNA (28). Finally, there is evidence indicating that spermine can bind to phospholipids (41). Such a binding may stabilize various membranous structures thereby explaining some of the results already mentioned (35, 37).

Since spermine releases ribosomal enzymes, binds strongly to RNA and probably to lipids, it may be rewarding to inquire further into the role it and other polyamines may play *in vivo* in the release of newly synthesized proteins from ribosomes and the transport of these proteins across the lipoprotein membrane of the endoplasmic reticulum to the intracisternal spaces (*cf.* 42). In this respect, it should be mentioned that various polyamines, including spermine, were found to increase the production of various exoenzymes, including amylase, by *B. subtilis* (43). Moreover, a similar effect was reported for a factor with polyamine properties which was isolated from these bacterial cells (43).

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